PHARMACOKINETIC, PHARMACODYNAMIC, AND TOXICOLOGICAL STUDIES ON NEW ANTI-ARRHYTHMIC AGENTS FROM SELECTED 3,7-DIHETERABICYCLO[3.3.1]-NONANE DERIVATIVES

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Dedicated to

MY WIFE, Hao Chen

MY DAUGHTER, Chen Chen

MY SON, Vinson Chen

PREFACE

This dissertation is composed of 12 chapters, most of which is a complete and independent manuscript, prepared in accordance with the guidelines of respective journals to which they were submitted. Chapter I is a review of the literature of antiarrhythmic agents and a justification of the need for new antiarrhythmic agents. Several HPLC methods for the determination of BRB-I-28, GLG-V-13, and their metabolite(s) in blood and urine are described in Chapter II-IV. Chapter II has been published in the *Journal of Chromatography Biomedical Application*, while Chapters III and IV have been published in the *Journal of Liquid Chromatography*. Chapter V, dealing with pharmacokinetics and plasma protein binding of BRB-I-28 in dogs, has been published in *Drug Investigation*. Chapter VI dealing with *in vitro* and *in vivo* studies of 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane has been submitted for publication in *Drug Metabolism and Disposition*. Chapter VII is concerned with the oral, intravenous pharmacokinetics, and protein binding of GLG-V-13 in dogs and rabbits and has been submitted for publication in *Arzneimittel Forschung/Drug Research*. Chapters VIII and IX deal with the effects of BRB-I-28 and GLG-V-13 on ATPase activities and mitochondrial respiration. Chapters VIII and IX have been published and submitted for publication in *Research Communication in Chemical Pathology and Pharmacology*, respectively. The acute and subchronic toxicity profiles of BRB-I-28 and GLG-V-13 are detailed in Chapters X and XI. Chapter XII is the summary and conclusions.

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CHAPTER I

LITERATURE REVIEW

Introduction

There are 1,000,000 deaths annually from cardiovascular disease in the USA in which about 300,000-400,000 are sudden or unexpected in nature (Kaplan, 1986; Gillum, 1989; Goldberg, 1989; Myerburg, 1992). It has been established that sudden cardiac arrest from ventricular fibrillation and arrhythmias is the most common cause of death (Lown, 1979; Janse and Wit, 1989; Olshausen et al., 1991). The management of these potentially lethal arrhythmias can be achieved by various approaches such as implantation of electrical defibrillators, surgical intervention and pharmacological treatment with antiarrhythmic drugs (Podrid, 1987; Fisch and Surawicz, 1991). The most frequently used mode of treatment for cardiac arrhythmia is with antiarrhythmic drugs (Graboys et al., 1982), although some available antiarrhythmic agents are neither universally effective nor safe.

Classification of Antiarrhythmic Drugs

Several attempts to classify the antiarrhythmic drugs were made in the last decade, but none were generally accepted (Nattel, 1991; Vaughan Williams, 1992).

The most common and extensively described classification of antiarrhythmic drugs is Vaughan Williams' classification (Millar and Vaughan Williams, 1981; Vaughan Williams, 1984; 1992) which is based on the fundamental electrophysiological effects of drugs on various cardiac tissues (Table 1).

TABLE 1

VAUGHAN WILLIAMS CLASSIFICATION

Class		Action	Drugs
I		Sodium channel blockade	
	Ia	Moderate phase 0 depression, slow conduction, prolong repolarization	Quinidine, procainamide, disopyramide, ajmaline, sparteine, cibenzoline, pirmenol, moricizine
	Ib	Minimal phase 0 depression, shorten repolarization	Lidocaine, phenytoin, mexiletine, aprindine, tocainide,
	Ic	Marked phase 0 depression, slow conduction, little effect on repolarization	Flecainide, encainide, lorcainide, indecainide, propafenone
II		ß-blockers	Propranolol, acebutolol, esmolol
III		Prolong action potential duration (APD)	Amiodarone, sotalol, bretylium, tedisamil
IV		Calcium channel blockade	Verapamil, diltiazem, nifedipine
V		Cl^- channel blockade	Alinidine

Class Ia antiarrhythmic drugs inhibit i_{Na} depress phase-0 depolarization.

Therefore, the amplitude, overshoot, and $\dot{V}_{max}$ of phase 0 in myocytes are decreased in a dose-dependent manner. Class Ia drugs prolong action potential duration (APD), slow conduction at high concentration, lengthen the effective refractory period (ERP), with the exception of moricizine which shortens the APD in Purkinje fibers (Bigger and Hoffman, 1990). Class Ia drugs decrease the slope of phase-4 depolarization and shift the threshold voltage toward zero (Bigger and Hoffman, 1990). These effects are due to use-dependent blockade of fast sodium channels and slowing of their rate of reactivation. At therapeutic doses, class Ia agents prolong the P wave, the PR intervals, QRS intervals and QT intervals of the ECG (Symanski & Gettes, 1993). Widening of the QRS complex in the ECG is a useful index for monitoring class Ia therapy. Because of their effects on ERP and conduction, class Ia drugs can abolish reentry arrhythmias.

Class Ib antiarrhythmic drugs inhibit the fast sodium current, shorten APD in Purkinje fibers and ventricular muscle, but cause no change in atrial fibers, resulting in lengthening ERP relative to APD (Vaughan Williams, 1984). Because these drugs reduce the APD, the QT interval may also be decreased. However, the drugs in class Ib have no effects on QRS duration or conduction during normal sinus rhythm. Unlike the class Ia drugs, which bind to both normal and depolarized myocardial cells, class Ib drugs have a more pronounced effect in areas of ischemic myocardium than in normal myocardium (Lazzara, et al., 1978). The effects of these drugs on sodium channels produce the least degree of rate- or use-dependency in normal heart. Class Ib drugs can abolish ventricular reentry, either by causing two-way block or by improving conduction.

Class Ic antiarrhythmic drugs bind tightly to and block sodium channels, and

therefore, reduce the $\dot{V}_{max}$ and overshoot of action potentials in normal and abnormal cardiac fibers (Woosley, 1991). They produce the greatest use-dependency and slow conduction and have only slight effects on APD and repolarization. Like class Ib agents, class Ic do not change the ST segments and heart rate. However, they produce an increase in the PR interval and the QRS duration. Propafenone is somewhat unique in that it has weak ß-adrenergic- and calcium antagonist properties in addition to its sodium channel blocking properties (Funck-Brentano, 1990).

Class II antiarrhythmic drugs are ß-adrenergic blockers (Vaughan Williams, 1984). Most of their antiarrhythmic properties can be explained by selective blockade of ß adrenergic receptors. The class II antiarrhythmic drugs can totally abolish automaticity in the His-Purkinje system. Like lidocaine and phenytoin, propranolol increases outward background current in Purkinje fibers, leading to decreased automaticity. Class II antiarrhythmic drugs produce: (a) increased ERP in SA and AV nodes; (b) reduced spontaneous phase 4 diastolic depolarization in both sodium- and calcium-dependent action potentials; and (c) decreased in Vmax and conduction velocity in SA and AV nodes (Bigger and Hoffman, 1990). In ECG, ß-blockers lengthen the PR interval, but have no effects on the duration of the QRS complex. At higher concentrations, propranolol and acetolol have inhibitory effects on phase-0 depolarization. ß-Blockers may provide protection against arrhythmias by decreasing myocardial oxygen consumption and reducing myocardial ischemia (Karagueuzian et al., 1987).

Class III antiarrhythmic drugs have different pharmacological properties, but they share the ability to prolong the APD and ERP in Purkinje fiber and ventricular muscle fibers. The class III drugs interfere with the repolarizing currents, carried

mainly by potassium (Colatsky et al., 1990). Amiodarone also has use-dependent blockade on the sodium channels, and therefore, reduce $\dot{V}_{max}$ of the action potential upstroke (Sheldon et al., 1989). It also has α- and ß-adrenergic receptor and calcium channel blocking activity (Symanski & Gettes, 1993). Sotalol is a ß-adrenergic receptor blocking agent that decreases automaticity (Bigger and Hoffman, 1990). Amiodarone and sotalol, but not bretylium, cause a significant increase in the PR interval. However, all three drugs prolong the QT intervals. Only amiodarone increases the duration of QRS. Amiodarone, bretylium and sotalol are thought to terminate reentry arrhythmias by markedly prolonging ERP (Bigger and Hoffman, 1990).

Class IV antiarrhythmic drugs are calcium channel blockers. They block the L-type calcium channels in the surface membrane and reduce the slow calcium-dependent inward current, which is responsible for depolarization of SA- and AV-nodes. The effect on the calcium channels by class IV drugs is use-dependent (Bigger and Hoffman, 1990). The major effect of verapamil or diltiazem is to decrease the conduction velocity through the AV node and increase its ERP. The calcium channel blocking agents do not influence $\dot{V}_{max}$ of action potential upstroke, ventricular conduction, or the QRS complex on the ECG, but they do shorten APD and ERP, leading to shortening the QT interval.

The class V agents are chloride channel blockers. Alinidine prolongs APD in the atrium and ventricle; does not increase K conductance or block sodium channels, and is not a competitive antagonist of ß-receptors. Class V drugs reduce the slope of the slow diastolic depolarization in pacemaker cells of the sino-atrial node (Millar and Vaughan Williams, 1981).

Limitations of the Vaughan Williams Classification

The Vaughan Williams classification has recently received criticism (Rosen, 1991; Woosley, 1991; Vaughan Williams, 1992). The major limitations are: (1) drugs belonging to the same class differ in their interaction with the given channels; (2) most antiarrhythmic drugs fall into more than one class; (3) classifications are based on the data from healthy cardiac tissues; and (4) the classification neglects the differences in the effects of antiarrhythmic drugs on different types of cardiac tissues, etc. Therefore, several alternative classifications, e.g. classification based on molecular level, and/or on effects on human arrhythmias have been proposed (Rosen, 1991; Vaughan Williams, 1991; Colatsky, 1992; Rosen, 1992; Vaughan Williams, 1992), but these alternative classifications are still in the developmental stage. With a deeper understanding of the mechanisms of arrhythmias and the actions of antiarrhythmic drugs, better classifications of antiarrhythmic drugs than the existing one will appear in the near future.

Selection of Antiarrhythmic Drugs

Class Ia antiarrhythmic drugs slow conduction velocity, prolong refractoriness, and decrease the automatic properties of sodium-dependent conductive tissues of the heart. Therefore, class Ia agents can be very effective in the treatment of automatic tachycardias by depressing the rate of spontaneous impulse generation of atrial or ventricular foci. In reentry tachycardias, these drugs depress conduction and prolong refractoriness, transforming the area of unidirectional block into a bidirectional block.

Clinically, class Ia drugs are broad-spectrum antiarrhythmics, being effective for both supraventricular and ventricular arrhythmias.

Class Ib drugs such as lidocaine and phenytoin have facilitative actions on cardiac conduction by shortening refractoriness with little effect on conduction velocity. Lidocaine, tocainide and mexiletine are considerably more effective for ventricular arrhythmias than for supraventricular arrhythmias.

Class Ic drugs, flecainide and encainide, are very effective in suppressing premature ventricular depolarization with a low incidence of side effects, but they increase mortality in patients with myocardial infarction (Echt et al., 1991; Rogers et al., 1989, 1992). Therefore, class Ic agents are rarely used for treating arrhythmias.

Class II drugs, ß-adrenergic receptor antagonists, are most useful in tachycardias of the SA and AV nodes, because the SA and AV nodes are heavily influenced by adrenergic innervation. These agents are also helpful in slowing ventricular response in supraventricular tachycardias by their effects on the AV node.

Class III antiarrhythmic drugs prolong refractoriness in atrial and ventricular fibers. Bretylium is very effective in ventricular fibrillation, whereas, it is rarely effective in ventricular tachycardia. In contrast, amiodarone and sotalol are effective in most tachycardias. Amiodarone and sotalol also depresses SA and AV nodal conduction by direct adrenergic blockade.

Class IV drugs, calcium channel blockers (verapamil and diltiazem) inhibit calcium entry into the cell, thus slow conduction, prolong refractoriness, and decrease automaticity of the SA and AV nodes. Class IV antiarrhythmic drugs are very effective against tachycardias with origins in the SA and AV nodes.

For many patients, a single antiarrhythmic drug may not adequately suppress

ventricular arrhythmia, and an important approach is the use of drug combinations (Greenspan, 1986; Hirsowitz, et al. 1986; Deedwania, 1987; Duff, 1987; Marchlinski, 1988; Awaji and Hashimoto, 1993; Dorian et al., 1993). Two or more partially effective agents used together may result in synergistic antiarrhythmic effects and enhance arrhythmia suppression. Additionally, the use of two drugs permits dose reduction of each agent without compromising the antiarrhythmic effect (Kawamura et al. 1990). This combination diminishes the potential for drug toxicity and improves patient compliance (Podrid, 1987; Patt, 1988).

Whether a single or combination of antiarrhythmic drug(s) is used, the ideal approach to the treatment of cardiac rhythm disorders depends on the proper identification of the arrhythmia, an understanding of the factors involved in the pathogenesis of the arrhythmia, a knowledge of the mechanism of action of the available pharmacologic agents, and an understanding of the clinical effects of the antiarrhythmic agents. The selection of antiarrhythmic drugs remains empirical, without adequate guidelines for selection (Podrid. 1987). A more quantitative approach that may ultimately lead to a more rational selection of drug therapy for cardiac arrhythmias should be based on the modes of actions of antiarrhythmic drugs on the monophasic action potential recorded from ischemically injured myocardial tissue (O'Donoghue and Platia, 1991).

Pharmacokinetics of Antiarrhythmic Drugs

Importance of Pharmacokinetic Studies

Pharmacokinetics quantitates the time course of absorption, distribution,

metabolism and excretion of substances or medicinal agents. Pharmacokinetics has evolved during the last three decades from a purely mathematical description of the time course of drug concentrations in the body to a discipline that is fully integrated with pharmacology, toxicology and clinical therapeutics (Peck et al., 1992; Smith, 1993). Consequently, the collection, analysis, and presentation of data on pharmacokinetic, metabolic and toxicological studies have become an important part of the new drug development process. Comprehensive pharmacokinetic information is a regulatory requirement for investigational new drug submission (IND) and new drug application (NDA) (Balant et al., 1990; Tse and Jaffe, 1991).

Support for Pharmacology. It is well recognized that the intensity and duration of the pharmacologic effect of the drug are functions, not only of the intrinsic activity of the drug, but also of its pharmacokinetic characteristics. Pharmacokinetic data are often useful in the interpretation of drug effects. Appropriate pharmacokinetic data can indicate whether the drug is poorly absorbed or undergoes first-pass effects, resulting in low therapeutic blood levels. This information is important for making decisions such as improving drug absorption by alteration in the salt form or drug formulation, investigation of the possibility of prodrugs, or abandonment of the oral route of administration. Knowledge of the pharmacologically efficacious systemic concentration in animal models can be utilized to guide later studies in humans.

Support for Toxicology. The most important function of animal pharmacokinetic studies is in support of drug-safety evaluation. Determination of pharmacokinetic characteristics during the course of toxicology studies may aid in the interpretation of certain toxicologic findings. For example, tissue distribution data

may be useful in explaining organ specific toxic manifestations.

Prediction of Human Pharmacokinetics. Preclinical data on drug absorption and disposition can support and guide decisions concerning initial trials in humans. For many drugs, the extent of absorption is very consistent between different animal species and man, but, the actual form of the drug reaching the systemic circulation may differ among species due to the large qualitative and quantitative differences in the metabolism of a drug among species. Thus, interspecies variations in important pharmacokinetic parameters, such as absolute bioavailability, clearance, and elimination half-life, can occur for the same compound. Such variations can make it difficult to make valid, direct extrapolation of animal disposition data to man. However, an allometric theory has been developed to estimate human pharmacokinetics by interspecies scaling, which is based on similarities in anatomy, physiology, and biochemistry among animal models and man (Boxenbaum & D'souza, 1990).

Development of New Dosage Forms and Formulations. Animal models are often employed to provide support in the development of new dosage forms or formulations. Initially, pharmacokinetic characteristics of a drug can serve as the basis for selection of a suitable dosage form. This knowledge can be used to determine the need for such specific dosage forms as enteric-coated or controlled-release units. Additionally, animals are often used as a screen for new dosage forms in order to eliminate unnecessary human trials with different formulations. These studies can reduce the cost and time necessary for developing a new formulation as well as unnecessary human exposure to test compounds.

Analytical Techniques

The interpretation of pharmacokinetic data ultimately depends upon the accurate quantitation of drugs and their metabolites in biological fluids. Without a selective, specific, and sensitive analytical method for the determination of a drug and its metabolite(s), significant errors may be introduced into evaluations of pharmacokinetic data. Thus, the analysis of therapeutic agents is still a challenge for analytical chemists because the measurement of low-level concentrations of therapeutic agents and their metabolic products in biological fluids remains extremely difficult. The compounds are usually found in very low concentrations with many other compounds and endogenous substances which may cause analytical interferences. Most analytical methods, such as dye methods, UV and visible spectrophotometry, and fluorimetry, are not sensitive and selective enough to identify an individual compound from the others. Other methods, such as microbiological assays and immunological methods (such as ELISA), have sometimes been used but are not widely employed in the measurements of concentrations of a drug and metabolite(s).

The high degree of sensitivity with radioactivity labelled compounds is mainly used for drug concentrations in tissues of animals and in animal pharmacokinetic studies. However, poor specificity and ethical problems raised by the use of radiolabelled substances in humans has limited its widespread use.

Mass spectral and NMR analysis are usually not used in pharmacokinetic studies, but they are important techniques for the elucidation of metabolite structures. However, gas chromatography-mass spectrum (GC-MS), and more recently high-performance liquid chromatography-mass spectrum (HPLC-MS), provide rapid and

sensitive techniques in characterizing drug metabolites.

Chromatographic techniques are widely used in pharmacokinetic studies. Thin-layer chromatography (TLC) remains a useful tool for the detection of metabolites, particularly in urine, but this method has generally been replaced by HPLC. Gas-liquid chromatography (GLC) methods are sensitive, precise, and rapid and can easily be automated, but the method is significantly restricted to parent drugs and some volatile metabolites. GC-MS techniques are important tools for the identification of drug metabolite(s). HPLC is the method of choice during drug development. It is recommended that formal pharmacokinetic studies be performed with HPLC, especially when dealing with steric drugs (Balant et al., 1990). HPLC has been utilized to analyze almost all antiarrhythmic drugs (Midha and Butterfield, 1979).

Whenever an analytical method is developed for use in pharmacokinetic studies, it must be fully validated, since method validation can significantly influence the pharmacokinetic data evaluation (Pachla et al., 1986). Any method should be validated to ensure that precise and accurate data are obtained before sample analysis is undertaken. The validation method should include the following performance characteristics: drug stability, sensitivity, selectivity, recovery, linearity, calibration curve precision and accuracy, and method precision and accuracy (Balant, et al., 1990).

Pharmacokinetic Profiles

The pharmacokinetic disposition of most antiarrhythmic drugs can be described by an open two-compartment model after intravenous administration and

a one-compartment model after oral administration with the antiarrhythmic effects being best correlated with drug content in the peripheral compartment (Welling and Tse, 1985). Several important pharmacokinetic parameters of the most commonly used antiarrhythmic drugs are summarized in Table 2 (Karagueuzian, 1987; Gilman et al., 1990). Pharmacokinetic profiles indicate that different antiarrhythmic drugs have individual pharmacokinetic characteristics. Only after understanding the pharmacokinetic profiles can individualized drug treatment be achieved. Pharmacokinetic profiles of a drug must be completed at the time of registration in order to provide a valid basis for safety and efficacy issues. It must be updated with new information during the post-marketing phase when unexplainable side-effects are observed, new pharmaceutical forms are developed, and new data are collected from new dosage regimens. The relevant pharmacokinetic studies must be performed using the same design as those used in preclinical study (Balant et al., 1990).

Pharmacokinetics can be limited in the absence of pharmacodynamic data. Integration of animal pharmacokinetic and pharmacodynamic data in drug development is very important (Peck, 1992; Smith, 1993). In considering species variation in dose response relationships, pharmacokinetic differences are considered as the major factor. Moreover, the variation in dose response may actually reflect differences in the pharmacodynamic response per se, due to various forms of receptor types expressed in individual species. It has been concluded that pharmacodynamics and safety evaluation must be performed, not just by inter-species plasma concentration comparisons, but by assessment of the ratios between doses and concentrations producing desired effects tolerated by animals (Balant et al., 1990).

TABLE 2

SOME PHARMACOKINETIC PARAMETERS OF COMMONLY USED ANTIARRHYTHMIC DRUGS

Drugs	F (%)	$t_{1/2}$ (h)	Binding (%)	V (L/kg)	Cl_B (ml/min/kg)	U.E (%)	R. E.	T. C. (μg/ml)
Quinidine	70-80	5-7	80-90	2.0-3.5	4.7±1.8	18±5	H/R	2-6
Procainamide	75-90	2.5-5.0	10-20	10-20	-	67±8	H/R	4-15
Disopyramide	70-95	4-8	50-80	0.8-2.0	1.2±0.4	55±6	H/R	2-6
Lidocaine	20-40	1-2	65-75	1-2	9.2±2.4	2±1	H	1.5-6
Phenytoin	85-95	6-24	89±23	0.5-0.8	-	2±8	H/R	10-20
Mexiletine	80-95	6-12	60-75	5-12	6.3±2.7	4-15	H	0.7-2
Tocainide	90-95	12-15	10-30	1.5-3.0	2.6±0.5	38±7	H	4-10
Flecainide	90-95	13-20	35-45	8-10	5.6±1.3	43±3	H	0.3-2.5
Encainide, PM	85-95	8-11	70-80	2.5-4.0	2.6±0.6	39±6	H/R	-
EM	20-30	1-3	70-80	2.5-4.0	25±8	5±2	H	-
Lorcainide	1-65	3-7	80-90	6.4±2.4	17.5±2.8	<2	H	0.1
Propranolol	15-35	3-5	80-95	4.3±0.6	16±5	<0.5	H	0.02
Propafenol	5-50	10-32	97	-	-	<1%	H/R	-

TABLE 2
(Cont'n)

Acebutolol	20-50	2-4	20-30	1.2±0.3	6.8±0.8	40±11	H/R	-
Esmolol	-	0.1-0.2	50-60	1.9±1.3	170±70	<1	H/R	-
Amiodarone	22-88	15-100d	95-97	70-150	1.9±0.4	0	H	0.5-2.5
Sotalol	100	10-15	Negligible	-	-	-	H/R	-
Bretylium	15-20	5-10	Negligible	4-8	10±1.9	77±15	R	0.5-2.0
Verapamil	20-40	4-12	95-99	1.5-5.0	15±6	<3	R/H	0.05-0.15
Diltiazem	30-55	2.5-5	70-85	3.1±1.2	12±4	<4	H/R	-

F = Bioavailability; V = Volume of distribution; CL_B = Body Clearance; U. E. = Urinary Excretion; R. E. = Route of Elimination; H = Hepatic; R = Renal; T. C. = Therapeutic Concentration; PM = Poor Metabolizers; EM = Extensive Metabolizers.

The combined data from pharmacokinetics and pharmacodynamics represent a challenging field for drug research. It is also obvious that more efforts are needed for the assessment and simultaneous analysis of kinetics and dynamics of drugs.

Plasma Protein Binding

Plasma protein binding is of pharmacological significance in many aspects. Plasma protein binding may (1) facilitate drug absorption and drug transport; (2) influence drug activity by reducing the proportion of the dose available to sites of action in tissues; and (3) decrease the availability of drug molecules for metabolism and excretion. However, plasma protein binding for a drug may be altered in the various pathological conditions and in the presence of other drugs. Therefore, information on plasma protein binding is useful at a very early stage of drug development.

Plasma protein binding can be determined by equilibrium dialysis, ultracentrifugation, ultrafiltration, partition equilibrium, gel filtration, spectroscopic, and isotopic techniques. Equilibrium dialysis is the most common and preferable method (Balant et al., 1990). Two important study methods are widely used: (1) "*in vitro-in vitro*" studies, where the binding of new drugs to animal and human plasma protein is determined by using "spiked samples", and (2) "*ex vivo-in vitro*" studies, where plasma protein binding is evaluated by using the sample obtained from subjects exposed to the drug. The latter technique can avoid the possible qualitative and/or quantitative alterations in plasma proteins and influence by the presence of metabolites. However, most protein binding data reported come from "*in vitro-in*

vitro" studies. Protein binding of antiarrhythmic drugs ranges from 0-100% (Table 2). Protein binding may be very useful for predicting drug-drug interactions between strongly bound drugs, since displacement of drugs from their plasma protein binding site(s) is the most frequent cause of an unwanted increase in the free fraction of a pharmacologically active moiety, leading to an alteration in the volume of distribution.

Metabolism

Phase I Metabolism. Two of the most important enzyme systems involved in Phase I metabolism are: (1) cytochrome P-450 system or the mixed function oxidase (MFO), which catalyzes oxidative reactions such as hydroxylation, dealkylation, epoxidation, deamination, sulfoxidation, desulfuration, dehalogenation, *N*-hydroxylation, and reductive reactions such as aromatic nitro reduction, and (2) the flavin-containing monooxygenase (FMO) system, which mainly catalyzes the metabolism of S- and N-containing compounds via S- and N-oxidation (Wislocki et al., 1980; Ziegler, 1988).

Phase II Metabolism. Phase II reactions are conjugating reactions which generally convert the parent compound or Phase I metabolite(s) into more water soluble products. Several types of conjugating reactions including glucuronidation, sulfation, methylation, acetylation, amino acid conjugation, and glutathione conjugation, are present in animals.

Although most drug metabolites are less biologically active than their parent compounds, some Phase I and II metabolites are more toxic. Therefore, drug metabolism is not simply "detoxification". In fact, the desired pharmacologic actions of some drugs are wholly attributed to their metabolites. Likewise, the toxic effects

of some drugs may be due in whole or in part to metabolic products.

Drug metabolism study is a part of drug development and safety evaluation. These data are helpful in understanding the biological action of a drug and are FDA required. Such data can also provide important information to guide chemical modifications, such as pro-drug synthesis, in order to increase drug stability and bioavailability. Usually, metabolic studies are conducted separately from the formal pharmacokinetic studies, and the chemical nature and the concentrations of metabolites in biological fluids should be determinated (Balant et al., 1990). Since drug metabolism has significant species differences, the metabolism of a new drug must be investigated in different species. It is very important to determine the pharmacokinetic characteristics of a metabolite, in addition to those of the parent drug, although assessment of the pharmacokinetics of a metabolite after the administration of the parent drug can be quite difficult because of the complex kinetic relationships between the parent drug and its metabolite (Chan, 1982; Cheng, 1992). However, without *in vitro* metabolism studies the interpretation of most *in vivo* metabolism data is virtually impossible. In fact, *in vitro* studies of drug metabolism, including liver perfusion, subcellular preparations, isolated enzymes, primary cells in culture, cell lines and fetal hepatocytes in culture, should be studied before the mechanisms of drug metabolism are extensively studied *in vivo*. (Balant et al., 1990).

The metabolites of antiarrhythmic drugs can have significant clinic importance (Kates et al., 1984). Only after understanding the pharmacological and pharmacokinetic profiles of both the parent drug and its metabolites is the individualized therapy possible. Table 3 summarizes the metabolic pathways and

TABLE 3

METABOLISM OF ANTIARRHYTHMIC DRUGS

Drugs	Metabolism Pathways	Metabolite(s)	Antiarrhythmic Activities of Metabolites
Quinidine	Aliphatic hydroxylation	3-Hydroxyquinidine	same as parent drug
	Aromatic hydroxylation	2'-Oxyquinidine	less than parent
	O-dealkylation	*O*-Demethylquinidine	same as parent compound
Procainamide	*N*-acetylation	*N*-Acetylprocainamide	same as parent drug, but by a different mechanism
	Hydrolysis	*p*-Aminobenoic acid	no
Disopyramide	Mono-*N*-dealkylation	*N*-Deisopropyldisopyramide	25-50% of parent drug; anticholinergic (toxic effect)
Sparteine	*N*-oxidation	*N*-Oxidation product	-
Lidocaine	Mono-*N*-dealkylation	Monoethylglycinexylidide (MEGX)	some
	Bi-*N*-dealkylation	Glycinexylidide (GX)	25% of parent drug; convulsion action (toxic effect)
Diphenyhydantoin	Parahydroxylation	5-Phenylhydantoin	no
Mexiletine	*N*-methylation + hydroxylation	*para*-*H*ydroxymexiletine	no

TABLE 3 (Con't)

Aprindine	*N*-dealkylation	*N*-Deethylaprindine	same as parent drug
Flecainide	*N*-dealkylation	*N*-Deethylflecainide	no
Encainide	*O*-demethylation	*O*-Demethylencainide (ODE)	50 times more
	O-demethylation + methoxylation	3-Methoxy-*O*-demethylencainide (MODE)	same as parent compound
	N-demetylation	*N*-Demethylencainide (NDE)	less
Lorcainide	*N*-dealkylation	Norlorcainide	same as parent compound
	Aromatic hydroxylation	*para*-hydroxylorcainide	-
	O-methylation	*O*-Methylorcainide	-
Indecainide	*N*-dealkylation	*N*-Deisopropyl indecainide	10% of parent drug
Propranolol	Aromatic hydroxylation	4-Hydroxypropranol	less
Amiodarone	Mono-*N*-dealkylation	Deethylamiodarone	less
	Bi-*N*-dealkylation	Bi-*N*-dealkylationamiodarone	-
Sotalol	no	-	-
Verapamil	*N*-dealkylation	Norverapamil	less

activity of metabolites of some common antiarrhythmic drugs (Hartenstein and Wagner, 1986; Karagueuzian et al., 1987). The metabolism of many antiarrhythmic drugs has been extensively studied. It is interesting that most metabolites of antiarrhythmic drugs possess some degree of antiarrhythmic properties. Dealkylation is the most important metabolic pathway for most antiarrhythmic drug because they are N- or O-containing compounds (Hartenstein and Wagner, 1986; Karagueuzian et al., 1987). Hydroxylation is also common for selected antiarrhythmic drugs.

Biochemical Mechanisms of Antiarrhythmic Drugs

During the last decade, several new antiarrhythmic drugs have been introduced for treatment of various cardiac arrhythmias. The electrophysiological mechanisms and pharmacological effects of these antiarrhythmic drugs have been widely studied (Bigger and Hoffman, 1990). However, little is known about the biochemical mechanisms of these antiarrhythmic drugs. Since mitochondrial function and sodium pump activity are significantly affected by ischemia induced by myocardial infarction (Jennings and Reimer, 1981), most biochemical studies are usually directed towards understanding the effects of antiarrhythmic drugs on myocardial mitochondrial function and sodium pump activity.

Effects of Antiarrhythmic Drugs on Mitochondrial Respiration

Mitochondrial function is crucial for the living cell. At least 80% of energy produced in mitochondria is used to drive various activities of a cell. Enzymes of the respiratory chain, which are embedded in the inner mitochondrial membrane, are

essential for oxidative phosphorylation in which two processes occur, namely (a) electron transfer via the respiratory chain and (b) ATP production coupled to the electron transport.

Myocardial ischemia causes a decrease in the supply of oxygen and nutrition to cells. Consequently, there is a marked decrease in energy production in mitochondria with cessation of coronary artery flow. Moreover, a decrease in ATP production markedly affects mitochondrial function. Persistent mitochondrial dysfunction is suggested to lead directly to cellular death (Jennings and Reimer, 1981; Hanaki et al., 1992).

It has been suggested that antiarrhythmics inhibit many aspects of mitochondrial function (Almontrefi and Dzimiri, 1990). The effects may be caused by the action of an antiarrhythmic drug on mitochondrial membranes by altering the activities of membrane proteins (channels and proteins). Antiarrhythmic drugs, such as perhexiline maleate, propranolol, lidoflazine, iproveratril, and amiodarone, inhibit mitochondrial function by acting on complex I of the mitochondrial respiratory chain. They inhibit the electron transport from flavin protein to CoQ (Lopes et al., 1977; Kluppel et al., 1978; Fortes et al., 1983a, 1983b, 1983c; Borba et al., 1984; Kluppel et al., 1986; Borba et al., 1987; Fromenty et al., 1990). Amiodarone is the only antiarrhythmic drug to inhibit complex II in addition to its inhibitory effects on complex I (Fromenty et al., 1990). All of these antiarrhythmic drugs also significantly inhibit mitochondrial ATPase activities. Inhibition of the mitochondrial respiratory chain and ATPase activity result in depletion of ATP. In animal studies, the protective effects of class I antiarrhythmic drugs, such as aprindine, disopyramide, flecainide, lidocaine, mexiletine, pentisomide, propafenone, and amiodarone on

myocardial mitochondria against ischemia, have been well characterized (Nokin et al., 1987; Hanaki et al., 1992). However, inhibitory effects on mitochondrial function may also be responsible for cell injury (Powis et al., 1990). Therefore, the pharmacological/toxicological consequence of these effects are still uncertain, and worthy of further investigation.

Effects of Antiarrhythmic Drugs on Na^+,K^+-ATPase

Na^+,K^+-ATPase is an integral transmembrane enzyme responsible for maintenance of Na^+ and K^+ gradients in mammalian cells (Skou, 1992; Skou and Esmann, 1992). Na^+,K^+-ATPase consists of two nonidentical, noncovalently associated subunits α and ß which are encoded by separate genes (Liu and Gick, 1993). The α subunit has a MW of 112,000 and is responsible for catalysis of ATP. Tissue-specific expression of three α isoforms, α_1, α_2 and α_3, are associated with Na^+,K^+-ATPases which have different sensitivities to cardiac glycosides (Kent et al., 1987) and different affinity for intracellular Na^+ (Doohan and Rasmussen, 1993). The smaller ß subunit (MW 35,000) is present in equimolar amounts with the α subunit and may facilitate the assembly of the αß heterodimer in the plasma membrane (Geering, 1990). ß-Subunit isoforms ($ß_1$, $ß_2$ and $ß_3$) are suggested to play a role in the assembly of the Na^+,K^+-ATPase molecule and its transport to the membrane (Horisberger et al., 1991; Doohan and Rasmussen, 1993). By maintaining constant gradients of Na^+ and K^+ across the plasma membrane of a wide variety of cells, this pump directly or indirectly controls cell functions (Hoffman and Forbush, 1983; Weer, 1985; Skou, 1992; Skou and Esman, 1992). In the heart, Na^+,K^+-

ATPase is important for the regulation of cell volume, maintenance of transmembrane voltage gradients, and generation of ion gradients for transport of calcium and other solutes. Therefore, the Na/K pump is referred to as an electrogenic pump (Gadsby, 1984).

In humans, under pathological conditions such as overloaded and failing hearts, the Na^+,K^+-ATPase pump numbers, activity, and/or gene expression are decreased (Zahler, 1993). In dog ventricular muscle infarcted by clamping a major coronary artery, there is a sharp decrease (up to 90%) in the number of high-affinity ouabain binding sites in the α_3 isoform and no change in the affinity or activity of the α_1 isoform (Decollogne, 1993).

It has become increasingly evident that multiple ionic effects of antiarrhythmic drugs may contribute to their antiarrhythmic actions (Bigger, 1984). Therefore, the antiarrhythmic actions of a single class or an individual agent may occur by more than one mode of interaction. Possible mechanisms of action include direct interactions with certain receptors, channels, and exchange proteins in which Na^+,K^+-ATPase is the most likely target enzyme (Ijzerman and Soudijn, 1989). Effects on Na^+,K^+-ATPase may contribute to some pharmacological actions such as positive inotropic effects displayed by digitalis. However, this phenomenon may also be related to the proarrhythmic effects. The positive inotropic effects resulting from inhibition of sodium pump activity have been extensively investigated (Thomas et al., 1990). However, the relationships between inhibitory actions of cardiotonic steroids on myocardial sodium pump and their positive inotropic actions still remain a subject of controversy (Thomas et al., 1990; Akera and Ng, 1991). A moderate inhibition of the sodium pump by digitalis does not cause a corresponding increase of intracellular

Na^+ ion concentration. Instead, a slight increase in intracellular Na^+ ion concentration increases the activity of the remaining pump units leading to an increase in Na-Ca exchange to produce the positive inotropic effects (Hansen, 1984; Thomas et al., 1990). On the other hand, proarrhythmic effects of antiarrhythmic drugs has been suggested as due to the malfunction of the electrogenic sodium pump (Rosen and Wit, 1987; Thomas et al., 1990). When the degree of sodium pump inhibition is excessive, a large increase in intracellular Na^+ occurs, resulting in Ca^{2+} overloading of the sarcoplasmic reticulum. At this condition, Ca^{2+} then enters the mitochondria which switch from producing ATP to pumping out the entering Ca^{2+} resulting in a depletion of ATP. When ATP needed for contractility and the maintainance of membrane activity becomes inadequate, negative inotropic effect and arrhythmia result (Thomas et al., 1990). This is probably the mechanism underlying the biochemical responses leading to digitalis-induced arrhythmias.

The correlation between Na^+,K^+-ATPase activity and shape of action potential is poorly understood. In a number of cell types, it is well established that hyperpolarization observed after an overdrive-stimulation is due to an activation of electrogenic Na^+,K^+-ATPase system (Thomas, 1972). Therefore, effects on the sodium pump may make some contribution to change in the electrophysiological properties of antiarrhythmic drugs in addition to direct effects on specific channels.

Class I antiarrhythmic drugs interact with the Na^+,K^+-ATPase activity in different ways. Most class I antiarrhythmic drugs show inhibitory actions similar to that of ouabain, a specific inhibitor of the Na/K-ATPase activity (Smith et al., 1984).

Class Ia drugs such as quinidine, procainamide and class Ib drugs such as lidocaine and tocainide have been found to produce concentration dependent

inhibition on Na^+,K^+-ATPase (Almotrefi and Dzimiri, 1990; Dzimiri and Almotrefi, 1991a). However, they are less potent than ouabain.

A class Ic drug, lorcainide, inhibits the myocardial Na^+,K^+-ATPase activity *in vitro* in a manner similar to that of ouabain (Almotrefi and Dzimiri, 1991a). However, the effective inhibitory concentration range for lorcainide is higher than its therapeutic levels. The inhibitory potency is enhanced significantly by reducing the K^+ concentration level of the incubation medium (Almotrefi and Dzimiri, 1991b). In contrast to other class I drugs, the class Ic drug encainide exerts stimulatory effects, rather than inhibitory effects on Na^+,K^+-ATPase activity at therapeutic concentrations (Almotrefi and Dzimiri, 1990).

A class II antiarrhythmic drug, propranolol significantly inhibits Na^+,K^+-ATPase activity at therapeutically relevant concentrations (Cook et al., 1983). This effect may have some therapeutic importance.

The effects of a class III antiarrhythmic agent (Amiodarone) on Na,K-ATPase activity have been widely studied (Broekhuysen et al., 1972; Prasada et al., 1986; Aomine, 1989; Dzimiri and Almotrefi, 1990a; 1991b; Almotrefi and Dzirimi, 1991c). For instance, at physiological concentrations, amiodarone inhibited the enzymatic hydrolysis of ATP by Na^+,K^+-ATPase system in a concentration-dependent fashion with an inhibitory activity range similar to that exhibited by ouabain (Broekhuysen et al., 1972). The interaction of amiodarone with the Na^+,K^+-ATPase system may possibly explain some of its pharmacological actions (Dzimiri and Almotrefi, 1990b). However, amiodarone had no inotropic effects and therefore no correlation between cardiotonic action and reduced Na^+,K^+-ATPase activity was expected. Moreover, some toxic effects were found to be mediated partly by its interaction with the sodium

pump (Rosen and Wit, 1987). Therefore, inhibitory action is probably pertinent to some of the cardiac properties shared by both amiodarone and cardiotonic steroids, such as their tendency to induce cardiac arrhythmias (Dzimiri and Almontrefi, 1991b; 1991c).

Antiarrhythmic Drugs and Membrane Channels

Sodium, potassium, calcium and chloride channels are very important in the maintenace of membrane potential as well as in the genesis of an action potential of myocardial cells. Therapeutic/toxic effects of most of the antiarrhythmic drugs are due to their specific effects on one or more of the above ion channels.

Voltage-dependent sodium channels play a crucial role in generating fast action potentials (Cohen and Barchi, 1992). Sodium channels may be in one of three different states, resting, open, or inactivated state (Hodgkin and Huxley, 1952). Class I agents have weak interactions with resting sodium channels of normal resting membrane. However, class I agents can produce use-dependent inhibition on certain states of sodium channels (Steinberg, 1986). Lidocaine interacts with open and inactivated channels, while quinidine and amiodarone preferentially interact with open and inactivated channels, respectively. Competitive studies have added support to the notion that certain class I drugs act on a common receptor site, but possess selective affinities for sodium channels at different states. Most class I antiarrhythmic drugs exhibit rate- or use-dependent effects. This means that the depression of $\dot{V}_{max}$ is greater at a faster heart rate. This phenomenon is consistent with the modulated receptor hypothesis of drug interactions with the sodium channel (Hondeghem and

Katzung, 1977). According to this model, drugs bind preferentially to the activated or inactivated a specific channel compared with the resting channel. The recovery from a block depends on the dissociation kinetics of the particular drug. The end result of use dependence is that drug effects, both antiarrhythmic and proarrhythmic, vary with the rates of stimulation. Other factors, such as ischemia, also modulate drug effects by altering the state of the sodium channel. Drugs with high affinity for the resting state could be toxic (Woosley, 1991). The modulated receptor theory uses a mathematical approach to calculate drug affinities to the three theoretical states of the channel. The guarded receptor model assumes that the affinity of a drug for the receptor is constant, but the diffusional constraints on the access and egress of a drug between its binding site within the channel and its pools in the membrane and cytoplasm are different (Woosley, 1991).

Potassium channel blockers and their use as antiarrhythmic drugs have received a great deal of attention recently (Bacaner, 1986; Narahashi, 1992; Wilde, 1993). At least seven different potassium channels have been described in the heart and are thought to play a role in normal repolarization (Woosley, 1991; Narahashi and Herman, 1992). Potassium currents, predominantly the delayed rectifier, have been identified as an important target for antiarrhythmic drug action. Blockade of the potassium currents during the plateau phase of the action potential is considered to be the mechanism of action by some antiarrhythmic drugs which prolong the refractory period (Roden, 1988). Besides the effects on sodium channels, most class I agents also have been demonstrated to block the potassium channels (Colatsky et al. 1990). Class Ia agents, such as quinidine and disopyramide, are potent nonselective inhibitors of potassium channels. Class Ib agents, such as lidocaine, do

not affect potassium channels. Class Ic agents flecainide, encainide, and propafenone were found to be potent, relatively selective blockers of potassium channel (Ik) (Duan and Nattel, 1993). Available data suggest that most currently available class III agents exert their effects on repolarization by blocking one or more potassium channels, other channels, and membrane receptors (Colatsky and Follmer, 1989; Colatsky et al., 1990). Recently, several new agents have been developed that not only affect action potential duration selectively but affect particular cardiac repolarizing K^+ currents selectively (Dukes et al., 1990; Kantor, 1990; Gwilt, 1991; Isomoto, 1993; Rees and Michael, 1993). These include tedisamil, a class III/I antiarrhythmic agent, which was found to block not only the delayed rectifier but also the transient outward potassium current (I_{to}), with a minimal effect on the fast and slow inward currents (Dukes and Morad, 1989; Dukes et al., 1990). E-4031 could be considered as a selective blocker of the delayed rectifying K^+ current (I_k) without any effect on the other potassium currents (Follmer, 1990; Isomoto, 1993). Other newer agents include glibenclamide, which blocks the ATP-dependent K^+ channel [$I_{k9ATP)}$] (Kantor, 1990); RP58866, blocker of an inwardly rectifying K^+ current (I_{k1}) (Rees and Michael, 1993); UK66,914, which blocks the delayed rectifying K^+ current (I_k) (Gwilt, 1991); and sematilide hydrochloride, which is blocker of the delayed rectifying K^+ current (I_k) (Sager, 1993).

Voltage-activated calcium channels play important roles in a variety of functions of excitable cells. These include generation of action potentials, control of bursting and pacemaker activity, secretion of neurotransmitter and hormones, and excitation-contraction coupling. Four different types of calcium channels (T-, N-, L- and P-types) have been characterized, in which the L-type calcium channel is

important in cardiac arrhythmias. Drugs that specifically modulate the activity of calcium channels could potentially be used in the treatment of cardiovascular disorders. Some of them have been successfully developed as therapeutic agents. These agents include verapamil, nifedipine, and diltiazem which are being used in the treatment of angina, paroxysmal supraventricular tachyarrhythmias, atrial fibrillation, hypertension, and cardioplegia. Verapamil is an antagonist of L-type calcium channel, and part of it's binding site is located in the sixth transmembrane segment (S_6) in the fourth repeat of the protein (Narahashi et al., 1992, Rampe, 1993). Blockade of calcium channels is unlikely to play a role in the prevention or suppression of most clinical ventricular arrhythmias (Akhtar, 1989; Woosley, 1991), but it may play a role in those arrhythmias generated from SA node and AV node, since action potentials in SA and AV nodes are dependent upon calcium not sodium channels. Verapamil, however, also blocks other cardiac ion channels, including delayed rectifier K^+ channels. This effect is concentration and voltage dependent and is attributed to open channel blockade. Verapamil produces potassium channel blockade by binding to the inner pore of voltage-dependent K^+ channels of the myocardium (Rampe, 1993).

The isolation of organ-specific sodium, potassium, and calcium channels or cloning of specific ion channels from human cardiac tissues for screening new antiarrhythmic drugs promises to aid in the development of new antiarrhythmic agents (Roberts, 1990; Rampe, 1993). Another novel approach to the development of pharmacotherapy for arrhythmias should include testing new agents for their ability to alter specific anion/cation channels in the ischemic myocardial cell and screening them for their antiarrhythmic activities in whole animal model (Curtis, 1993).

Toxicity of Antiarrhythmic Drugs

Most of the currently available antiarrhythmic agents are less than ideal in the therapeutic setting, since clinically significant adverse reactions are common during drug therapy for ventricular arrhythmias (Velebit, et al., 1984; Nygaard et al., 1986; Woosley, 1987; Bigger and Hoffman, 1990) (Table 4). Use of class I agents has always been limited by serious side effects such as proarrhythmic and negative inotropic effects (Podrid, et al., 1987; Woosley, 1991). Negative inotropic effect of class I antiarrhythmic drugs may be due to blockade of inward sodium currents. Therefore, the intracellular calcium ion content in functioning myocardial cells is reduced (Schlepper, 1989). Furthermore, the cardiac arrhythmia suppression trial (CAST) demonstrated that flecainide and encainide increase mortality in patients with recent myocardial infarction (Rogers et al., 1989; Echt, 1991; Rogers et al., 1992). Other clinical trials demonstrated that the routine use of class I antiarrhythmic agents, after myocardial infarction, was associated with increased mortality (Morganroth, 1992; Teo et al., 1993). Class II agents cause significant hypotension and are associated with left ventricular failures (Bigger and Hoffman, 1990). Class III agents and Class IV agents have marked systemic toxic effects as shown in Table 4.

Toxicological Studies of Potential Therapeutic Agents

As one can see from Table 4, all antiarrhythmic drugs have the potential to produce systemic toxicity. Therefore, detailed characterization of toxicity of the drug can be of significant therapeutic importance. Once the efficacy, specificity, and

TABLE 4

TOXIC EFFECTS OF ANTIARRHYTHMIC DRUGS

Drugs	Toxic effects
Class I	negative inotropic and proarrhythmic effects
Quinidine	cardiotoxicity, GI disorder, cinchonism, hypersensitivity
Procainamide	cardiotoxicity, lupus-like syndrome, GI and CNS disorder
Disopyramide	anticholinergic actions, dry mouth, constipation, GI disorder
Lidocaine	dizziness, respiratory arrest, convulsion, confusion, seizure and coma
Phenytoin	nystagmus, dizziness, ataxia, stupor, and coma
Mexiletin	GI upset (nausea, vomiting etc), CNS symptoms (tremor)
Tocainide	tremor, dizziness, headache, anorexia, vomiting,
Encainide	increase mortality, dizziness, tremor, nausea
Flecainide	increase mortality, CNS disorder, GI disorder
Propafenone	granulocytopenia, a lupus-like syndrome
Class II	
Propranolol	bronchospasm, angina, cardiac arrhythmia and myocardial infarction, hypotension, nightmares, insomnia
Class III	
Amiodarone	bradyarrhythmias, hypothyroidism, pulmonary toxicity, hepatic injury, photosensitivity, pigmentation
Sotalol	heart failure, proarrhythmias, bradycardia
Class IV	
Verapamil	heart failure, hypotension, bradycardia, GI disorder
Diltiazem	heart failure, hypotension, bradycardia, GI disorder

bioavailability of the new drug are well characterized, toxicological studies on the drug are essential for its further development. A comprehensive approach to toxicity testing proceeds in stepwise pattern from acute, subacute, and subchronic leading ultimately to chronic tests.

Acute Toxicity. At the beginning of drug development, short term toxicity tests are considered supplements to chronic studies in identifying and assessing potential human risks. Acute toxicity comprises adverse effects occurring within a short time of administration of a single dose of a test compound. Acute toxicity studies can provide information about the magnitude of toxicity, sex differences in toxic responses, the tissues and organs affected, and the speed of recovery in relationship to dosage and time after dosing. In addition, such studies can provide dosing information for subsequent subacute and subchronic studies.

Subacute and Subchronic Toxicity. The purpose of investigating subacute or subchronic toxicity is to establish the effects of repeated exposure to the test compound by regular administration to animals, usually daily for at least 14 days. The data provide additional information, including sex-related differences, target organs, and dose levels in relation to toxicological responses and are necessary for planning chronic studies. During the period of drug administration, animals are observed daily to detect signs of toxicity. Periodic recordings of the effects of a drug on body weight, food and water consumption, hematological tests, and serum clinical chemistry should be evaluated. Target organs and tissues are examined grossly and microscopically for the signs of toxicity.

Chronic Toxicity. Chronic toxicity studies are those in which the animals are exposed to the drug repeatedly for 3 months to several years. These studies must be

performed before safety and efficacy studies (Phase III) are done and must be completed before the drug is marketed. Throughout the chronic studies, all animals are observed for changes in appearance and behavior, neurologic effects, body weight, rate of weight gain, food and water consumption. Hematology, clinical chemistry, urinalysis, and histopathology are also evaluated. These studies can provide information about sex-differences, target organ(s) involved and therapeutic margins. In addition, teratogenesis, carcinogenesis, mutagenesis, immunotoxicity and behavioral toxicity of the drug must be examined.

Rationale for Seeking New Antiarrhythmic Drugs

Since the CAST study (a placebo-controlled, randomized study designed to test the hypothesis that pharmacological suppression of asymptomatic or mildly symptomatic ectopic activity with a variety of agents would improve prognosis after myocardial infarction), a critical reexamination of the adequacy of existing therapies for the control of cardiac arrhythmias has been done. Consequently, drugs that act by mechanisms other than sodium channel blockade, especially the class III antiarrhythmic agents, are currently receiving renewed interest as possible alternative therapies (Campbell, 1993). The Electrophysiology Study versus Electrocardiographic Monitoring (ESVEM) Trial (Mason et al., 1989), Basel Antiarrhythmic Study of Infarcted Survival (BASIS) (Burkart, et al., 1990), Canadian Amiodarone Myocardial Infarction Arrhythmia Trial (CAMIAT) (Cairns, 1991), and the Cardiac Arrest in Seattle: Conventional Versus Amiodarone Drug Evaluation (CASCADE) study (Greene et al., 1993) trials demonstrated that the class III agents, sotalol and amiodarone, had a better outcome in preventing sudden death or the recurrence of

ventricular tachycardia as compared with conventional class I drugs. However, both agents have beta-blocking actions as well. Preliminary results suggest that the class III agents generally demonstrate greater efficacy than conventional class I agents in preventing ventricular arrhythmias occurring during acute ischemia or evoked by programmed electrical stimulation, while producing less cardiac depression than other antiarrhythmic drug classes (Colatsky et al. 1990). However, most currently available class III agents (for example, amiodarone and sotalol) were developed for different purposes and all possess several other actions and serious side effects. Neither amiodarone nor sotalol is a "pure" class III agent. Sotalol is also a non-cardioselective ß-blocker. Amiodarone is also a non-competitive α and ß adrenergic antagonist. Additionally, it has calcium antagonist properties and even some class I antiarrhythmic actions. This raised several questions concerning the importance of the class III action for their therapeutic effects. Presently, there are no specific antiarrhythmic agents available for clinical use which have very little systemic toxicity. Therefore, development and therapeutic application of antiarrhythmic agents with specificity of action and limited systemic toxicity is a continuing challenge to cardiovascular physiologists, pharmacologists, medicinal chemists, and cardiologists.

During the last decade, many new antiarrhythmic agents have been developed in order to increase the spectrum of antiarrhythmic properties and reduce the propensity for systemic toxic effects (Hartenstein & Wagner, 1986; Steinberg, et al. 1986; Arrowsmith and Cross, 1990). Following is a summary of pre-clinical antiarrhythmic properties of selected 3,7-diheterabicyclo[3.3.1]nonane derivatives.

Antiarrhythmic Properties of 3,7-Diheterabicyclo[3.3.1]nonanes

3,7-Diheterabicyclo[3.3.1]nonane (DHBCN) derivatives have been found to possess potential antiarrhythmic properties (Jeyaraman and Avila, 1981). Sparteine was the first compound which was found to have class I antiarrhythmic activities. However, serious toxic effects such as nervousness, convulsions, and loss of muscular control limited its reference. Since then, structure-activity relationships of bispidine (3,7-diheterabicyclo[3.3.1]nonanes), from the inner ring of the sparteine moiety, have been widely studied. This effort has resulted in several potential antiarrhythmic agents which are more effective and less toxic than those of sparteine. Subsequently, a series of 3,7-dialkylbispidine salts have been synthesized. Since most of these compounds have antiarrhythmic effects with a very low therapeutic index, they were considered to be of little therapeutic significance (Zisman, 1989; Garrison, 1993). Tedisamil, which belongs to the family of 3,7-diheterabicyclo[3.3.1]nonanes, has been developed as a class III antiarrhythmic agent. The pharmacological and pharmacodynamic properties of tedisamil, a typical DHBCN derivative, have been extensively characterized (Dukes and Morad, 1989; Beatch et al. 1991). During the last decade, Berlin and his co-workers have developed an unique synthetic methodology to obtain a series compounds which are classified as DHBCN derivatives (Zisman, 1989; Berlin et al., 1991; Garrison, 1993). Derivatives synthesized by Berlin and his associates have been systematically screened for their potential to suppress induced ventricular arrhythmia in anesthetized dogs by Dr. Scherlag of the University of Oklahoma Health Science Center (Bailey et al., 1984; Thompson et al., 1987; Scherlag et al., 1988; Smith et al., 1989). Several DHBCN

derivatives exhibited effective inhibition on induced ventricular arrhythmia in dog models and therefore are potential candidates for further development as newer agents for the treatment of life-threatening arrhythmias. Among the various DHBCN derivatives screened were BRB-I-28 (7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane $HClO_4$), and a soluble form of BRB-I-28 (7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane HCl), and GLG-V-13 {3-[4-(1*H*-imidazol-1-yl)benzoyl]-7-isopropyl-3,7-diazabicyclo-[3.3.1]nonane·2 $HClO_4$}. These agents were more effective in inhibiting sustained ventricular tachycardias than lidocaine. The hemodynamic, antiarrhythmic, and electrophysiological properties of BRB-I-28 and GLG-V-13 are summarized as follows.

Antiarrhythmic and Hemodynamic Properties

BRB-I-28, when administered intravenously at doses of 3 or 6 mg/kg, was found to be more effective in preventing induced reentrant ventricular tachycardia in dogs than lidocaine, a most commonly used class Ib antiarrhythmic drug in treating ventricular arrhythmias in acute myocardial infarction (Scherlag et al., 1988; Fazekas et al., 1993a; 1993b; 1993c). BRB-I-28 was also found to be effective in reducing the incidence of ventricular tachycardia in rat models (Pugsley et al., 1992). BRB-I-28 was unique in that it increased mean systemic arterial blood pressure in anesthetized dogs by 10-15% during sinus rhythm. Additionally, BRB-I-28 had no significant effects on ventricular automaticity, AV nodal conduction, and intra-atrial or His-Purkinje or QRS duration (Scherlag et al., 1988).

GLG-V-13 has been demonstrated to prevent sustained ventricular tachycardia induced by programmed electrical stimulation of infarcted dog heart (Fazakas et al.,

1993c). This compound increased the ventricular effective refractory period and QT duration especially increasing the atrial His-Purkinje and His-Purkinje-ventricle interval. Subsequent studies demonstrated that GLG-V-13 has low proarrhythmic activity, little cardiodepressant effects, and longer duration of pharmacological effects than BRB-I-28. This agent markedly prolongs the action potential duration (APD) and has combined class Ib and class III antiarrhythmic properties (Fazekas et al., 1992; 1993a; 1993b; 1993c; 1994).

Electrophysiological Properties of BRB-I-28 in Normal Myocardial Tissues

BRB-I-28 failed to alter either normal automaticity or abnormal automaticity (24-h infarct preparation) in intact canine heart while suppressing induction of sustained ventricular tachycardia with provocative pacing both one day and 4 days after left anterior descending coronary artery occlusion. The electrophysiological effects of BRB-I-28 have been studied in normal canine hearts, in intact canine myocardial tissue preparations, and in isolated myocytes from canine epicardium. BRB-I-28 reduced maximum phase 0 upstroke ($\dot{V}_{max}$) and slowed conduction in canine epicardium and Purkinje tissue, primarily by slowing the recovery of the sodium channel after activation/inactivation. $\dot{V}_{max}$ and conduction velocity were slowed only at rapid heart rates (HR >210 beats/min). The half-life ($t_{1/2}$) for recovery of $\dot{V}_{max}$ after inactivation in the presence of drug is relatively rapid ($t_{1/2}$ = 160±26 ms) and is consistent with the relatively low molecular weight and lipid solubility of the antiarrhythmic drug. Although BRB-I-28 fails to alter significantly the action potential duration (APD) in canine Purkinje fibers, canine epicardium, or canine endocardium, the rate-dependent slowing of cardiac conduction observed only

at rapid HR and the relatively rapid recovery of $\dot{V}_{max}$ from inactivation in the presence of BRB-I-28 indicate that it should be included in the group of class Ib antiarrhythmic drugs. These actions are similar to the electrophysiological actions of lidocaine, tocainide, and mexiletine (Patterson et al., 1991).

Electrophysiological Properties of BRB-I-28 in Injured Myocardium

BRB-I-28 prolonged refractoriness in ischemically injured epicardium, which is very similar to that caused by lidocaine (Patterson et al., 1993). It produced less rate-dependent epicardial delay and failed to facilitate reentry arrhythmia formation. BRB-I-28 produced similar rate-dependent prolongation of HV intervals in the normal His-Purkinje system, and produced both tonic and use-dependent conduction block in the ischemically injured His-Purkinje system (Patterson et al., 1991; 1993). In isolated superfused ventricular epicardium, BRB-I-28 reduced action potential amplitude and maximum phase 0 upstroke ($\dot{V}_{max}$) in normal and ischemically injured tissue, with marked tonic and use-dependent conduction block. Action potential duration was unaltered. In isolated, superfused, ischemically injured canine endocardium, BRB-I-28 reduced APA and $\dot{V}_{max}$ and prolonged refractoriness and conduction times in ischemically injured tissue without altering APD. Tonic block was more prominent, and use-dependent block was observed at lower drug concentrations in ischemically injured tissue. The data demonstrate selective conduction depression and prolongation of refractoriness for BRB-I-28 in ischemically injured tissues. Both use-dependent and tonic conduction block contribute to the decrease in conduction observed with BRB-I-28 in ischemically injured myocardium. More prominent tonic conduction block of BRB-I-28 was present in ischemically

injured epicardium and His-Purkinje tissue than in ischemically injured left ventricular epicardium (Patterson et al., 1993).

Mechanism of Action of BRB-I-28

The relative affinity of BRB-I-28 to bind with open (activated) versus closed (inactivated) sodium channels of the normal or ischemic myocardial tissue is unknown at present time. Experimental results suggest that BRB-I-28 binds to inactivated sodium channels and delays the recovery of inactivated channels to the resting state. The effects of BRB-I-28 in ischemically injured myocardial tissues could be mediated by either (a) increased proportion of sodium channels in the inactivated state with membrane depolarization or (b) increased duration for sodium channels in the inactivated state with prolongation of APD. This effect may be the basis of antiarrhythmic action of BRB-I-28 (Patterson et al., 1991; 1993).

Pharmacokinetics of BRB-I-28 in Rats

A preliminary pharmacokinetic study of the ^{14}C-labelled BRB-I-28 in rats showed that the elimination half life of radioactivity from the blood was approximately 5.5 hours (Alavi et al., 1991). Oral dosing resulted in rapid and extensive absorption (bioavailability was estimated to be 80%) and peak concentrations within 30 minutes after administration. These results suggest that the oral route of administration may be suitable for the therapeutic management of patients suffering from cardiac arrhythmias. Tissue distribution studies have demonstrated extensive distribution of radioactivity into highly perfused organs, particularly liver, kidney, and heart. These observations correlate well with the high

volume of distribution at steady state (Vd_{ss} 3.7 L/kg). Levels of radioactivity in brain and perirenal fat were low in comparison with other tissues and decreased steadily with time after administration. Poor penetration into highly perfused brain tissue indicates the presence of a transport barrier between blood and brain and suggests that central neurotoxicity may not limit the therapeutic use of these agents. Indeed, preliminary toxicological studies have failed to demonstrate any gross neuro-behavioral toxic signs after oral administration of superpharmacological doses to rats. Thin-layer chromatographic analysis of urine samples collected from rats with administration of ^{14}C-BRB-I-28 indicated that most of the original dose is excreted in the form of metabolites.

Specific Objectives of the Proposed Research Project

As one can see from the above discussion, BRB-I-28 and GLG-V-13 are two potential antiarrhythmic agents. Studies on the preclinical pharmacokinetics, pharmacodynamics and toxicology of these two DHBCN derivatives are of paramount importance in the development of these two agents as new antiarrhythmic agents. The objectives of the proposed thesis research project are:

1. To develop a rapid, specific, and sensitive reversed-phase high performance liquid chromatographic (HPLC) method for determination of BRB-I-28, GLG-V-13, and their metabolites in biological fluids.

2. To characterize absorption, distribution, metabolism and excretion (pharmacokinetic) profiles of BRB-I-28 and GLG-V-13 in dogs using HPLC method and to identify major metabolites in biological fluids using mass spectral (MS) and

nuclear magnetic resonance (NMR) spectroscopy.

3. To determine the effects of BRB-I-28 on myocardial microsomal Na^+,K^+-ATPase and Mg^{2+}-ATPase activities related to its positive inotropic, hypertensive, and antiarrhythmic/toxicological properties.

4. To carry out toxicological, pathological, and clinical chemistry profiles of BRB-I-28 and GLG-V-13 administered to mice for a period of 45 and 90 days.

Methodology

The pharmacokinetic, pharmacodynamic and toxicologic studies on these new antiarrhythmic agents were carried out at the molecular, cellular, organ and whole animal level. The biochemical basis for the cardiotonic effects of BRB-I-28, GLG-V-13, and their derivatives was characterized at subcellular levels using guinea pig myocardial microsomal Na^+,K^+-ATPase, Mg^{2+}-ATPase, Ca^{2+}-ATPase, and mitochondria. The pharmacokinetic and toxicological studies on BRB-I-28 and GLG-V-13 were performed in whole animals and in tissues. Metabolism studies were carried out *in vivo* in the whole animal (dogs and rats) and *in vitro* at the hepatic microsomal level. Various analytical methods such as chromatography and biochemical techniques have been used in this research.

References

Alavi, F.K., Clarke, C. R., Sangiah, S., Berlin, K. D., Zisman, S., Chen, C. L., Garrison, G. L., Scherlag, B. J., and Lazzara, R. Disposition of BRB-I-28 (7-benzyl-7-aza-3-thiabicyclo[3.3.1]nonane hydroperchlorate) a novel antiarrhythmic agent. Drug Invest. 3: 317-323, 1991.

Akera, T., and Ng, Y. C. Digitalis sensitivity of Na^+,K^+-ATPase, myocytes and the Heart. Life sciences. 48: 97-106, 1991.

Akhtar, M., Tchou, P. and Jazayeri, M. Use of calcium channel entry blockers in the treatment of cardiac arrhythmias. Circulation. 80: IV-31-IV-39, 1989.

Almotrefi, A. A., and Dzimiri, N. Effects of quinidine, lidocaine and encainide on guinea pig myocardial Na^+,K^+-ATPase activity. Res. Commun. Chem. Pathol. Pharmacol. 69: 253-256, 1990.

Almotrefi, A. A., and Dzimiri, N. Influence of lorcainide on microsomal Na^+,K^+-ATPase in guinea-pig isolated heart preparation. Br. J. Pharmacol. 102: 530-532, 1991a.

Almotrefi A. A. , and Dzimiri, N. Variations in potassium concentration modify the inhibitory effect of lorcainide on myocardial Na^+,K^+-ATPase activity. Br. J. Pharmacol. 104: 793-796, 1991b.

Almotrefi, A. A., and Dzirimi, N. The influence of potassium concentration on the inhibitory effects of amiodarone on guinea pig microsomal Na^+,K^+-ATPase. Pharmacol. Toxicol. 69: 140-143, 1991c.

Aomine, M. Suggestive evidence for inhibitory action of amiodarone on Na^+,K^+-pump activity in guinea pig heart. Gen. Pharmacol. 20: 491-496, 1989.

Arrowsmith, J. E. and Cross, P. E. Antiarrhythmic agents. Annual Reports in Medicinal Chemistry. 25: 79-88, 1990.

Awaji, T., and Hashimoto, K. Antiarrhythmic effects of combined application of class I antiarrhythmic drugs; addition of low-dose mexiletine-enhanced antiarrhythmic effects of disopyramide and aprindine in various-rate canine ventricular tachycardias, J. Cardiovasc. Pharmacol. 21: 960-966, 1993.

Bacaner, M. B., Clay, J. R., Shrier, A., Brochu, R. Potassium channel blockade: a mechanism for suppressing ventricular fibrillation. Proc. Natl. Acad. Sci USA. 83: 2223-2227, 1986.

Bailey, III, B. R., Berlin, K. D., Holt, E. M., Scherlag, B. J., Lazzara, R., Brachmann,

J., van der Helm, D., Powell, D. R., Pantaleo, N., Ruentiz, P. C., Synthesis, conformational analysis, and antiarrhythmic properties of 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane-9-one, 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane hydroperchlorate, and 7-benzyl-9-phenyl-3-thia-7-azabicyclo[3.3.1]nonane-9-ol hydroperchlorate and derivatives: single crystal X-ray diffraction analysis and evidence for chair-chair and chair-boat conformers in the solid state. J. Med. Chem. 27: 758-767, 1985.

Balant, L. P., Roseboom, H., and Gundert-remy, U. M. Pharmacokinetic criteria for drug research and development. Advances in Drug Research. 19: 1-138, 1990.

Beatch, G. N., Abraham, S., MacLeod, B. A., Yoshida, N. R., and Waker, M. J. Antiarrhythmic properties of tedisamil (KC8857), a putative transient outward K^+ current blocker. Br. J. Pharmacol. 102: 13-18, 1991.

Berlin, K. D., Scherlag, B. J., Clarke, C. R., Otiv, S. R., Zisman, S. A., Sangiah, S., and Mulekar, S. V. Salts of 3-azabicyclo[3.3.1]nonanes as potential antiarrhythmic agents, and precursors thereof. U.S. Patent 5,084,572, 1992: Chem. Abstr. 115: 114550c, 1991.

Bigger, Jr. J. T. Antiarrhythmic treatment: an overview. Am. J. Cardiol. 53: 8B-16B, 1984.

Bigger, Jr. J. T., and Hoffman, B. F. Antiarrhythmic drugs. In "The Pharmacological Basis of Therapeutics" (Gilman, A. G. et al. eds), Eighth Eds, pp 840-873, Pergamon Press, New York, 1990.

Borba, H. R., Silveira, O., Kluppel, M. L. W., Lopes, L. C. V., Souza, V. B. V., and Campello, A. P. Aspectos bio quimicos do mecanismo de acao de drogas antiarritmicas sobre mitocondria. VI. Modificacoes conformacionais. Arq. Biol. Tecnol. 27: 37-51.

Borba, H. R., Lopes, L. C. V., Silveira, O., Campello, A. P., and Kluppel, M. L. W. Biochemical aspects of the mechanism of action of antiarrhythmic drugs on mitochondria. VIII. Effects on Ca^{2+} transport. Res. Commun. Chem. Pathol. Pharmacol. 55: 59-65, 1987.

Boxenbaum, H., and D'Souza, R. W. Interspecies pharmacokinetic scaling. Biological design and neoteny. Advances in Drug Research. 19: 139-196, 1990.

Broekhuysen, J. Clinet, M. and Delisee, C. Action of amiodarone on guinea pig heart sodium and potassium activated adenosine triphosphatase. Comparison with ouabain. Biochem. Pharmacol. 21: 2951-2960, 1972.

Burkart, F., Pfisterer, M., Kiowski, W. Effects of antiarrhythmic therapy on mortality in survivors of myocardial infarction with asymptomatic complex ventricular arrhythmias: Basel Antiarrhythmic Study of Infarct Survival (BASIS). J. Am. Coll.

Cardiol. 16: 1711-1718, 1990.

Cairns, J. A. Post-myocardial infarction mortallity in patients with ventricular premature depolarizations; Canadian Amiodarone Myocardial Infarction Arrhythmia Trial Pilot Study. Circulation. 84: 550-557, 1991.

Campbell, T. J. Class III antiarrhythmic action: the way forward? The Medical Journal of Australia. 158: 732-733, 1993.

Chan, K. K. A simple integrated method for dug and derived metabolite kinetics. An application of the statistical moment theory. Drug Metab. Dispos. 10: 474-479, 1982.

Cheng, H. A general method for calculating the mean transit times and distribution rate parameters of catenary metabolites. Biopharm. Drug Dispos. 13: 179-186, 1992.

Cohen, S. A., and Barchi, R. L. Cardiac sodium channel structure and function. Trends in cardiovascular Medicine. 4: 133-140, 1992.

Colatsky, T. J., Foller, C. H., and Starmer, C. F. Channel specificity in antiarrhythmic drug action. Circulation. 82: 2235-2242, 1990.

Colatsky, T. J., and Follmer, C. H. K^+ channel blockers and activators in cardiac arrhythmias. Cardiovasc Drug Rev. 7: 199-209, 1989.

Colatsky, T. J., Tollmer, C. H., Starmer, C. F. Channel specificity in antiarrhythmic drug action. Mechanism of potassium channel block and its role suppressing and aggravating cardiac arrhythmias. *Circulation*. 82: 2235-2242, 1990.

Colatsky, T. J. The Sicilian gambit and antiarrhythmic drug development. Cardiovascu. Res. 26: 562-565, 1992.

Cook, L. S., Straub, K. D., Doherty, J. E., Whittle, J. L., and Baker, B. J. Digitalis-sensitive Na^+,K^+-ATPase: lack of a direct catecholamine-mediated stimulation in bovine myocardial tissue. J. Cardiovasc. Pharmac. 5: 446-449, 1983.

Curtis, M. J., Garlick, P. B., and Ridley, P. D. Anion manipulation, a novel antiarrhythmic approach: mechanism of action. J. Mol. Cell Cardiol. 25: 417-436, 1993.

Decollogne, S., Bertrand, I. B., Ascensio, M., Drubaix, I., and Lelievre, L. G. Na^+,K^+-ATPase and Na/Ca exchange isoforms: physiological and physiopathological relevance. J. Cardiovasc. Pharmacol. 22(suppl. 2): S96-S98, 1993.

Deedwania, P. L., Olukotum, A. Y., Kupersmith, J., Jenkins, P., Golden, P. Beta blockers in combination with class I antiarrhythmic agents. Am. J. Cardiol. 60: 21B-28B, 1987.

Dhalla, N. S., Singal, P. K., Panagia, V., Harrow, J. A. C., Anand-Srivatava, M. B., and Beamish, R. E. Progress and problems in understanding the involvement of calcium in heart function. Can. J. Physiolo. Pharmacol. 62: 867-873, 1984.

Doohan, M. M., and Rasmussen, H. H. Myocardial cation transport. Journal of Hypertension. 11: 683-691, 1993.

Dorian, P., Newman, D., Berman, N., Hardy, J., Mitchell, J. Sotalol and type IA drugs in combination prevent recurrence of sustained ventricular tachycardia. J. Am. Coll. Cardiol. 22: 106-113, 1993.

Duff, H. J., Michell, L. B., Manyari, D., Wyse, D. G. Mexiletine-quinidine combination: electrophysiological correlates of a favorable antiarrhythmic interaction in humans. J. Am. Coll. Cardiol. 10: 1149-1156, 1987.

Dukes, I. D. and Morad, M. Tedisamil inactivates transient outward K^+ current in rat ventricular myocytes. Am. J. Physiol. 257 (Heart Circ. Physiol. 26): H1746-H1749, 1989.

Dukes, I. D., Cleeman, L., Morad, M. Tedisamil blocks the transient outward and delayed rectifier K^+ currents in mammalian cardiac and glian cells. J. Pharmacol Exp. Ther. 254: 560-569, 1990.

Duan, D., and Nattel, S. Potassium channel blocking properties of propafenone in rabbit atrial myocytes. J. Pharmacol. Exp. Ther. 264: 1113-1123, 1993.

Dzimiri, N., and Almotrefi, A. Actions of amiodarone in guinea pig myocardial ATPase preparations. Eur. J. Pharmacol. 183: 1772-1773, 1990a.

Dzimiri, N. and Almotrefi, A. A. Comparative effects of procainamide, tocainide and lorcainide on Na^+,K^+-ATPase in guinea pig heart preparations. Gen. Pharmacol. 22: 403-406, 1991a.

Dzimiri N., and Almotrefi, A. A. Amiodarone: biochemical evidence for its interaction with myocardial Na^+,K^+-ATPase in guinea pig microsomal preparations. Biochemical Pharmacol. 41: 470-472, 1991b.

Echt, D. S. and the CAST Investigators. Mortality and morbidity in patients receiving encainide, flecainide, or placebo. The Cardiac Arrhythmia Suppression Trial. N. Engl. J. Med. 324 (12): 781-788, 1991.

Fazekas, T., Mabo, P., Berlin, K. D., Garrison, G. L., Patterson, E., Scherlag, B. J., Lazzara, R. Comparative electrophysiological effects of a new chemical class of antiarrhythmic agents, 3,7-diheterabicyclo[3.3.1]nonanes, in the 1-4-day-old infarcted dog heart. The Physiologists. 35: 221, 1992.

Fazekas, T., Mabo, P., Berlin, K. D., Garrison, G. L., Patterson, E., Scherlag, B. J.,

Lazzara, R. A Lidokaines a BRB-I-28 osszehasonlito szivelektrofiziologiai vizsgalata experimentalis szivizom infarktusban. Cardiologia Hungarica, 22: 17-21, 1993a.

Fazekas, T., Scherlag, B. J., Mabo, P., Berlin, K. D., Garrison, G. L., Chen, C. L., Sangiah, S., Patterson, E., Lazzara, R. The comparative antiarrhythmic and proarrhythmic activity of a 3,7-diheterobicyclo[3.3.1]nonane, BRB-I-28, and lidocaine in the 1-4-day-old infarcted dog heart. Acta Physiologica Hungarica. 81: 289-299, 1993b.

Fazekas, T., Scherlag, B. J., Mabo, P., Patterson, E., Berlin, K. D.,, Garrison, G. L. Egy uj refrakter periodust megnyujto antiaritmias vegyulet, a GLG-V-13 szivelektrofiziologiai jellemzese. Cardiologic Hungarica. 22: 9-15, 1993c.

Fazekas, T., Scherlag, B. J., Mabo, P., Berlin, K. D., Garrison, G. L., Patterson, E., Lazzara, R. Electrophysiological characterization of a new heart rate-lowering and refractoriness prolonging antiarrhythmic agent, GLG-V-13, in the 1-4-day-old infarcted dog heart. Acta physiologica Hungarica. submitted, 1994.

Fisch, C. and Surawicz, B (eds). Cardiac electrophysiology and arrhythmias. Elsevier, New York, 1991.

Follmer, C. H. and Colatsky, T. J. Block of delayed rectifier potassium current, IK, by flecainide and E-4031 in cat ventricular myocytes. Circulation. 82: 289-293, 1990.

Fortes, J. C., Kluppel, M. L. W., Lopes, L. C. V., and Campello, A. P. Biochmical aspects of the mechanism of action of antiarrhythmic drugs on mitochondria. III. Effect on NADH-succinate cytochrome c reductase (Complex I-II-III). Arq. Biol. Tecnol. 26: 23-28, 1983a.

Fortes, J. C., Kluppel, M. L. W., Silveira, O., Lopes, L. C. V., and Campello, A. P. Biochemical aspects of the mechanism of action of antiarrhythmic drugs on mitochondria. IV. Effect on mitochondrial swelling. Arq. Biol. Tecnol. 26: 419-425, 1983b.

Fortes, J. C., Kluppel, M. L. W., Silveira, O., Lopes, L. C. V., and Campello, A. P. Biochmical aspects of the mechanism of action of antiarrhythmic drugs on mitochondria. V. Effect on the oscillatory states. Arq. Biol. Tecnol. 26: 545-556, 1983c.

Fromenty, B., Fisch, C., Berson, A., Letterson, P., Larrey and Pessayre, D. Dual effect of amiodarone on mitochondrial respiration. Initial protonophoric uncoupling effect followed by inhibition of the respiratory chain at the levels of complex I and complex II. J. Pharmacol. Exp. Ther. 255: 1377-1384, 1990.

Funck-Brentano, C., Kroemer, H, K., Lee, J. T., Roden, D. M., Drug therapy: profenone. New England Journal of Medicine. 322: 518-525, 1990.

Gadsby, D. C. The Na/K pump of cardiac cells. Ann Rev. Biophys. Bioeng. 13: 373-98, 1984.

Garrison, G. L. Selected derivatives of 3,7-diheterabicyclo[3.3.1]nonanes which posses multi-class antiarrhythmic activities. Ph.D. Dissertation. Oklahoma State University. 1993.

Geering, K. J. Subunit assembly and functional maturation of Na^+,K^+-ATPase. J. Membrane Biol. 115: 109-121, 1990.

Gilman, A. G., Rall, T. W., Nies, A. S., Taylor, P. (ed). The Pharmacological Basis of Therapeutics, 8th edition, Pergamon Press, New York, 1990.

Gillum, R. F. Sudden coronary death in the United States, 1980-1985. Circulation. 79: 756-765, 1989.

Goldberg, J. Declining out-of-hospital sudden coronary death rates: Additional pieces of the epidemiologic puzzle. Circulation, 79: 1369-1373, 1989.

Graboys, T. B., Lown, B., Podrid, P. J., Desilva, R. Long term survival of patients with malignant ventricular arrhythmias with antiarrhythmic drugs. Am. J. cardiol. 50: 437-443, 1982.

Greene, H. L. and the CASCADE Investigators. Randomized antiarrhythmic drug therapy in survivors of cardiac arrest (The CASCADE Study). Am. J. Cardiol. 72: 280-287, 1993.

Greenspan, A. M., Spielman, S. R., Horowitz, L. N. Combination antiarrhythmic drug therapy for ventricular tachyarrhythmias. PACE. 9: 565-5766, 1986.

Gwilt, M., Dalrymple, H. W., Blackburn, K. J., Dickinson, R. P., Cross, P. E., Higgins, A. J. Electrophysiologic properties of UK66,914, a novel class III antiarrhythmic agent. J. Cardiovasc. Pharmacol. 17: 376-385, 1991.

Hanaki, Y., Sugiyama, S., Hieda, N., Taki, K., Hayashi, H., Ozawa, T. Cardioprotective effects of various class I antiarrhythmic drugs in canine hearts. J. Am. Coll. Cardiol. 14: 219-224, 1992.

Hansen, O. Interaction of cardiac glycosides with (Na + K) activated ATPase. A biochemical link to digitalis-induced inotropy. Pharmacological Reviews. 36: 143-163, 1984.

Hartenstein, J. and Wager, B. Antiarrhythmic agents. In "Cardiovascular Drugs" (Bristol, J. A., ed), pp 305-415. A Wiley-Interscience Publication, John Wiley & Sons, New York, 1986.

Hirsowitz, G., Podrid, P. J., Lampert, S., Stein, J., Lown, B. The role of bata blocking

agents as adjunct therapy to membrane stabilizing drugs in malignant ventricular arrhythmia. Am. Heart J. 111: 625-631, 1986.

Hodgkin, A. L., and Huxley, A. F. A quantitative description of membrane current and its application to conduction and excitation in nerve. J. Physiol (Lond). 117: 500-544, 1952.

Hoffman, J. F. and Forbush, B. (eds). Structure, mechanism, and function of Na/K Pump. Current Topics in Membranes and Transport. Vol. 19, Academic Press, New York.

Hondeghem, L. M. and Katzung, B. G. Time- and Voltage-dependent interactions of antiarrhythmic drugs with sodium channels. Biochim. Biophys. Acta. 472: 373-398, 1977.

Horisberger, J. D., Lemas, V., Kraehenbuhl, J. P., and Rossier, B. C. Structure-function relationship of Na^+,K^+-ATPase. Annu. Rev. Physiol. 53: 565-584, 1991.

Ijzerman, A. P., and Soudijn, W. The antiarrhythmic properties of ß-adrenoceptor antagonists. Trend Pharmacol. Sci. 10: 31-36, 1989.

Isomoto, S., Shimizu, A., Konoe, A. Kaibara, M., Centurion, O. S., Fukatani, M., and Yano, K. Electrophysiologic effects of E-4031, a new class III antiarrhythmic agent, in patients with supraventricular tachyarrhythmias. Am. J. Cardiol. 71: 1464-1467, 1993.

Janse, M. J., and Wit, A. L. Electrophysiological mechanisms of ventricular arrhythmias resulting from myocardial ischemia and infarction. Physiol. Rev. 69: 1049-1169, 1989.

Jennings, R. B., and Reimer, K. A. Lethal myocardial ischemic injury. Am. J. Pathol. 102: 241-255, 1981.

Jeyaraman, R., and Avila, S. Chemistry of 3-azabicyclo[3.3.1]nonanes. Chem. Rew. 81: 149-174, 1981.

Kantor, P. F., Coetzee, W. A., Carmeliet, E. E., Dennis, S. C., Opie, L. H. Reduction of ischemic K^+ loss and arrhythmias in rat hearts: effect of glibenclamide, a sulfonylurea. Circ. Res. 66: 478-485, 1990.

Kaplan, H. R. Perspectives: New cardiovascular drugs. In "Cardiovascular Drugs", (Bristol, J. A., eds), pp. 417-438, A Wiley-Interscience Publication, John Wiley & Sons, New York, 1986).

Karagueuzian, H. S., Singh, B. N., and Mandel, W. J. Antiarrhythmic drugs: model of action, pharmacokinetic properties, and clinical application. In"Cardiac Arrhythmias. Their Mechanisms, Diagnosis, and Management". (Mandel, W. J., eds),

pp 697-737, J. B. Lippincott Company, Philadelphia, 1987.

Kates, R. E., Woosley, R. L., and Harrison, D. C. Clinic importance of metabolites of antiarrhythmic drugs. Am. J. Cardiol. 53: 248-251, 1984.

Kawamura, T., Kodama, I., Toyama, J., Hayashi, H., Saito, H., Yamada, K. Combined application of class I antiarrhythmic drugs causes "additive", "reductive", or "synergistic" sodium channel block in cardiac muscles. Cardiovascular research, 24: 925-931, 1990.

Kent, R. B., Emanuel, J. R., Nariah, Y. B., Levenson, R. L., and Housman, D. E. Ouabain resistance conferred by expression of cDNA for a murine Na,K-ATPase α subunit. Science. 234: 901-903, 1987.

Kluppel, M. L. W., Silveira, O., Lopes, L. C. V., and Campello, A. P. Biochemical aspects of the mechanism of action of antiarrhythmic drugs on heart mitochondria. II. Enzymatic activities. Arq. Biol. Tecnol., 21: 13-18, 1978.

Kluppel, M. L. W., Borba, H. R., Silveira, O., Lopes, L. C. V., and Campello, A. P. Biochemical aspects of the mechanism of action of antiarrhythmic drugs on mitochondria. VII. Effect on energy-linked reactions and on membrane potential. Cell Biochem. Func. 4: 289-296, 1986.

Lazzara, R., Hope, R. R., EI-Sherif, N., Scherlag, B. J. Effects of lidocaine on hypoxic and ischemic cardiac cells. Am. J. Cardiol. 41: 872-879, 1978.

Liu, F. H., and Gick, G. Regulation of Na,K-ATPase ß1 mRNA content by thyroid hormone in neonatal rat cardiac myocytes. Celluar and Molecular Biology Research. 39: 221-229, 1993.

Lopes, L. C. V., Silveira, O., Kluppel, M. L. W., and Campello, A. P. Biochemical aspects of the mechanism of action of antiarrhythmic drugs on heart mitochondria. I. Oxygen uptake and oxidative phosphorylation. Arq. Biol. Tecnol. 20: 7-14, 1977.

Lown, B. Sudden cardiac death: the major challenge confronting contemporary cardiology. Am J. Cardiol. 43: 313-338, 1979.

Mason, J., and the ESVEM Investigators. The ESVEM Trial. Circulation. 79: 1354-1360, 1989.

Marchlinski, F. E., Buxton, A. E., Kindwall, K. E. Comparison of individual and combined effects of procainamide and amiodarone in patients with sustained ventricular tachyarrhythmias. Circulation. 78: 583-591, 1988.

Midha, K. K., and Butterfield, A. G. Drugs acting on the cardiovascular system. In "GLC and HPLC Determination of Therapeutic Agents". (Tsuji, K. eds). pp1015-1047, Marcel Dekker, New York, 1979.

Millar, J. S., and Vaughan Williams, E. M. Anion antagonism--a fifth class of antiarrhythmic action? Lancet. i:1291-1293, 1981.

Morganroth, J. Proarrhythmic effects of antiarrhythmic drugs: evolving concepts. Am. Heart J. 123: 1137-1139, 1992.

Myerburg, R. J., Kessler, K. M., and Castellanos, A. Sudden cardiac death: Structure, function, and time dependence of risk. Circulation. 85(suppl I): I-2-I-10, 1989

Narahashi, T., and Herman, M.D. Overview of toxins and drugs as tools to study excitable membrane ion channels. Methods in Enzymology. 207: 620-643, 1992

Nattel, S. Antiarrhythmic drug classifications. A critical appraisal of their history, present status, and clinical relevance. *Drugs*. 41 (5): 672-701, 1991.

Nokin, P., Jungbluth, L., Mouton, J. Protective effects of amiodarone pretreatment on mitochondrial function and high energy phosphates in ischemic rat heart. J. Mol. Cell Cardiol. 19: 603-614, 1987.

Nygaard, T. W., Sellers, T. D., Cook, T. S., and Dimarco, J. P. Adverse reactions to antiarrhythmic drugs during therapy for ventricular arrhythmias. J. Am. Med. Assoc. 256: 55-57, 1986.

O'Donoghus, S. and Platia, E. V. Monophasic action potential recordings: evaluation of antiarrhythmic drugs. Progress in Cardiovascular diseases. 36: 1-14, 1991.

Olshausen, K. V., Witt, T., Pop, T., Treese, N., Bethge, K., Meyer, J. Sudden cardiac death while wearing a Holter monitor. Am J. Cardiol. 67: 381-386, 1991.

Pachla, L. A., Wright, D. S., and Reynolds, D. L. Bioanalytic considerations for pharmacokinetic and biopharmacokinetic studies. J. Clin. Pharmacol. 26: 332-335, 1986.

Patt, M. V., Grossbard, C. L., Graboys, T. B., Lown, B. Combination antiarrhythmic therapy for management of malignant ventricular arrhythmia. Am. J. cardiol. 62: 18I-21I, 1988.

Patterson, E., Scherlag, B. J., Berlin, K. D., and Lazzara, R. Electrophysiological action of BRB-I-28 in canine myocardial tissues. J. Pharmacol. Exp. Ther. 259: 558-565, 1991

Patterson, E., Scherlag, B. J., Berlin, K. D., Lazzara, R. Electrophysiologic actions of BRB-I-28 in ischemically injured canine myocardium. J. Cardiovasc. Pharmacol. 21: 637-646, 1993.

Peck, C. C., Barr, W. H., Benet, L. Z. et al. Conference report: Opportunities for

integration of pharmacokinetics, pharmacodynamics and toxicokinetics in rational drug development. J. Pharm. Sci. 81: 605-610, 1992.

Podrid, P. J. Antiarrhythmic drug selection. Ann. Rev. Med. 38: 1-37, 1987.

Podrid, P. J., Lampert, S., Graboys, T. B., Blatt, C. M., and Lown, B. Aggravation of arrhythmia by antiarrhythmic drugs-incidence and predictors. Am. J. Cardiol. 59, 38-44, 1987.

Powis, G., Olsen, R., Standing, J. E., Kachel, D., and Martin II, W. J. Amiodarone-mediated increase in intracellular free Ca^{2+} associated with cellular injury to human pulmonary artery endothelial cells. Toxicol. Appl. Pharmacol. 103: 156-164, 1990.

Prasada Rao. K. S., Rao, S. B., Camus, P. H., and Mehendale, H. M. Effects of amiodarone on Na^{+},K^{+}-ATPase and Mg^{2+}-ATPase activities in rat brain synaptosomes. Cell Biochem. Funct. 4: 143-151, 1986.

Pugsley, M. K., Walker, M. J. A., Garrison, G. L., Howard, P. G., Lazzara, R., Patterson, E., Penz, W. P., Scherlag, B. J., and Berlin, K. D. The cardiovascular and antiarrhythmic properties of a series of novel sparteine analogs. Proc. West. Pharmacol. Soc. 35: 87-91, 1992.

Rampe, D., Wible, B., Fedida, D., Dage, R. C., and Brown, A. M. Verapamil blocks a rapidly activating delayed rectifier K^{+} channel cloned from human heart. Molecular Pharmacology, 44: 642-648, 1993.

Rees, S. A., and Curtis, M. J. Specific Ik_1 blockade: a new antiarrhythmic mechanism? effect of RP58866 on ventricular arrhythmias in rat, rabbit, and primate. Circulation. 87: 19791989, 1993.

Roberts, R. Cardiovascular research and its beckoned call for molecular biology. In "Molecular Biology of the Cardiovascular System", ((Roberts, R., and Schneider, M. D., ed), pp 1-11, Wiley-Liss, A John Wiley & Sons, Inc., Publication, New York, 1990.

Roden, D. M., Bennett, P. B., Snyders, D. J., Balser, J. R., Hondeghem, L. M. Quinidine delays IK action in guinea pig ventricular myocytes. Cir. Res. 62: 1055-1058, 1988.

Rogers, W. J., and the Cardiac Arrhythmia Suppression Trial II Investigators. Effect of the antiarrhythmic agent morcizine on survival after muocardial infarction. N. Engl. J. Med. 327: 227-233, 1992

Rogers, W. J., and the Cardiac Arrhythmia Suppression Trial (CAST) Investigators. Preliminary report: effect of encainide and flecainide on mortality in a randomized trial of arrhythmia suppression after myocardial infarction. N. Engl. J. Med. 321: 406-412, 1989.

Rosen, M. R., and Task Force of the Working Group on arrhythmias of the European Society of Cardiology. The sicilian gambit. A new approach to the classification of antiarrhythmic drugs based on their actions on arrhythmogenic mechanisms. Circulation. 84: 1831-1851, 1991.

Rosen, M. R., Janse, M. J., Schwartz, P. J. The Sicilian gambit: A response to Drs Colatsky and Harrison. Cardiovasc Res. 26: 568-570, 1992.

Rosen, M. R., and Wit, A. L. Arrhythmogenic actions of antiarrhythmic drugs. Am. J. Cardiol. 59: 10E-18E, 1987.

Sager, P. T., Nademanee, K., Antimisiaris, M., Pacifico, A., Pruitt, C., Godfrey, R., Singh, B. N. Antiarrhythmic effects of selective prolongation of refractoriness. Electrophysiologica actions of sematilide HCl in humans. Circulation. 88: 1072-1082, 1993.

Scherlag, B. J., Patterson, E., Lazzara, R., Bailey, B. R., Thompson, M. D., and Berlin, K. D. Comparative electrophysiological and hemodynamic actions of BRB-I-28 and lidocaine in the normal and infarcted dog heart. J. Electrophysio. 2: 461-477, 1988.

Schlepper, M. Cardiodepressive effects of antiarrhythmic drugs. Eur Heart J. 10(suppl E): 73-80, 1989.

Sheldon, R. S., Hill, R. J., Cannon, N. J., and Duff, H. J. Amiodarone: biochemical evidence for binding to a receptor for class I drugs associated with the rat sodium channel. Circulation Res. 65: 477-482, 1989.

Skou, J. C. The Na-K pump. News in Physiological Sciences. 7: 95-100, 1992.

Skou, J. C., and Esmann, M. The Na,K-ATPase. J. Bioenerg. Biomembr. 24: 249-261, 1992.

Smith, D. A. Integration of animal pharmacokinetic and pharmacodynamic data in drug safety assessment. Eur. J. Drug Metab. Pharmacokinet. 18: 31-39, 1993.

Smith, T. W., Antman, E. M., Friedman, P. L., Blatt C. M. and Marsch, J. D. Digitalis glycosides: mechanism and manifestation of toxicity. Prog. Cardiovasc. Dis. 26: 495-540, 1984.

Steinberg, M. I., Lacefield, W. B., and Robertson, D. W. Class I and III antiarrhythmic drugs. Annual Reports in Medicinal Chemistry. 21: 95-108, 1986.

Symanski J. D. and Gettes, L. S. Drug effects on the electrocardiogram, a review of their clinical importance. *Drugs*. 46 (2): 219-248, 1993.

Teo, K. K., Yusuf, S., and Furberg, C. D. Effects of prophylactic antiarrhythmic drug

therapy in acute myocardial infarction. An overview of results from randomized controlled trials. J. Am. Med. Assoc. 270: 1589-1595, 1993.

Thomas, R., Gray, P. and Andrews, J. Digitalis: its mode of action. receptor, and structure-activity relationships. Advances in Drug Research. 19: 311-562, 1990.

Thomas, R. C. Electrogenic sodium pump in nerve and muscle cells. Physiol. Rev. 52: 563-594, 1972.

Thompson, M. D., Smith, G. S., Berlin, K. D., Holt, E. M., Scherlag, B. J., van der Helm, D., Muchmore, S. W., Fidelis, K. A. Synthesis and antiarrhythmic properties of novel 3-selena-7-azabicyclo[3.3.1]nonanes and derivatives. Single-crystal X-ray diffraction analysis of 7-benzyl-3-selena-7-azabicyclo[3.3.1]nonane-9-one and 7-benzyl-3-selena-7-azabicyclo[3.3.1]nonane hydroperchlorate. J. Med. Chem. 30: 780-788, 1987.

Tse, F. L. S. and Jaffe J. M. (ed). Preclinical Drug Disposition a laboratory Handbook. Marcel Dekker, Inc., New York, 1991.

Vaughan Williams, E. M. A Classification of antiarrhythmic actions reassessed after a decade of new drugs. J. Clin. Pharmacol. 24: 129-147, 1984.

Vaughan Williams, E. M. Classifying antiarrhythmic actions: by facts or speculation. J. Clin. Pharmacol. 32: 964-977, 1992.

Vaughan Williams, E. M. Significance of classifying antiarrhythmic actions since the cardiac arrhythmia suppression trial. J. Clin. Pharmacol. 31: 123-135, 1991.

Velebit, V., Podrid, P., Lown, B., Cohen, B. H., and Graboys, T. B. Aggravation and provocation of ventricular arrhythmias by antiarrhythmic drugs. Circulation. 65: 886-894, 1984.

Weer, P. D. Cellular sodium-potassium transport. In "The Kidney: Physiology and Pathology", (Sedin, D. W., and Giebisch, G., eds), pp. 31-48. Raven Press, New York, 1985.

Welling, P. G., and Tse, F. L. S. (eds). Pharmacokinetics of cardiovascular, central nervous system, and antimicrobial drugs. The Royal Society of Chemistry, Burlington House, London, 1985.

Wilde, A. A. M. Role of ATP-sensitive K^+ channel current in ischemic arrhythmias. Cardiovascular Drugs and Therapy. 7: 521-526, 1993.

Wislocki, P. G., Miwa, G. T., and Lu, A. Y. H. Reaction catalyzed by the cytochrome p-450 system. In "Enzymatic Basis of Detoxication" (Jacoby, W. B. eds), pp. 135-182, Academic Press, New York, 1980.

Woosley, R. L. Antiarrhythmic drugs. Annu. Rev. Pharmacol. Toxicol. 31: 427-455, 1991.

Woosley, R. L., and Roden, D. M. Pharmacological causes of arrhythmogenic actions of antiarrhythmic drugs. Am. J. Cardiol. 59: 19E-25E, 1987.

Working group on arrhythmias of the European society of cardiology. The sicilian Gambit. A new approach to the classification of antiarrhythmic drugs based on their actions on arrhythmogenic mechanisms. Circulation. 84: 1831-1851, 1991.

Zahler, R., Gilmore-Hebert, M., Baldwin, J. C., Franco, K., and Benz, Jr. E. J. Expression of α isoforms of the Na,K-ATPase in human heart. Biochemica et Biophysica, 1149: 189-194, 1993.

Ziegler, D. M. Flavin-containing monooxygenase. Drug Metab. rev. 19, 2-32, 1988.

Zipes, D. P. Proarrhythmic effects of antiarrhythmic drugs. Am. J. Cardiol. 59: 26E-31E, 1987.

Zisman, S. A. A study of stereochemical and substituent effects on antiarrhythmic activity of selected 3-azabicyclo[3.3.1]nonanes and derivatives. Ph.D. Dissertation. Oklahoma State University. 1989.

CHAPTER II

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF BRB-I-28, A NOVEL ANTIARRHYTHMIC AGENT, IN DOG PLASMA AND URINE

Abstract

A sensitive reversed-phase HPLC technique with UV detection has been developed to determine the concentration of BRB-I-28 (I), a novel antiarrhythmic agent,in dog plasma and urine. The mobile phase was acetonitrile-methanol-37.5 m*M* phosphate buffer, pH 6.8-triethylamine (50:50:75:0.1 v/v). After alkalinization with NaOH, the compound was extracted from dog plasma and urine with chloroform. The extraction recovery was 83% from plasma and 84% from urine. Good linearity ($r>0.996$) was observed throughout the range of 0.1-12.0 μg/ml (plasma) and 0.1-8.0 μg/ml (urine). Intra-and inter-assay variabilities were less than 4%. Limits of quantitation were 0.08 μg/ml in plasma and in urine, respectively. HPLC analysis of plasma and urine samples from a dog treated with I demonstrated that the method was accurate and reproducible.

Introduction

BRB-I-28 (7-benzyl-7-aza-3-thiabicyclo[3.3.1]nonane·$HClO_4$, I) has been shown to possess effective antiarrhythmic properties [1,2] and exhibits electrophysiological properties typical of class Ib antiarrhythmic drugs [3]. The basis of some electrophysiological effects of antiarrhythmic properties of I could possibly be due to its inhibitory effects on myocardial Na^+,K^+-ATPase and Mg^{2+}-ATPase activities [4].

The pharmacokinetic and tissue distribution profiles of I in rats have been characterized using a radioisotope technique [5]. However, this method cannot be used to determine accurately the amount of I in biological fluids, because it measures the total amount of both parent compound and its metabolites. There are no other analytical methods currently available for this analysis. This paper describes a rapid, selective, and sensitive HPLC technique for the determination of this compound in biological fluids, including plasma and urine. Using this method, pharmacokinetic profiles and metabolites of I in dogs are being characterized.

Materials and Methods

Chemicals

All the reagents used in this study were HPLC grade, and deionized, distilled water was used throughout the work [Milli-Q™ Water system, Millipore Corp., Marlborough, MA]. Acetonitrile, methanol, chloroform, propanol and potassium phosphate monobasic were obtained from Fisher Chemicals (Fair Lawn, NJ) and

triethylamine was from Pierce Chemical Co. (Rockford, IL). Compounds I and SAZ-VII-23 [3-benzoyl-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane·$HClO_4$, II], the internal standard, were synthesized via a type of Mannich reaction starting from 4-thianone [1].

HPLC Analysis

The HPLC system consisted of a Waters 501 HPLC pump, Waters U6K universal liquid chromatography injector with a 2ml injection loop, a Model 484 Tunable Absorbance Detector controlled by a Baseline 810 Chromatography Work Station with a NEC Powermate Sx plus a computer, and NEC Pinwriter P5200 (Millipore, Milford, MA). A 250 x 4.6 mm Ultramex 5 C_6 (5 μm) and a 30 x 4.6 mm Ultramex 5 C_6 guard column (5 μm) were purchased from Phenomenex (Torrance, CA). The mobile phase was acetonitrile-methanol-37.5 m*M* phosphate buffer, pH 6.8-triethylamine (50:50:75:0.1 v/v). The mobile phase was filtered through a 0.5 um Millipore filter and degassed before use. The column was eluted under isocratic conditions utilizing a flow rate of 1.2 ml/min at ambient temperature. The detection wavelength was 261 nm for I.

Extraction of I from Dog Plasma and Urine

For the determination of I, II was used as the internal standard. To 250 μl of dog plasma was added 25μl of 10 μg/ml of internal standard. After alkalinization with 100 μl of 5 M NaOH, five ml of chloroform was added, and the mixture was mixed for 3 min. Following centrifugation (1000 x g 10 min), the organic phase was transferred to a clean test tube. The supernatant was reextracted with 1 ml of

chloroform. The combined chloroform extracts were evaporated to dryness under a stream of N_2. The residue was reconstituted in 50 μl of methanol, and 35 μl of this solution was injected for HPLC analysis. Extraction of I from urine was similar to that from plasma, except one half ml of dog urine was used and diluted with 4 ml of water before extraction.

Extraction Recovery

The samples (n = 5) were prepared to give final concentrations of 1 μg/ml and 4 μg/ml in plasma and in urine, respectively. Using the extraction procedure, the samples were extracted in the absence of the internal standard. The organic layer was evaporated, and the residues were reconstituted in methanol. The ratio of the peak area of I extracted over that of unextracted equivalent concentrations of drug under identical chromatographic conditions was calculated as extraction recovery.

Calibration Curves

Various concentrations of I were freshly prepared in methanol prior to each assay. A calibration curve was generated to confirm the linear relationship between the peak-area ratio and the concentration of the drug in the samples. Appropriate amounts of I standard were added to give concentrations ranging from 0.1 to 12.0 μg/ml in plasma and from 0.1 to 8.0 μg/ml in urine. The compounds were stable in methanol at 4 °C for at least one month. Plasma and urine samples with known concentrations of I were extracted as previously described, and standard curves were generated by plotting peak-area ratios (drug/internal standard) against drug

concentrations tested. Each standard curve was replicated five times. Linear regression analysis of standard curve was performed using the computer program PHARM/PCS [6].

Intra- and Inter-assay Accuracy and Precision

To determine intra-assay accuracy and precision for measurements of concentrations of materials, I and its internal standard were added to plasma and urine (n = 6), and the concentrations were calculated using a standard curve. The percentage of the mean concentration determined over the mean concentration added was taken as the accuracy of the method. Inter-assay accuracy and precision were determined similarly over 6 consecutive days. Precision was estimated by determing the inter-assay coefficient of variations (C.V.).

Results and Discussion

Chromatographic Separation

Several combinations of acetonitrile, methanol, buffer (with different pH) and triethylamine were evaluated as possible mobile phases. It was determined that the combination described in the HPLC analysis was found to be the most suitable for separating I. Varying proportions of triethylamine in the mobile phase change both retention time and sharpness of peak of compound I. The pH of the mobile phase is a very important factor influencing the elution of I. If the mobile phase pH is lower, the retention time for I is shorter, but there is also a concomitant decrease in sensitivity. Neither acetonitrile nor methanol alone is suitable as the strong

solvent.

The chromatographic behavior of I in the new Ultremax C_6 column is unique. Several purchased columns with supposedly the same materials gave erratic results. A new column did not provide acceptable separation for I. We consistently obtained a symmetric peak with a prolonged leading shoulder peak and low sensitivity with each of three new columns. The retention time for I increased after preconditioning the column with 10 L of mobile phase. The sensitivity reached a maximum and a symmetrically sharp peak appeared with the retention time of more than 12 min. Only this column was used to assay I in the biological fluids. Therefore, precautions should be taken in interpreting the results when a new column is used to separate I. The reason for the variation in column performance is unknown.

Extraction

Using trichloroacetic acid (TCA) to precipitate proteins decreased the absolute recovery. This may be caused by decomposition of I. The use of chloroform to precipitate proteins and to extract compound I directly from plasma offered great advantage; fewer pollutant peaks were found. Anticoagulants, such as EDTA and heparin, did not affect the extraction recovery. It was necessary to dilute the urine before extraction, because this procedure reduced the accumulation of pollutants on the column. Extraction recoveries were 83% from plasma and 84% from urine, respectively.

Standard Curves

Five consecutive standard curves for pure I analyzed on separate days

demonstrated a linear relationship between concentration and peak area. The standard curves obtained from extraction of dog plasma and urine containing known amounts of I were linear ($r>0.996$) over the concentration ranges tested. The range of coefficient of variations was between 1-17%. The regression equations were: $Y = -0.0218 + 10.37X$ for plasma and $Y = 0.0100 + 5.855X$ for urine, respectively, in which Y was drug recovered in μg/ml and X was the peak area ratio (drug/internal standard). The limits of quantitation of I were 0.08 μg/ml for plasma and for urine, respectively.

Precision and Accuracy

The results obtained indicated that the values of intra- and inter-assay coefficient of variance (C.V.) in plasma and urine were less than 4%. Accuracy of this method was 96-101%.

Applications to Dog Samples

Compound I, dissolved in ethanol solution (50%), was administered intravenously to an adult, male and healthy mongrel dog at dose of 10 mg/kg. Blood samples (ca. 5 ml) were collected by vein puncture. The samples were heparinized and centrifuged. The plasma fractions were stored at -20 °C until analyzed. Urine was collected by catheter and stored at -20 °C until analyzed. The plasma and urine samples were stable even at 4 °C for one month. The internal standard (II) was added to the dog plasma and urine samples, and samples were extracted as previously described. Representative HPLC profiles of the plasma and urine samples of a dog given I (10 mg/kg) intravenously are shown in Figure 1 and 2.

The plasma concentration-time profiles of I in one dog given intravenous dose of 10 mg/kg are shown in Fig. 3.

The results show that the HPLC method described herein is suitable for pharmacokinetic studies of this novel antiarrhythmic agent. Studies of the pharmacokinetic and metabolite(s) profiles of I in dogs are in progress.

References

1. B. R. Bailey, K. D. Berlin, E. M. Holt, B. J. Scherlag, R. Lazzara, J. Brachmann, D. van der Helm, D. R. Powell, N. S. Pantaleo and P. C. Ruenitz, J. Med. Chem. 27 (1984) 758.

2. B. J. Scherlag, E. Patterson, R. Lazzara, B. R. Baily, M. D. Thompson and K. D. Berlin, J. Electrophysiol. 2 (1988) 461.

3. E. Patterson, B. J. Scherlag, K. D. Berlin and R. Lazzara, J. Pharmacol. Exp. Ther. 259 (1991) 558.

4. C. L. Chen, S. Sangiah, K. D. Berlin, G. L. Garrison, W. Dunn, Y. Nan, B. J. Scherlag, R. Lazzara and E. Patterson. The FASEB Journal 6 (1992) Abstracts, Part II: #5363

5. F. K. Alavi, C. R. Clarke, S. Sangiah, K. D. Berlin, S. A. Zisman, C. L. Chen, G.L. Garrison, B. J. Scherlag and R. Lazzara, Drug Invest. 3 (1991) 317.

6. R. J. Tallarida and R. B. Murray, Linear Regression I. In: Manual of pharmacologic calculations with computer programs. Springer-Verlag, New York, 1986, p10.

Figure Legends

Figure 1. HPLC profile of control plasma (a) and plasma sample 5 min after i.v. dose of 10 mg/kg of I (b). See experimental for chromatographic conditions.
Peaks: 1 = II, internal standard, 2 = I. The estimated concentration of I was 3.6 μg/ml.

Figure 2. HPLC profile of control urine (a) and urine sample 1 hr after i.v. dose of 10 mg/kg of I (b). See experimental for chromatographic conditions.
Peaks: 1 = II, internal standard, 2 = I. Arrows show possible metabolites.
The estimated concentration of I was 11.5 μg/ml.

Figure 3. Plasma I concentration profile after i.v. dose of 10 mg/kg in one dog.

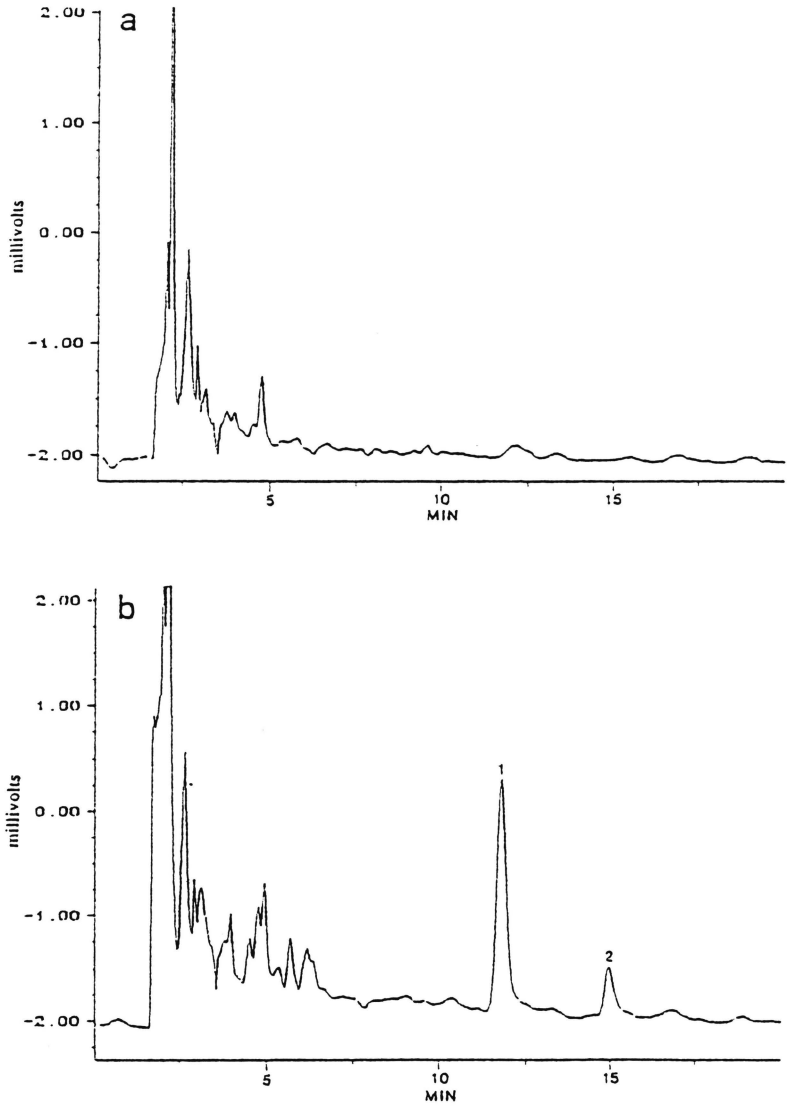
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Figure 1

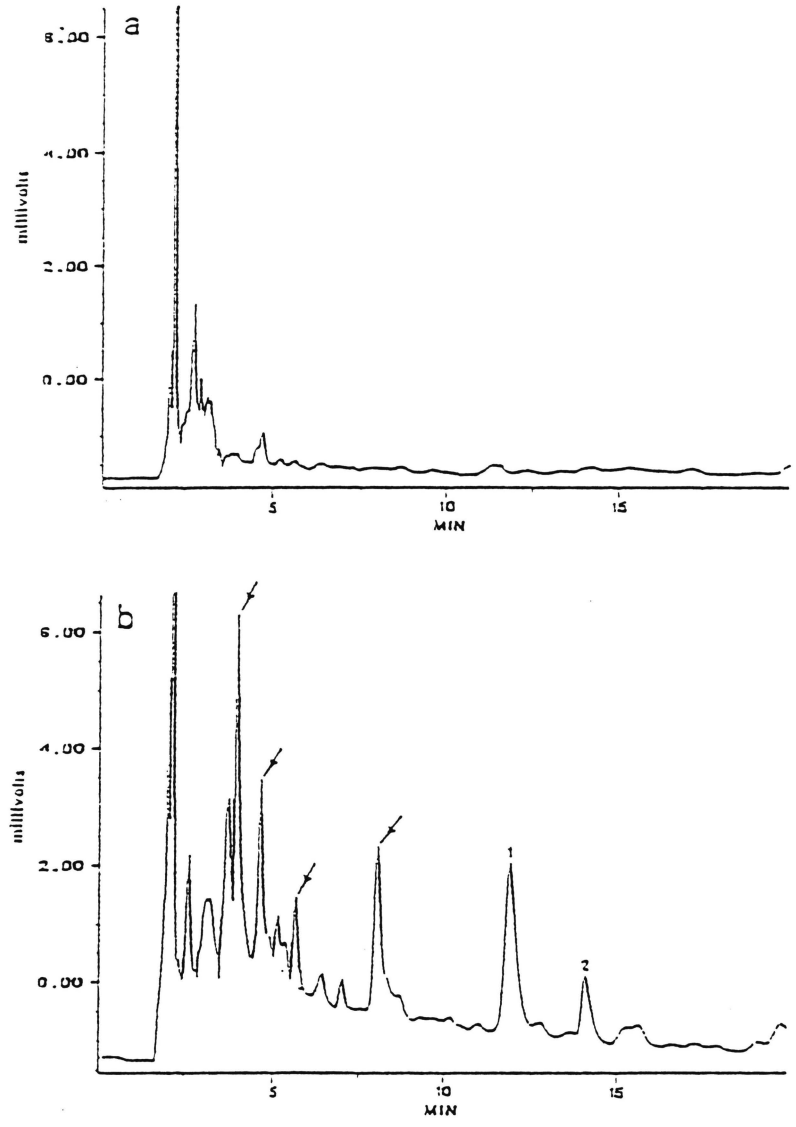


Figure 2

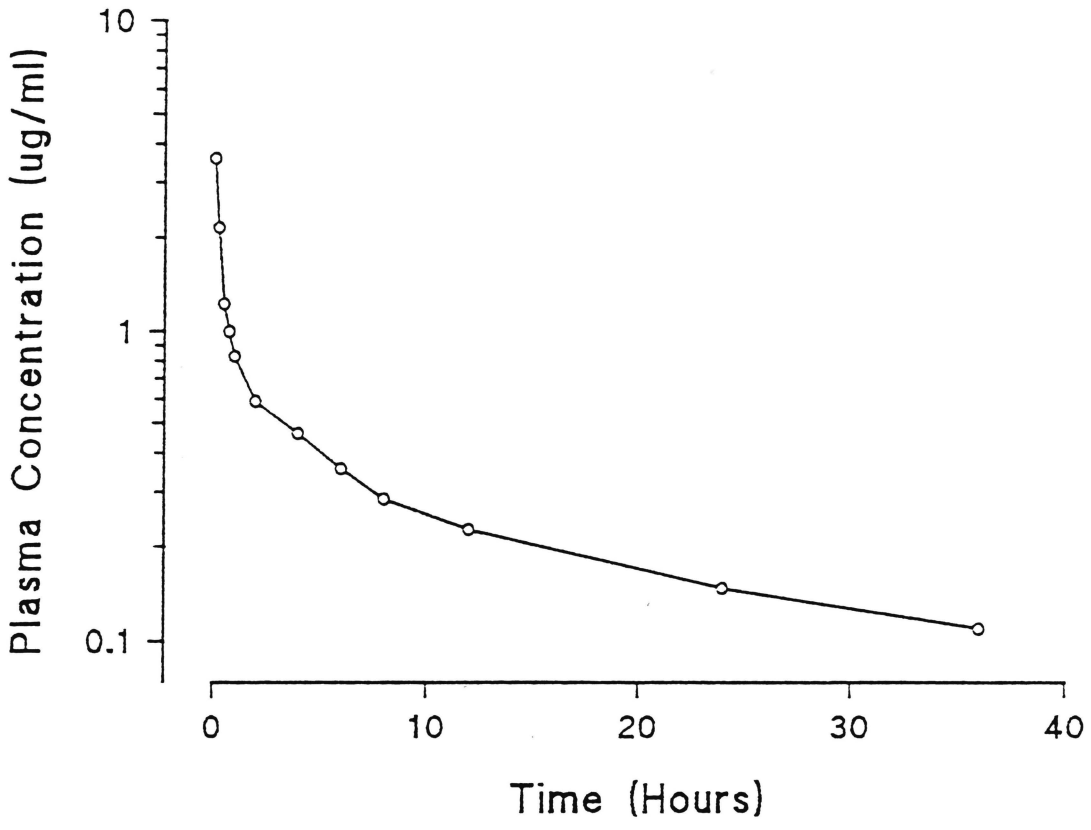


Figure 3

CHAPTER III

DETERMINATION OF GLG-V-13, A NOVEL ANTIARRHYTHMIC AGENT, IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Abstract

A sensitive reversed-phase HPLC technique with UV detection has been developed to determine the concentration of GLG-V-13 {3-[4-(1H-imidazol-1-yl)benzoyl]-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane dihydroperchlorate} (I), a novel combined class Ib and class III antiarrhythmic agent, in dog plasma and urine. Alkalinized plasma and urine samples were extracted with chloroform, and the extracts were reconstituted in methanol. An aliquot was injected on to a Waters HPLC system with a 250 x 4.6 mm Ultramex 5 C_6 analytical column (5 μm) and 30 x 4.6 mm Ultramex 5 C_6 guard column (5 μm). The elute was detected by a UV detector at 256 nm. Acetonitrile-methanol-37.5 m*M* phosphate buffer, pH 6.8 (27:27:46 v/v) containing 3.6 m*M* triethylamine was used as the mobile phase. The average extraction recovery was 89% from plasma and 93% from urine. Good linearity ($r>0.999$) was observed throughout the range of 8-8000 ng/ml in plasma and in urine with the quantitation limit of 8 ng/ml. Intra- and inter-assay variabilities were less than 4%. HPLC analysis of plasma and urine samples from a dog treated with I demonstrated that the method was accurate and reproducible. Preliminary

pharmacokinetic results showed that the plasma concentration-time curves fitted a two compartment open model with slow elimination ($t_{1/2\beta}$ 3.0827 h^{-1}), wide distribution (V_c 2.389 L/kg and $V_{d(ss)}$ 3.6808 L/h·kg), and long mean residual time (MRT 4.7632 h), respectively. It seems that there is a difference in disposition of this compound in pathological dogs compared to a normal one.

Introduction

3,7-Diheterabicyclo[3.3.1]nonane (DHBCN) derivatives have been found to possess potential antiarrhythmic properties.[1-5] Several DHBCN derivatives exhibited antiarrhythmic activity in animal models and therefore are viable candidates for the treatment of life-threatening disorders in humans who experience sudden heart attacks or major infarctions of the heart.[6-8] GLG-V-13, 3-[4-(1H-imidazol-1-yl)benzoyl]-7-isopropyl-3,7-diazabicyclo[3.3.1] nonane dihydroperchlorate (I) (Figure 1), has been demonstrated to increase the ventricle effective refractory period, to prolong QT duration (increase atria Hispurkinje and Hispurkinje-ventricle interval), and to prevent sustained ventricular tachycardia induced by programmed electrical stimulation of infarcted dog heart.[9-10] Compound I was found to have combined class Ib and class III antiarrhythmic activity without hemodynamic depressant effect.[9-10]

The longer duration of pharmacological effects, low proarrhythmic activity, apparent lack of cardiodepressant effects, and combined class Ib and class III antiarrhythmic actions of I make this compound a very promising candidate as an antiarrhythmic agent. Thus, there is merit in characterizing the pharmacokinetics of I in animals. To date, there are no analytical methods currently available for analyzing compound I in biological fluids. We now describe a rapid, selective, and sensitive HPLC technique for the determination of this compound in biological fluids, including plasma and urine. Using this method, preliminary pharmacokinetic profiles of I in dogs were characterized.

Materials and Methods

Chemicals

All the reagents used in this study were HPLC grade, and deionized, distilled water was used throughout the work [Milli-Q™ Water system, Millipore Corp., Marlborough, MA]. Acetonitrile, methanol, chloroform, and potassium phosphate monobasic were obtained from Fisher Chemicals (Fair Lawn, NJ), and triethylamine was purchased from Pierce Chemical Co. (Rockford, IL). Compound I and SAZ-VII-22 {3-(4-chlorobenzoyl)-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane, II}, as an internal standard (I.S.), were synthesized by established methods.[11-12]

Standard Solution and Internal Standard

A stock solution of compound I was prepared in methanol at a concentration of 0.4 mg/ml. This stock solution was then diluted further to yield appropriate working solutions for the preparation of the calibration standards.

A stock solution of the I.S. was prepared in methanol (10 μg/ml) for addition to plasma and urine samples.

Extraction Procedures

For the determination of I, II was used as the I.S. To 250 μl of dog plasma or urine was added 25μl of 10 μg/ml of the I.S.. After alkalinization with 100 μl of 5 *M* NaOH, five ml of chloroform was added, and the mixture was mixed for 3 min. Following centrifugation (1000 x g 10 min), the organic phase was transferred into a clean test tube. The supernatant was reextracted with 1 ml of chloroform. The

combined chloroform extracts were evaporated to dryness under a stream of N_2. The residue was reconstituted in 50 μl of methanol, and 35 μl of this solution was injected for HPLC analysis.

Apparatus and Chromatographic Conditions

The HPLC system consisted of a Waters 501 HPLC pump, Waters U6K universal liquid chromatography injector with a 2 ml injection loop, a Model 484 Tunable Absorbance Detector controlled by a Baseline 810 Chromatography Work Station with a NEC Powermate Sx plus a computer, and an NEC Pinwriter P5200 (Millipore, Milford, MA).

The analytical column used was a 250 x 4.6 mm Ultramex 5 C_6 (5 μm) and the guard column was a 30 x 4.6 mm Ultramex 5 C_6 (5 μm). Both columns were purchased from Phenomenex (Torrance, CA).

The mobile phase was acetonitrile-methanol-37.5 m*M* phosphate buffer, pH 6.8-triethylamine (27:27:46 v/v) containing 3.6 m*M* triethylamine. The mobile phase was filtered through a 0.5 μm Millipore filter and degassed before use. The column was eluted under isocratic conditions utilizing a flow rate of 1.2 ml/min at ambient temperature. The detection wavelength was 256 nm.

Extraction Recovery

The samples (n = 5) were prepared to give final concentrations of 100 ng/ml and 4000 ng/ml of compound I and 3 μg/ml of the I.S. in plasma and in urine, respectively. Using the extraction procedure described previously, the samples were extracted in the absence of the I.S. The organic layer was evaporated, and the

residues were reconstituted in methanol. The ratio of the peak area of I extracted over that of unextracted equivalent concentrations of agent under identical chromatographic conditions was calculated as extraction recovery.

Calibration Curves

A calibration curve was generated to confirm the linear relationship between the peak-area ratio and the concentration of the agent in the samples. Twenty five μl of a suitable standard solution of compound I and 25 μl of the I.S. working solution were added to drug-free plasma and urine to give compound I concentrations at 8, 40, 80, 200, 400, 800, 2000, 4000, and 8000 ng/ml. Plasma and urine samples with known concentrations of I and the I.S. were extracted as previously described, and standard curves were generated by plotting peak-area ratios (agent/I.S.) against drug concentrations tested. Each standard curve was replicated five times. Linear regression analysis of standard curve was performed using the computer program PHARM/PCS. [13] The concentrations of I in unknown plasma and urine samples were calculated by interpolating the peak-height ratios with the calibration curve.

Intra- and Inter-assay Accuracy and Precision

To evaluate the intra-assay accuracy and precision, I and the I.S. were added to drug-free plasma and urine samples (n = 6) at concentrations of 80 and 4000 ng/ml. These standard samples were prepared and stored at -20 °C, and analyzed with the unknown samples. The concentrations were calculated using a standard curve. The percentage of the mean concentration determined over the mean concentration added was taken as the accuracy of the method, and the coefficient

of variance was used as an index of precision. The inter-assay accuracy and precision were determined similarly over 6 consecutive days. Precision was estimated by determing the inter-assay coefficient of variations (C.V.).

Stability of Compound I

Compound I was added to free-drug dog plasma and urine to a final concentration of 100 and 4000 ng/ml, respectively. An aliquot of plasma or urine was extracted immediately as described above for the determination of compound I, while other aliquots of plasma or urine were stored frozen at -20 °C or exposure to light at room temperature (25±2 °C). Each month, an aliquot of frozen plasma and urine sample was thawed, extracted and analyzed as described above to evaluate the stability. For the determination of stability of I exposed to light at room temperature, a one day experimental duration was performed (one sample/two hour interval).

Drug Administration and Sampling

One experiment was carried out 24-96 hours after the two-stage ligation of the left anterior descending coronary artery in an anesthetized beagle dog. Programmed premature stimuli or rapid intermittent 3-beat-bursts (240-420/min) were delivered to the right ventricle to induce sustained monomorphic ventricular tachycardia. After administration of 3 mg/kg of compound I via an i.v. bolus injection, blood samples (± 5 ml) were collected via vein puncture at 5, 10, 15, 30, 45, 60, 75, 90 and 120 min. Another experiment was carried out on a normal unanesthetized beagle dog with an intravenous bolus injection of 6 mg/kg of I with blood samples (± 5 ml) being collected up to 12 hr. The samples were heparinized and centrifuged at 20000 x g

for 10 min. The plasma fractions were stored at -20 °C until analyzed. Urine was collected by catheter and stored at -20 °C until analyzed.

Results and Discussion

Extraction Efficiency

Alkalinization of plasma and urine samples increased extraction efficiency with the pH value for the solution at 12 showing the best extraction recovery. Most of compound I was in a nonionized state at pH value of 12. The use of chloroform to precipitate proteins and to extract compound I directly from plasma offered marked advantage since fewer peaks were found for contaminants. Using trichloroacetic acid (TCA) to precipitate proteins decreased the absolute recovery. This may be due to decomposition of I. This situation was similar to that of some other DHBCN derivatives.[14-16] Anticoagulants, such as EDTA and heparin, did not affect the extraction recovery. Extraction recoveries of compound I were 87.5-91.2% from plasma and 92.2-93.6% from urine, respectively (Table 1). The recoveries of the I.S. were 85% from plasma and 95% from urine.

Chromatographic Separation

Several combinations of acetonitrile, methanol, buffer (with different pH) and triethylamine were evaluated as possible mobile phases. It was determined that the combination described herein was found to be the most suitable for separating I. Under the described chromatographic conditions, a good separation of compound I and its I.S. was achieved. The retention times were 11.40±0.44 and 19.07±0.73 min,

respectively. At the retention time, the compound I and its I.S. were eluted without an interference peak from the blank plasma and urine (Figures 2 and 3). Varying proportions of triethylamine in the mobile phase changed both retention time and sharpness of the peak for compound I. The pH of the mobile phase is a very important factor influencing the elution of I. If the pH of the mobile phase was below 6.8, the retention time for I was short. There was a concomitant decrease in sensitivity. Neither acetonitrile nor methanol alone was suitable as the strong solvent. These observations were very similar to that with other DHBCN derivatives. [14-16] Therefore, varying the ratio of methanol-acetonitrile-triethylamine-phosphate buffer appears to be the best combination for separating and analyzing DHBCN derivatives in biological fluids. For example, methanol-acetonitrile-phosphate buffer-triethylamine (50:50:75:0.1) was used to analyze BRB-I-28[14] and methanol-acetonitrile-phosphate buffer (28.5:28.5:43 v/v) containing 4 m*M* triethylamine was used for analyze SAZ-VII-22 and SAZ-VII-23.[15, 16]

Assay Validation

Linearity. Five consecutive standard curves for pure I analyzed on separate days demonstrated a good linear relationship between concentration and peak area. The standard curves obtained from extraction of both dog plasma and urine containing known amounts of I were linear over the concentration ranges tested (8-8000 ng/ml). The range of coefficient of variations was between 1-17%. The calibration curves were found to be linear and could be described by the regression equations: $Y = -0.01492 + 0.5127X$ ($r = 0.9996$) for plasma and $Y = -0.04057 + 0.6557X$ ($r = 0.9995$) for urine, respectively, in which Y was the agent recovered in

μg/ml, and X was peak area ratio (agent/internal standard). The limits of quantitation of I were 8 ng/ml in plasma and in urine, respectively. This sensitivity has proved useful in the analysis of pharmacokinetic data of dog plasma and urine after administration of compound I.

Precision and Accuracy. The results obtained indicate that intra- and inter-assay coefficient of variance (C.V.) in plasma and urine were less than 4%. The accuracy of this method was 94-99% (Table 1). These results suggest that the procedures described as above are satisfactory with respect to both accuracy and precision.

Stability of Compound I

It was found that the free amine of compound I turned yellow when exposed to light for 2-3 hr, while compound I (disalt) was not sensitive to light. This experimental observation showed that I was very stable in dog plasma and urine at -20 °C for at least 6 months and was also stable when exposed to light at room temperature (25±2 °C) for one day. Possibly compound I may be present as the disalt or monosalt in the biological fluid.

Preliminary Pharmacokinetic Studies

The plasma and urine samples were extracted and analyzed as previously described. Representative HPLC profiles of the plasma and urine samples of a dog given I (6 mg/kg) intravenously are shown in Figure 2 and 3.

The plasma concentration-time profiles of I in dogs given intravenous dose of 3 and 6 mg/kg are shown in Figure 4. Data fitting and pharmacokinetic parameters

calculations were carried out using the Boomer program.[17] The plasma concentration change can be best described as a two compartment model with the equation:

$$C = A_1 e^{-\alpha t} + A_2 e^{-\beta t}.$$

The calculated pharmacokinetic parameters after iv bolus injection of 6 mg/kg is shown in Table 3. The results suggest that compound I has a very low elimination rate with $t_{1/2\beta}$ 3.0827 h^{-1} and longer mean residual time (MRT 4.7632 h), which is consistent with the duration of pharmacological effects. It was interesting that the disposition of I was different in the beagle dog under normal and pathological conditions. In a pathological dog, the plasma concentration of I during 45-60 min was higher than the plasma concentration during 20-30 min. This may be due to the enterohepatic circulation. The exact mechanism is unknown.

The results showed that the HPLC method described above has a lower quantitation limit of 8 ng/ml using a 250 μl sample. As shown in this report, this method is suitable for pharmacokinetic studies of this novel antiarrhythmic agent. Studies of the pharmacokinetic and metabolite(s) profiles of I in animals are in progress.

References

1. R. Jeyaraman, S. Avila, Chemical Review., 81: 149-174 (1981).

2. J. Hartenstein and B. Wager, "Antiarrhythmic agents," in Cardiovascular Drugs, J. A. Bristol ed., John Wiley & Sons, New York, 1986, pp. 305-415.

3. B. R. Bailey, K. D. Berlin, E. M. Holt, B. J. Scherlag, R. Lazzara, J. Brachmann, D. van der Helm, D. R. Powell, N. S. Pantaleo and P. C. Ruenitz, J. Med. Chem., 27: 758-767 (1984).

4. M. D. Thompson, G. S. Smith, K. D. Berlin, E. M. Holt, B. J. Scherlag, D. van der Helm, S. W. Muchmore, K. A. Fidelis, J. Med. Chem., 30: 780-788 (1987).

5. G. S Smith, M. D. Thompson, K. D. Berlin, M. E. Holt, B. J. Scherlag, E. Patterson, R. Lazzara, Eur. J. Med. Chem., 25: 1-8 (1990).

6. S. A. Zisman, Ph.D. Dissertation. Oklahoma State University. 1989.

7. G. L. Garrison, Ph.D. Dissertation. Oklahoma State University. 1993.

8. B. Oexle, J. Weinich, H. Antoni, J. Moll. Coll. Cardiol., 19 (Supplement III): 194 (Abstract) (1987)

9. T. Fazekas, P. Mabo, K. D. Berlin, B. J. Scherlag, R. Lazzara, The Physiologist., 35: 221 (Abstract) (1992).

10. T. Fazekas, B. J. Scherlag, P. Mabo, K. D. Berlin, G. L. Garrison, C. L. Chen, S. Sangiah, E. Patterson, R. Lazzara, Acta Physiologica Hungarica., 81: 289-299 (1993).

11. S. T. Zisman, K. D. Berlin, B. J. Scherlag, Org. Prep. Proc. Intern., 22: 255-264 (1990).

12. K. D. Berlin, G. L. Garrison, S. Sangiah, C. L. Chen, B. J. Scherlag, R. Lazzara, E. Patterson, Unpublished data, 1994.

13. R. J. Tallarida, R. B. Murray, Manual of Pharmacologic Calculations with Computer Programs, Springer-Verlag, New York, 1986.

14. C. L. Chen, B. A. Lesseley, C. R. Clarke, J. D. Roder, S. Sangiah, K. D. Berlin, G. L. Garrison, B. J. Scherlag, R. Lazzara, E. Patterson, J. Chromatogr. 583: 274-279 (1992).

15. C. L. Chen, J. D. Roder, S. Sangiah, H. Chen, K. D. Berlin, G. L. Garrison, B. J. Scherlag, R. Lazzara, E. Patterson, Anal. Lett., 26: 1125-1135 (1993).

16. C. L. Chen, J. D. Roder, S. Sangiah, H. Chen, K. D. Berlin, G. L. Garrison, B. J. Scherlag, R. Lazzara, E. Patterson, Analytical Sciences, 9: 429-431, 1993.

17. D. W. A. Bourne, Computer Methods and Programs in Biomedicine 29: 191-195 (1989).

TABLE 1

RECOVERY OF COMPOUND I ADDED TO DOG PLASMA AND URINE
(MEAN±SD, N = 5)

	Added (ng/ml)	Found (ng/ml)	Recovery (%)
Plasma			
	100	87.3 ± 4.5	87.3 ± 4.5
	4000	36480 ± 1320	91.2 ± 3.6
Urine			
	100	92.2 ± 5.3	92.2 ± 5.3
	4000	37420 ± 1800	93.6 ± 4.5

TABLE 2

INTRA-ASSAY (WITHIN-DAY) AND INTER-ASSAY (BETWEEN-DAY) PRECISION AND ACCURACY OF THE DETERMINATION OF COMPOUND I IN DOG PLASMA AND URINE

Concentration (ng/ml)		Accuracy (%)	C.V. (%)
Added	Found		
Plasma			
Intra-assay (n = 6)			
80	76.5	95.6	1.65
4000	3728	93.2	2.26
Inter-assay (n = 6)			
80	79.2	96.8	3.00
4000	3810	95.2	2.29
Urine			
Intra-assay (n = 6)			
80	76.0	95.0	3.14
4000	3780	94.5	1.52
Inter-assay (n = 6)			
80	77.6	97.0	3.53
4000	3960	99.0	1.38

* C.V. = coefficient of variance

TABLE 3

PHARMACOKINETIC PARAMETERS FOLLOWING I.V. BOLUS
6 MG/KG OF I TO ONE DOG

Parameters	Value
i.v. (6 mg/kg)	(n = 1)
A1 (mg/L)	0.844
A2 (mg/L)	1.669
α (h^{-1})	6.754
ß (h^{-1})	0.2248
K_{10} (h^{-1})	0.3326
K_{12} (h^{-1})	2.086
K_{21} (h^{-1})	4.575
$t_{1/2\alpha}$ (h)[a]	0.1026
$t_{1/2ß}$ (h)[a]	3.083
V_c (L/kg)[a]	2.389
$V_{d(area)}$ (L/kg)[a]	3.437
$V_{d(ss)}$ (L/kg)[a]	3.681
Cl_B (ml/h/kg)[a]	0.7727
AUC_{iv} (mg·h/L)	7.765
$AUMC_{iv}$ (mg/L)	36.984
MRT_{iv} (h)[a]	4.763

Abbreviations: K_{10}-first-order elimination rate constant; K_{12}, K_{21} are the first-order rate constants describing distribution between central (plasma) and peripheral compartment (tissues); $t_{1/2\alpha}$ is distribution half-life after iv; $t_{1/2ß}$ is elimination half-life after iv; V_c = volume of the central compartment; $V_{d(area)}$ is apparent volume distribution calculated using AUC; Vd(ss) is apparent volume of distribution at stead state; Cl_B is body clearance of the drug.

Figure Legends

Figure 1. Structures of GLG-V-13 (A) and the internal standard (B)

Figure 2. Representative chromatograms from (a) blank plasma and (b) plasma sample 8 hr after i.v. dose of 6 mg/kg of I. See experimental for chromatographic conditions.
Peaks: 1 = I, 2 = I.S.
The estimated concentration of I was 0.27 μg/ml.

Figure 3. Representative chromatograms from (a) blank urine and (b) urine sample 1 hr after i.v. dose of 6 mg/kg of I (b). See experimental for chromatographic conditions.
Peaks: 1 = I, 2 = I.S.
The estimated concentration of I was 5.1 μg/ml.

Figure 4. Plasma concentration profiles after i.v. dose of 6 and 3 mg/kg of compound I in a normal dog and in a pathological dog, respectively.

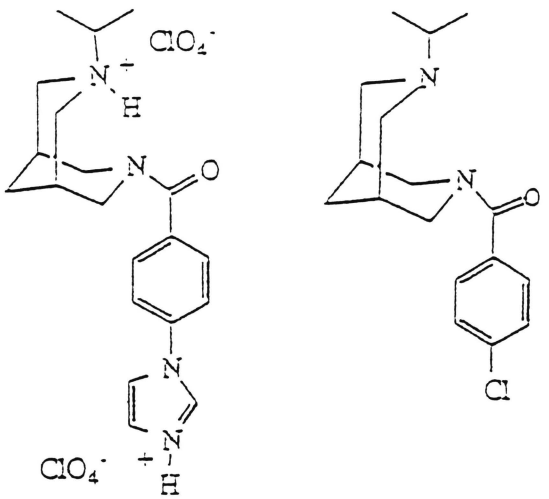


Figure 1

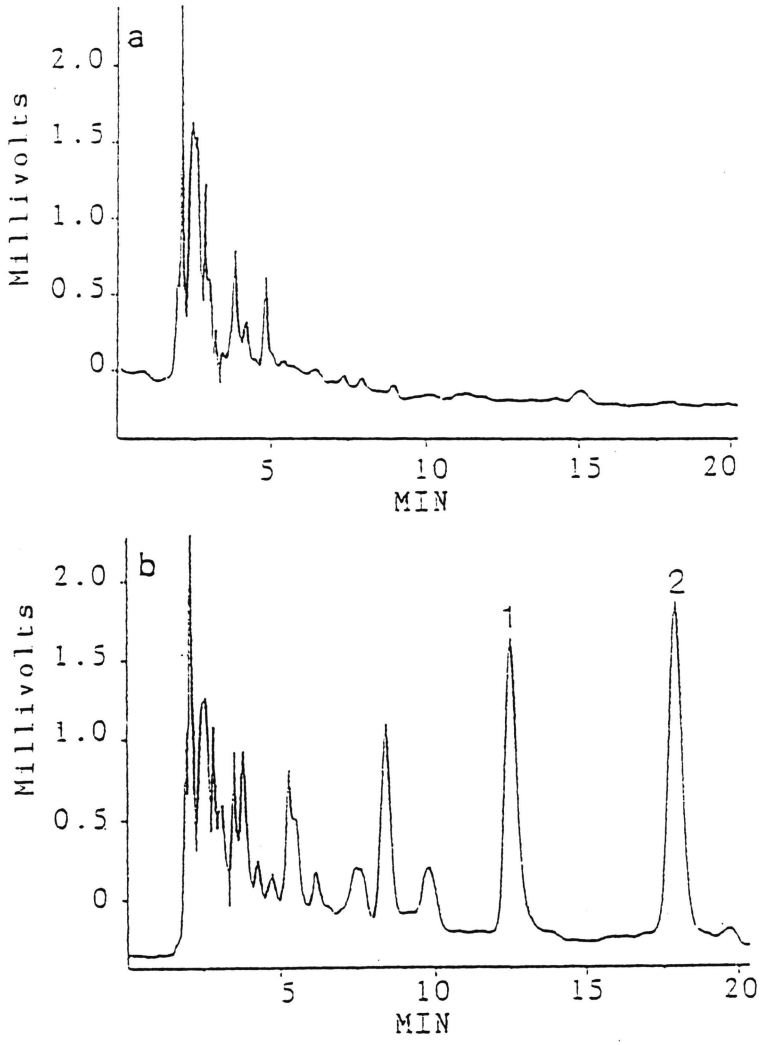

a
Millivolts
2.0
1.5
1.0
0.5
0
5
10
15
20
MIN
b
Millivolts
2.0
1.5
1.0
0.5
0
1
2
5
10
15
20
MIN

Figure 2

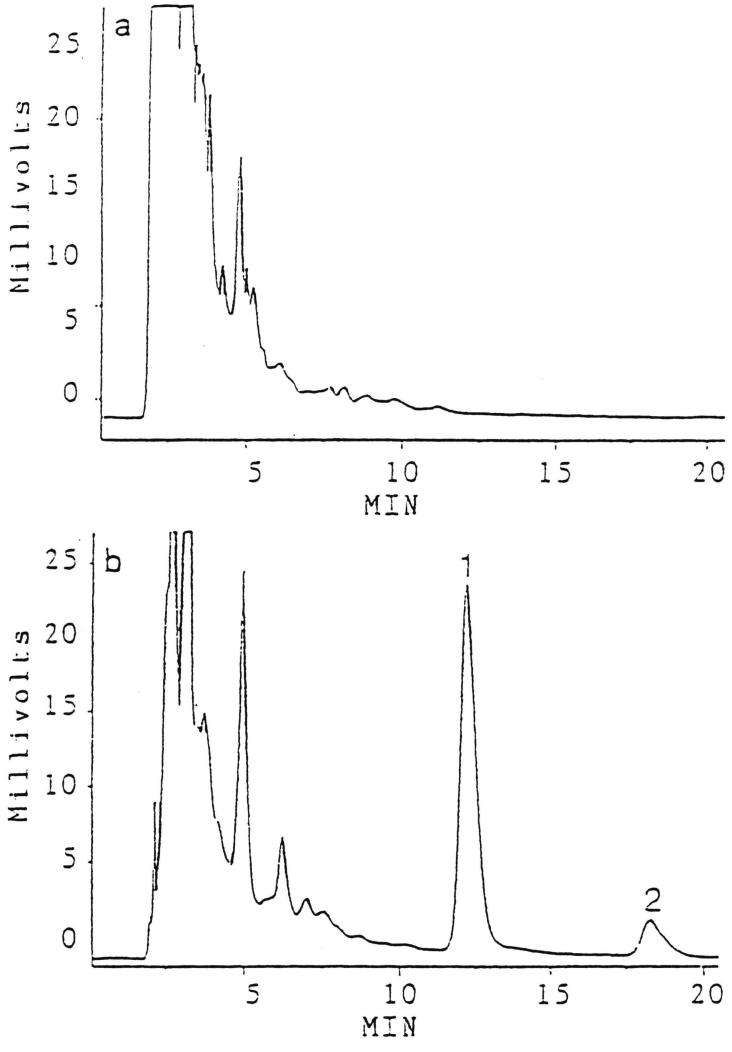


Figure 3

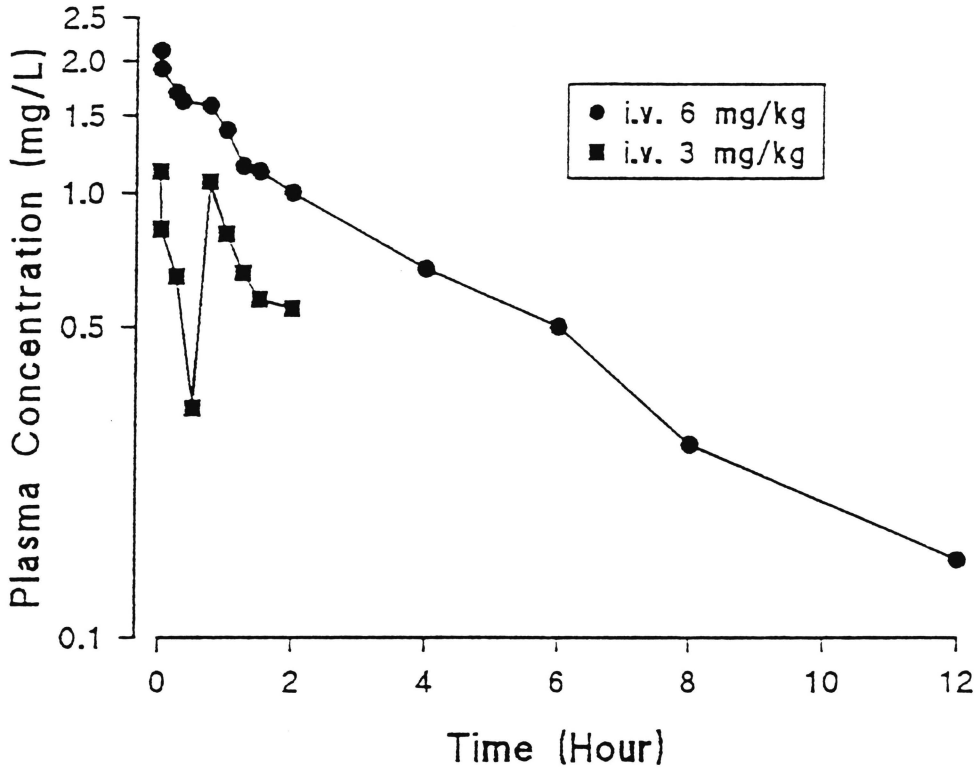

Plasma Concentration (mg/L)
2.5
2.0
1.5
1.0
0.5
0.1
0
2
4
6
8
10
12
Time (Hour)
i.v. 6 mg/kg
i.v. 3 mg/kg

FIgure 4

CHAPTER IV

SIMULTANEOUS DETERMINATION OF A NOVEL ANTIARRHYTHMIC AGENT, 7-BENZYL-3-THIA-7-AZABICYCLO[3.3.1]NONANE, AND ITS SULFOXIDATION METABOLITE IN PLASMA AND URINE BY HPLC

Abstract

A HPLC method was developed for the simultaneous determination of the concentrations of 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane (I) as the hydrochloride and the corresponding sulfoxide (II), the major metabolite, in dog plasma and urine. Plasma and urine samples were alkalinized and extracted with chloroform. An aliquot was injected on to a HPLC system with a C6 reversed-phase column and an UV detector. Acetonitrile-methanol-37.5 m*M* phosphate buffer, pH 6.8 (28:28:44 v/v) containing 4.0 m*M* triethylamine, was used as a mobile phase. The compounds I and II were detected at 261 nm. The extraction recovery for I and II was 85% and 94% from plasma and 89% and 91% from urine, respectively. Good linearity ($r>0.994$) was observed throughout the range of 0.1-10.0 μg/ml for I and 0.04-10 μg/ml for II in plasma and in urine. Intra- and inter-assay variabilities were less than 8%. The accuracy of this method was > 95% for both compounds, and the limits of quantitation were 0.08 μg/ml for I and 0.03 μg/ml for II in plasma, and in urine, respectively. This method was applied to determine plasma and urine concentrations of I and II simultaneously in a dog treated with I.

Introduction

7-Benzyl-3-thia-7-azabicyclo[3.3.1]nonane (I) (Figure 1) has been demonstrated to have effective antiarrhythmic properties[1-4] and less proarrhythmic activity than those of lidocaine in dog models.[5] This drug could be classified as class Ib antiarrhythmic drug.[6,7] The significant elevation of systemic arterial blood pressure at the effective antiarrhythmic dose of I during sinus rhythm in dogs[2,4,5] could possibly be due to its inhibitory effects on myocardial Na^+,K^+-ATPase and Mg^{2+}-ATPase activities and its positive inotropic effects on atrial and papillary muscles.[8] Reduced proarrhythmic effects and little cardiac depressant actions make this compound a very promising candidate as an antiarrhythmic agent.

A HPLC method for determining the concentrations of 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane·$HClO_4$ in biological fluids has been developed.[9] The pharmacokinetics and plasma protein binding of I·$HClO_4$ in rats[10] and of I·HCl in dogs[11] have been characterized. In dogs, parent compound I·HCl was found to be extensively metabolized to form a major metabolite, 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane-3-oxide (II)[12] (Figure 1). However, no analytical methods are currently available to analyze II in biological fluids. This paper describes a reversed-phase HPLC method for simultaneous determination of I and its major metabolite II in dog plasma and urine.

Materials and Methods

Chemicals

The compounds I and II were synthesized by established methods.[1,13] All

solvents used in this study were HPLC grade. All water used was purified through a Milli-Q™ water system (Millipore Corp., Marlborough, MA). Acetonitrile, methanol, chloroform, potassium phosphate monobasic (Fisher Chemicals, Fair Lawn, NJ), and triethylamine (Pierce Chemical Co. Rockford, IL) were used as purchased in this study. ß-Glucuronidase, crude solution (G 0876) from *Helix pomatia*, was purchased from Sigma Co. (St. Louis, MO). 3-Benzoyl-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane (III) was synthesized via a type of Mannich reaction starting from *N*-benzyl-4-piperidinone[14] and used as internal standard (I.S.).

Stock Solutions and Standards

Methanol stock solutions of I, II (1 mg/ml) and the I.S. (10 μg/ml) were stable for at least 2 months at 2-4 °C. Known amounts of the solution were added to 0.25 ml of drug-free plasma and 0.50 ml of drug-free urine to obtain calibration curves in the range of 0.1-10 μg/ml for I and 0.04-10 μg/ml for II. Several standards were prepared for each curve.

HPLC Analysis

The HPLC system consisted of a Waters 501 HPLC pump, a Waters U6K universal liquid chromatography injector with a 2 ml injection loop, a Model 484 Tunable Absorbance Detector controlled by a Baseline 810 Chromatography Work Station with a NEC Powermate Sx plus a computer, and a NEC Pinwriter P5200 (Millipore, Milford, MA). A 250 x 4.6 mm Ultramex 5 C_6 (5 μm) and 30 x 4.6 mm Ultramex 5 C_6 guard column (5 μm) were purchased from Phenomenex (Torrance, CA). The mobile phase was acetonitrile-methanol-37.5 m*M* phosphate buffer, pH

6.8 (28:28:44 v/v) containing 4.0 m*M* triethylamine. The mobile phase was filtered through a 0.5 um Millipore filter and degassed before use. The column was eluted under isocratic conditions utilizing a flow rate of 1.2 ml/min at ambient temperature. The detection wavelength was 261 nm for I and II.

Animal Study

An adult, male mongrel dog (10.5 kg) was pre-conditioned for 7 days and was housed in a controlled environment (12-hour light/12-hour dark photoperiod, 22±1 °C, 60±10% relative humidity). The dog was allowed free access to food and water. Twenty-four hours before the experiments, food was withdrawn. The dog had free access to water before and during the experiments. The dog had polyethylene cannulas implanted in the right and left cephalic veins for collection of blood samples. An experiment was initiated between 0900 and 1100 h. Compound I was dissolved in water immediately before each experiment, and a dose of 20 mg/kg was administrated orally. Blood samples (ca. 5 ml) were collected via the cephalic vein catheter at 0, 5, 10, 30, and 45 min and at 1, 2, 4, 6, 8, and 12 hr after administration of I. After each sampling, lost blood was replaced with an equal volume of sterile heparinized saline. Blood samples were heparinized, and centrifuged at 2000 x g for 10 min. Urine samples were collected from the dogs housed in a stainless steel metabolism cage at designated times, 0-2, 2-24, 24-48, and 48-72 hr, via a urinary catheter. Both plasma and urine samples were stored at -20 °C. Aliquots of 0.25 ml of plasma and of 0.5 ml urine were used for HPLC assay.

Extraction of I and II from Dog Plasma

Twenty five μl of internal standard III (10 μg/ml) were added to 250 μl of dog plasma. After alkalinization with 100 μl of 5 *M* NaOH, 5 ml of chloroform was added, and the mixture was mixed for 3 min. Following centrifugation (1000 x g 10 min), the organic phase was transferred into a clean test tube. The supernatant was reextracted with 1 ml of chloroform. The combined chloroform extracts were evaporated to dryness under a stream of N_2. The solid residue was reconstituted in 50 μl of methanol, and 35 μl of this solution was utilized for HPLC analysis.

Extraction of Total (Free + Conjugated) I and II from Dog Urine

A crude solution of ß-glucuronidase (100,000 units of ß-glucuronidase and 1000-5000 units of sulfatase per ml) was used to hydrolyze the glucuronidate and sulfate of I and II. For the determination of the total (free plus conjugated) concentration of I and II, aliquots of 0.5 ml of dog urine samples were subjected to enzymatic hydrolysis prior to extraction. The urine sample were adjusted to pH 5 with acetic acid, to which 0.1 ml of ß-glucuronidase crude solution was added, and then the solution was incubated at 37 °C for 24 hr. The hydrolysate solution was adjusted to pH ±12 with NaOH (2 *M*) and then extracted with chloroform to yield total I and II.

Extraction Recovery

The samples (n = 5) were prepared to give final concentrations of 0.2 and 4 μg/ml in plasma and urine, respectively. Using the extraction procedure cited above, the samples were extracted in the absence of III (I.S). The ratio of the peak area

of I and II extracted to that of unextracted equivalent concentrations of drugs under identical chromatographic conditions was calculated as extraction recovery.

Pharmacokinetic Analysis

Pharmacokinetic modelling and parameters were performed by PharmK program.[15]

Results and Discussion

Extraction Efficiency

The same procedures described previously for extraction of I from biological fluids[9] was used to extract simultaneously I and II from plasma and urine. Alkalinization of plasma and urine samples increases extraction efficiency. The use of chloroform to precipitate proteins and to extract compounds I and II directly from plasma offered great advantage in that fewer pollutant peaks were found. Anticoagulants, such as EDTA and heparin, do not affect the extraction recovery. Extraction recoveries of compound I were 79-92% from plasma and 78-95% from urine, while the data for compound II were 80-103% from plasma and 85-101% from urine, respectively (Table 1). The recoveries of I.S. were 85% from plasma and 90% from urine.

Chromatographic Separation

A modified chromatographic separation conditions[9] for I was used to separate I, II, and III (I.S). Minor changes in the combinations of acetonitrile, methanol,

buffer and triethylamine were evaluated as possible mobile phases. It was determined that the combination described in HPLC analysis was found to be the most suitable for separating I and II. Under the described chromatographic conditions (see HPLC analysis), a good separation of compound I, II and its internal standard was achieved. The retention times were 5.1±0.3 and 10.3±0.6 min for I and I.S., respectively. We were still unable to achieve a stable retention time for I,[9] but the retention time for I was determined to be 14-19 min. With these retention times the drug I, metabolite II, and the I.S. were eluted without any interference peaks originating in blank plasma and urine (Figure 2 and 3). The effects on separation of varying proportions of triethylamine and pH value in the mobile phase were similar to that in the analysis of I and other 3,7-diheterabicyclo[3.3.1]- nonane analogues. [9,16-18] The effect of these variables were not only on the retention time but also on the sharpness of the peaks for compounds I and II. Like other DHBCN (3,7-diheterabicyclo[3.3.1]nonane) analogues,[9,16-18] neither acetonitrile nor methanol alone was suitable as a strong solvent for the separation of I and II. It was found that minor changes in the combination of acetonitrile, methanol, triethylamine and buffer (pH 6.8) could provide a very good mobile phase for analyzing several DHBCN analogues. For example, acetonitrile-methanol-phosphate buffer (28.5:28.5:43 v/v) containing 4 m*M* triethylamine was used to analyze SAZ-VII-22 and SAZ-V-VII-23[16,17] and that (27:27:46 v/v) containing 3.6 m*M* triethylamine for GLG-V-13.[18]

Assay Validation

Linearity. Five consecutive standard curves for pure I and II analyzed on separate days demonstrated a good linear relationship between concentration and

peak area. The standard curves obtained from extraction of dog plasma and urine containing known amounts of I were linear ($r>0.996$) over the concentration ranges tested. The range of coefficient of variations was between 1-12%. The calibration curves were found to be linear (Table 1). The limits of quantitation of I and II were 80 ng/ml and 30 ng/ml for plasma and for urine, respectively. This sensitivity is adequate for use in the analysis of pharmacokinetics data of dog plasma and urine after administration of compound I or II.

Precision and Accuracy. The results obtained indicate that intra- and inter-assay coefficient of variance (C.V.) in plasma and urine was less than 8%. The accuracy of this method was 89-97%. These results suggested that the proposed procedure was satisfactory with respect to both accuracy and precision.

Applications to Dog Samples

The internal standard was added to the dog plasma and urine samples, and samples were extracted as previously described. Representative HPLC profiles of the plasma and urine samples of a dog given I (20 mg/kg) orally are shown in Figures 2 and 3.

The plasma concentration-time profiles of I and II in one dog given an oral dose of 20 mg/kg are shown in Figure 4 and are fitted to a one compartment open model. There were 3.22% and 15.6% of I and II, respectively, excreted into urine with respect to the oral dose. The area under the curve for II is 1.77 times of that of I, indicating that I undergoes extensive metabolism. A recent drug metabolism study[12] showed that I is present in the free state, while II is predominantly present in the form of glucuronide and/or sulfate conjugates in urine.

The results show that the HPLC method described above has a lower quantitation limit of 0.08 μg/ml for I and 0.03 μg/ml for II using a sample volume of 250 μl. As shown in this report, this method is suitable for simultaneous pharmacokinetic studies of this novel antiarrhythmic agent and its S-oxidation metabolite II. In addition, this method can also be used to isolate II and quantitate it from the biological samples containing only II.

References

1. B. R. Bailey, K. D. Berlin, E. M. Holt, B. J. Scherlag, R. Lazzara, J. Brachmann, D. van der Helm, D. R. Powell, N. S. Pantaleo and P. C. Ruenitz, J. Med. Chem., 27, 758-767 (1984).

2. B. J. Scherlag, E. Patterson, R. Lazzara, B. R. Bailey, M. D. Thompson and K. D. Berlin, J. Electrophysiol., 2, 461-477 (1988).

3. G. S. Smith, M. D. thompson, K. D. Berlin, E. M. Holt, B. J. Scherlag, E. Patterson and R. Lazzara, Eur. J. Med. Chem., 25, 1-8 (1990).

4. M. K. Pugsley, M. J. A. Walker, G. L. Garrison, P. G. Howard, R. Lazzara, E. Patterson, W. P. Penz, B. J. Scherlag and K. D. Berlin, Proc. West. Pharmacol. Soc. 35, 87-91 (1992).

5. a) T. Fazekas, B. J. Scherlag, P. Mabo, K. D. Berlin, G. L. Garrison, C. L. Chen, S. Sangiah, E. Patterson and R. Lazzara, Acta Physiologica Hungary., 81, 289-299 (1993). b) T. Fazekas, P. Mabo, K. D. Berlin, B. J. Scherlag, R. Lazzara, The Physiologist., 35, 221 (1992).

6. E. Patterson, B. J. Scherlag, K. D. Berlin and R. Lazzara, J. Pharmacol. Exp. Ther. 259, 558-565 (1991).

7. E. Patterson, B. J. Scherlag, K. D. Berlin and R. Lazzara, J. Cardiovasc. Pharmacol., 21, 637-646 (1993).

8. C. L. Chen, S. Sangiah, K. D. Berlin, G. L. Garrison, W. Dunn, Y. Nan, B. J. Scherlag, R. Lazzara and E. Patterson, Res. Commun. Chem. Pathol. Pharmacol., 78, 3-16 (1992).

9. C. L. Chen, B. A. Lesseley, C. R. Clarke, J. D. Roder, S. Sangiah, K. D. Berlin, G. L. Garrison, B. J. Scherlag, R. Lazzara and E. Patterson, J. Chromatogr. Biomed. Appl., 583, 274-279 (1992).

10. F. K. Alavi, C. R. Clarke, S. Sangiah, K. D. Berlin, S. A. Zisman, C. L. Chen, G. L. Garrison, B. J. Scherlag and R. Lazzara, Drug Invest., 3, 317-323 (1991).

11. C. L. Chen, S. Sangiah, J. D. Roder, H. Chen, K. D. Berlin, G. L. Garrison, B. J. Scherlag, R. Lazzara and E. Patterson, Drug Invest., 6, 237-243 (1993).

12. C. L. Chen, S. Sangiah, D. W. A. Bourn, J. D. Roder, H. Chen, K. D. Berlin, G. L. Garrison, K. M. Couch, B. J. Scherlag, R. Lazzara and E. Patterson, Drug Metab. Dispos., submitted 1994.

13. a) B. R. Bailey, Ph.D. Dissertation, Oklahoma State University, 1983; b) S.

A. Zisman, Ph.D. Dissertation, Oklahoma State University. 1989; c) G. L. Garrison, Ph.D. Dissertation, Oklahoma State University. 1993.

14. S. A. Zisman, K. D. Berlin, B. J. Scherlag, Organic Preparations and Procedures International., 22, 255-264 (1990).

15. D. R. Lu and F. Mao, J. Pharm. Sci., 82, 537-542 (1993).

16. C. L. Chen, S. Sangiah, J. D. Roder, H. Chen, K. D. Berlin, G. L. Garrison, B. J. Scherlag, R. Lazzara and E. Patterson, Analytical Sciences., 9, 429-431 (1993).

17. C. L. Chen, S. Sangiah, J. D. Roder, H. Chen, K. D. Berlin, G. L. Garrison, B. J. Scherlag, R. Lazzara and E. Patterson, Analytical Letters., 26, 1125-1135 (1993).

18. C. L. Chen, S. Sangiah, J. D. Roder, H. Chen, K. D. Berlin, G. L. Garrison, B. J. Scherlag and R. Lazzara, J. Liquid Chromatogr., 17, 913-927 (1994).

TABLE 1

LINEARITY AND RECOVERY OF COMPOUND I AND II ADDED TO PLASMA AND URINE

Samples	Range (μg/ml)	n	Linear Correlation[a]	r	CV[b] (%)	m[c]
I in plasma	0.1-10	35	Y= 0.05484+11.461X	0.995	1.2-10	85
I in urine	0.1-10	35	Y= 0.11655+5.5174X	0.994	3.5-9.7	89
II in plasma	0.04-10	35	Y=-0.19556+3.1716X	0.997	2.8-11	94
II in urine	0.04-10	35	Y=-0.10697+3.0815X	0.995	1.8-9.7	91

[a] Y was drug recovered in μg/ml, X was peak area ratio (drug/I.S.).
[b] CV = coefficient of variance
[c] m = mean recovery

Figure Legends

Figure 1. Chemical structure of I, II and III
I = 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane
II = 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane-3-oxide
III = 3-benzoyl-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane

Figure 2. Representative chromatograms from (a) blank plasma and (b) plasma sample 30 min after oral dose of 20 mg/kg of I to a dog.
See experimental for chromatographic conditions.
The estimated concentration of I and II was 1.2 μg/ml and 3.5 μg/ml, respectively. I.S. = Internal Standard

Figure 3. Representative chromatograms from (a) blank urine and (b) urine sample 1 hr after oral dose of 20 mg/kg of I to a dog.
See experimental for chromatographic conditions.
The estimated concentration (free + conjugated) of I and II was 3.1 μg/ml and 4.2 μg/ml, respectively. I.S. = Internal Standard

Figure 4. Plasma I and II concentration profile after oral dose of 20 mg/kg in one dog.

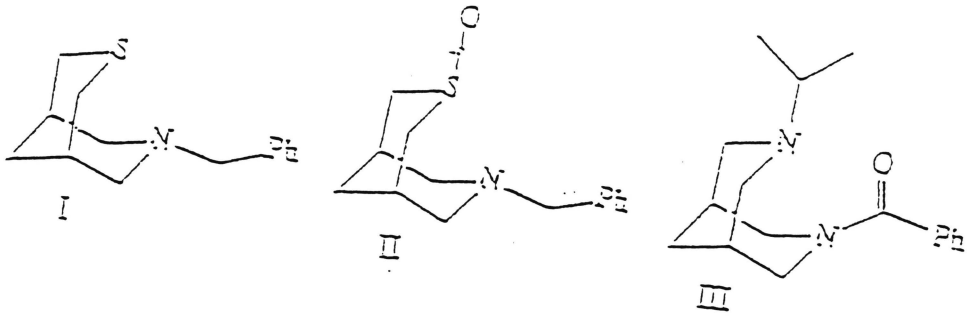


Figure 1

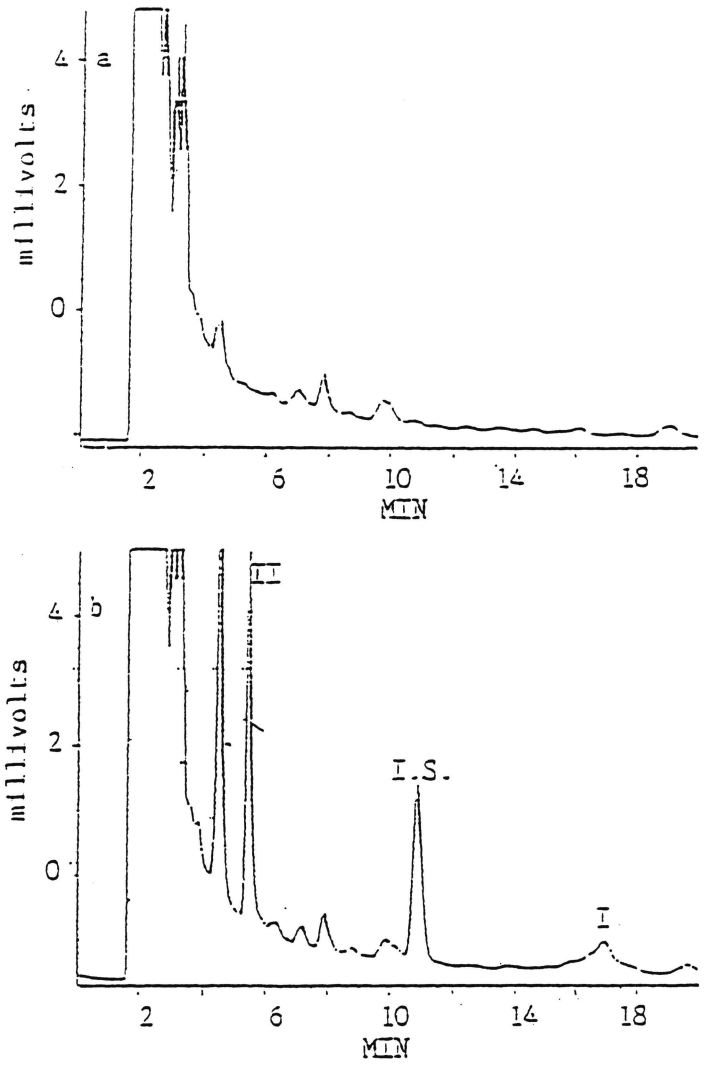

a
millivolts
4
2
0
2
6
10
14
18
MIN
b
millivolts
4
2
0
II
I.S.
I
2
6
10
14
18
MIN

Figure 2

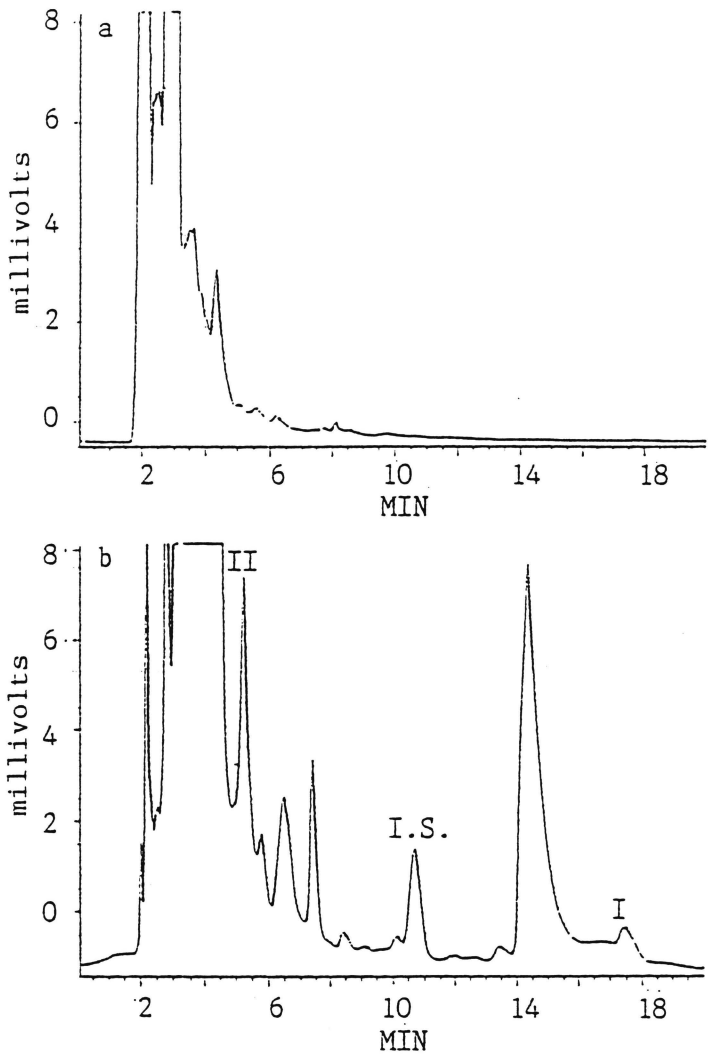


Figure 3

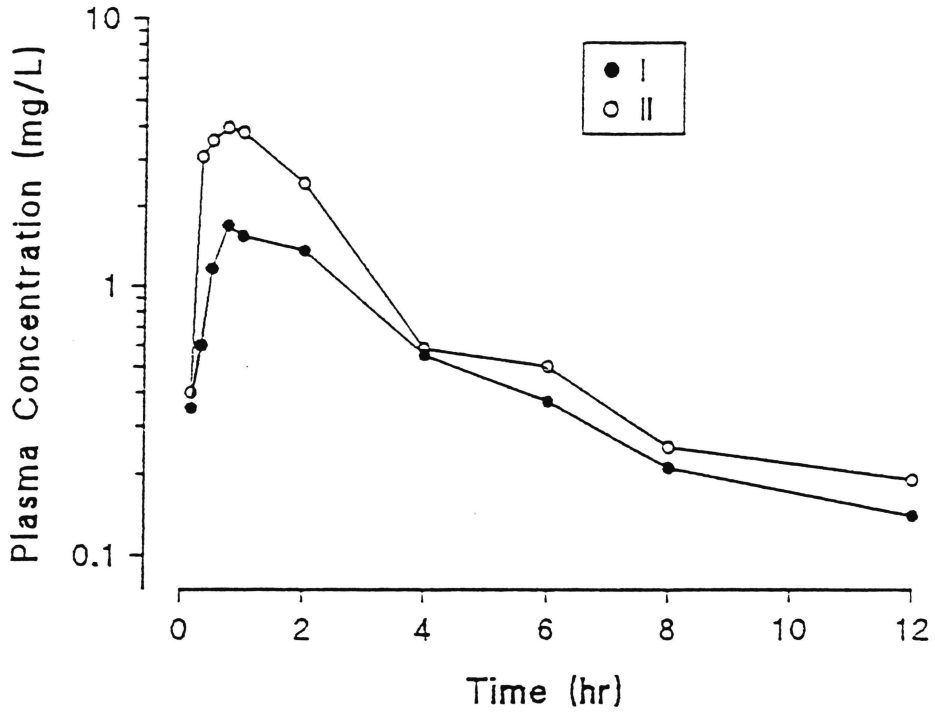


Figure 4

CHAPTER V

PHARMACOKINETICS AND PLASMA PROTEIN BINDING OF BRB-I-28, A NOVEL ANTIARRHYTHMIC AGENT, IN DOGS

Abstract

The pharmacokinetics and plasma protein binding of BRB-I-28, a novel antiarrhythmic agent, were investigated in dogs. The concentration of BRB-I-28 was determined by a reverse-phase high-performance liquid chromatographic assay. The plasma concentration-time profile of BRB-I-28, following an i.v. bolus dose of 10 mg/kg, can be adequately described by a two compartment open model. The distribution volume at steady state ($V_{d(ss)}$) ranged from 6.775 to 11.483 L/kg, while the total systemic clearance (Cl_B) ranged from 1.171 to 1.379 L/h/kg. The half-life of elimination ($t_{1/2ß}$) ranged from 2.472 to 6.182 h. Following i.v. dosing, approximately 1.85% of the parent compound was excreted in the urine (0-48 h). Changes in plasma concentrations, after oral administration of BRB-I-28 at 20 mg/kg, was best described by a 1-compartment open model. BRB-I-28 was rapidly absorbed (t_{max} of 1.22 h and C_{max} of 1.59 mg/L) with rapid elimination rate ($t_{1/2kel}$ ranged from 0.859 to 2.038 h^{-1}). Oral bioavailability was estimated to be 44.5%. Only 2.56% of the oral dose (20 mg/kg) was excreted via the urine (0-48 h). Protein binding was performed *in vitro* and *ex vivo* by equilibrium dialysis at 37 °C. *In vitro* binding of BRB-I-28 to plasma protein was 29.7±10.3% (from 4 to 16 mg/L). *ex vivo* binding

of BRB-I-28 to plasma protein was 24.0±8.7%. Extensive distribution of BRB-I-28 may due to its lower binding to plasma protein. Lower oral bioavailability and limited elimination of free parent BRB-I-28 in urine indicates that BRB-I-28 may undergo extensive metabolism which may be the reason for its shorter pharmacological effects. However, BRB-I-28 was found to have less proarrhythmic effects and no cardiodepressant actions, suggesting that this novel antiarrhythmic agent is worthy of further development.

Introduction

BRB-I-28 (7-benzyl-7-aza-3-thiabicyclo[3.3.1]nonane·HCl) (Figure 1) has demonstrated more effective antiarrhythmic properties (Bailey et al. 1984; Scherlag et al. 1988; Smith et al. 1990) and less proarrhythmic activity (Fazekas et al. 1993) than lidocaine and exhibits electrophysiological properties typical of class Ib antiarrhythmic drugs (Patterson et al. 1991; 1993). In addition, this antiarrhythmic agent was found to produce a significant elevation of systemic arterial blood pressure at the effective antiarrhythmic dose during sinus rhythm, in contrast to the hypotensive effect of lidocaine (Fazekas et al. 1993). The basis of some electrophysiological effects of antiarrhythmic properties of BRB-I-28 and its moderate hypertensive response could possibly be due to its inhibitory effects on myocardial Na^+,K^+-ATPase and Mg^{2+}-ATPase activities and its positive inotropic effects on atrial and papillary muscles (Chen et al. 1992).

The pharmacokinetic and tissue distribution profiles of BRB-I-28 in rats have been characterized using a radioisotope technique (Alavi et al., 1991). This method measured both the parent compound and its metabolite(s); therefore, the pharmacokinetic profiles of parent BRB-I-28 alone *in vivo* remain unknown. The purpose of these studies was to characterize disposition and protein binding of parent BRB-I-28 using a rapid, selective, and sensitive HPLC technique.

Materials and Methods

Chemicals

All the reagents used in this study were HPLC grade. Deionized water filtered

through a Milli-Q™ water system (Millipore Corp., Marlborough, MA) was used. Acetonitrile, methanol, chloroform, propanol and potassium phosphate monobasic (Fisher Chemicals, Fair Lawn, NJ) and triethylamine (Pierce Chemical Co. Rockford, IL) were used as obtained. BRB-I-28 was synthesized by the established method (Bailey et al. 1984). The SAZ-VII-23 (3-benzyl-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane·$HClO_4$) was synthesized via a Mannich reaction starting from 4-thianone (Zisman et al. 1990) and was used as an internal standard.

Animals and Sample Collection

Adult, male mongrel dogs were pre-conditioned for 7 days and were housed in a controlled environment (12-hour light/12-hour dark photoperiod, 22±1 °C, 60±10% relative humidity). The dogs were allowed free access to food and water. Twenty-four hours before the experiments, food was withdrawn. The dog had free access to water before and during the experiments. All animals had polyethylene cannulas implanted in the right and left cephalic veins (for drug administration and collection of blood samples). All experiments were initiated between 0900 and 1100h. BRB-I-28·hydrochloride was dissolved in water immediately before each experiment. Intravenous administration of BRB-I-28 (10 mg/kg) was done by bolus injection, and the cannulas were flushed immediately with 5 ml of sterile heparinized saline. Oral administration (20 mg/kg) was through gavage. After administration, blood samples (5 ml) were collected via one of the cephalic vein catheter at 0, 5, 10, 30, and 45 min, and at 1, 2, 4, 6, 8, and 12 hr after administration of BRB-I-28. After each sampling, the lost blood was replaced with an equal volume of sterile heparinized saline. Blood samples were heparinized, and centrifuged at 2000 x g for 10 min; plasma samples

were collected and stored at -20 °C. Urine samples were collected at designated times via a urinary catheter; after measuring the volume, the urine samples were stored at -20 °C. Aliquots of 0.25 ml of plasma and of 0.5 ml urine were used for HPLC assay.

Sample Extraction and HPLC Determination of BRB-I-28

BRB-I-28 concentrations in plasma, urine and buffer from equilibrium dialysis were determined using a previously reported reversed-phase high-performance liquid chromatographic method (Chen et al. 1992). To 250 μl of plasma samples or buffer was added 25 μl of 10 μg/ml of internal standard. After alkalinization with 100 μl of 5 *M* NaOH, five ml of chloroform was added, and the mixture was mixed for 3 min. Following centrifugation (1000 x g 10 min), the organic phase was transferred to a clean test tube. The supernatant was reextracted with 1 ml of chloroform. The combined chloroform extracts were evaporated to dryness under a stream of N_2. The residue was reconstituted in 50 μl of methanol, and 35 μl of this solution was injected for HPLC analysis. Extraction of BRB-I-28 from urine was similar to that from plasma, except one half ml of dog urine was used and diluted with 4 ml of water before extraction. The Waters HPLC system (Waters, Millipore Co., Milford, MA) was comprised of a pump (model 501), a tunable absorption detector (Waters 484) and chromatographic software (Maxima 820). A Ultramex 5 C_6 column (5 μm, 250 x 4.6 mm) and Ultramex 5 C_6 guard column (5 μm, 30 x 4.6 mm) purchased from Phenomenex (Torrance, CA) were used to resolve BRB-I-28 and SAZ-VII-23 (internal standard). The mobile phase was acetonitrile-methanol-37.5 m*M* phosphate buffer, pH6.8-triethylamine (50:50:75:0.1 v/v). The column was eluted under isocratic

conditions utilizing a flow rate of 1.2 ml/min at ambient temperature. The detection wavelength was 261 nm for BRB-I-28. Standard curves were prepared daily using peak area ratios of BRB-I-28 concentrations in plasma, urine and buffer to internal standard.

Pharmacokinetic Analysis

Data fitting and pharmacokinetic parameter calculations were carried out using the Boomer program (Bourne, et al. 1989). An appropriate pharmacokinetic model was chosen on the basis of lowest weighted squared residuals, lowest Akaike's information criterion (AIC) value, R-squared and correlation coefficiency (Akaike 1973; Boxenbaum et al. 1974; Yamaoka et al. 1978). The area under the curve (AUC) was calculated by the trapezoidal rule between first (0 hours) and last sampling times (Gibaldi et al. 1975). Systemic clearance (Cl_B) was determined by dividing the dose by the AUC. Mean residence time (MRT) was calculated by dividing the area under the first moment curve (AUMC) by AUC (Mayer et al. 1988). The volume of distribution at steady state (V_{dss}) was calculated as the product of Cls and MRT. The time (t_{max}) taken to achieve peak concentration (C_{max}) was calculated using differential calculus (Gibaldi et al. 1975). Bioavailability (F) was estimated by the following equation:

$$F\ (\%) = AUC_{po} \times Dose_{iv}/AUC_{iv} \times Dose_{po}$$

The results are presented as means and standard deviations, except for pharmacokinetic parameters which are not normally distributed are presented as medians (ranges).

Binding to Plasma Protein *In Vitro* and *Ex Vivo*

Plasma protein binding was determined at 37 °C by an equilibrium dialysis technique using a 5-cell model of Spectrum Equilibrium Dialyzer (Spectrum Medical Industries, Inc., Los Angles, CA). The cells were separated by a semipermeable membrane (Spectrum Medical Industries, Inc., Los Angles, CA), with a molecular weight cutoff of 3,500, which was rinsed in the above isotonic buffer for at least 24 hrs. For *in vitro* protein binding, one milliliter of plasma was dialyzed against 1 ml of an isotonic buffer solution of 0.05 *M* phosphate and 0.07 *M* NaCl, pH 7.4. The cells were incubated at 37 °C for 8 hrs with gentle shaking (12 rpm). BRB-I-28, at a concentration of 4, 8 and 16 μg/ml, was added to buffer outside. For *ex vivo* protein binding, 0.5 ml of plasma (5 min after intravenous administration of 10 mg/kg of BRB-I-28) was dialyzed against the same volume of above isotonic buffer solution. In preliminary studies, it was found that equilibrium was achieved within 4 hr. BRB-I-28 was stable at this condition. There was 101.1±5.0% total recovery of BRB-I-28 from both sides of the dialysis chamber, suggesting that no significant binding to the membrane occurred. After 8 hrs at equilibrium, 250 μl aliquot were taken from both chambers. The HPLC method described above was used to determine the concentrations of BRB-I-28 in the plasma side or buffer side. Standard curves were prepared daily using peak-area ratios of various BRB-I-28 concentrations in plasma or buffer prepared as above. A blank plasma sample was dialyzed in each binding experiment to assess the presence of endogenous or exogenous compounds that might interfere with the BRB-I-28 measurements. The percentage of binding was calculated as a ratio of (C_p-C_b) over (C_p).

Results

Oral and Intravenous Pharmacokinetic Profiles

The plasma BRB-I-28 concentration-time curves are presented in Figure 2. The concentration range of BRB-I-28 was 0.24-4.3 mg/L following iv 10 mg/kg (n = 5), and 0.14-1.8 mg/L following oral administration of 20 mg/kg (n = 5), respectively. The plasma concentration data of BRB-I-28, after intravenous administration at dose of 10 mg/kg and after oral administration of 20 mg/kg, were best fitted to the exponential equations shown below, respectively.

$$C = A_1e^{-\alpha t} + A_2e^{-ßt}$$

$$C = B_1e^{-kelt} - B_2e^{-kat}$$

The C is the plasma BRB-I-28 concentration; A_1, A_2, B_1 and B_2 represent mathematical coefficients; α and ß represent the hybrid rate constants for the distribution phase and the terminal elimination phase; and k_a and k_{el} represent the first order elimination absorption and elimination rates, respectively.

Pharmacokinetic parameters of oral and intravenous administration of BRB-I-28 are presented in Table 1. Following intravenous administration, disposition of BRB-I-28 was best described by a two-compartment open model with moderate rate of elimination $t_{1/2ß}$ (4.50 h) and extensive distribution ($V_{d(ss)}$ = 9.028 L/kg). Following oral administration, the change of plasma BRB-I-28 concentration with time was fitted to a one-compartment open model with rapid absorption (t_{max} was 1.22 h and MRT was 3.94 h) and rapid elimination ($t_{1/2kel}$ was 1.583 h). The oral bioavailability was estimated to be 44.5%.

Urinary Excretion

About 1.85% of the iv dose of 10 mg/kg was eliminated as free parent compound in urine (0-48 h). Only 2.56% of the po dose of 20 mg/kg of free parent BRB-I-28 was excreted in the urine (0-48 h). Most of the parent BRB-I-28 was eliminated before 2 hr (Table 2).

Protein Binding

The plasma protein binding of BRB-I-28 *in vitro* remained relatively constant over a concentration of 4-16 mg/L with the mean binding percentage of 28.9±9.1% (Table 3). The mean plasma protein binding percentage of BRB-I-28 *ex vivo* was 24.0±8.7% (Table 4). There was no significant difference between *in vitro* and *ex vivo* plasma protein binding.

Discussion

We previously demonstrated that when ^{14}C-labelled BRB-I-28, a novel antiarrhythmic agent, was administrated orally to rats at 10 mg/kg, there was rapid absorption with 80% bioavailability (Alavi et al. 1991). Our present results of $t_{1/2ka}$ = 0.544 (range from 0.413 to 0.799) and 44.5% oral bioavailability of parent BRB-I-28 indicate that, following oral absorption, BRB-I-28 may undergo extensive metabolism in dogs compared to that observed in rats. Extra peaks in HPLC profile of BRB-I-28 in dog urine collected after oral or intravenous administration suggest the presence of several possible metabolites (Chen et al. 1992), which further support our hypothesis of extensive metabolism of BRB-I-28.

The apparent volume distribution of BRB-I-28 in dogs, although larger than that of rats, is generally in agreement with previous reports (Alavi et al. 1991). The lower percentage of the parent BRB-I-28 bound to the plasma proteins, particularly albumin, facilitates the escape of most of the drug from the vascular compartments to other extravascular tissues and be eliminated rapidly from the body. These two systemic effects, such as lower plasma protein binding and faster elimination rate, created a lower concentration of BRB-I-28 in plasma, leading to higher volume of distribution in dogs. The relatively rapid elimination of BRB-I-28 is consistent with its short duration of action in suppressing the induction of sustained ventricular tachycardia in dogs (Scherlag et al. 1988; Fazekaz et al. 1993). It was interesting to find that two other derivatives of BRB-I-28 also have wide distribution in dogs (Chen et al. 1993a; 1993b).

In rats, BRB-I-28 was found to be extensive distributed in highly perfused organs such as liver, kidney and heart (Alavi et al. 1991). Based on our results in dogs, the values of K_{12}/k_{21} ratio and V_{ss}/V_c ratio, it is reasonable to assume that the drug may be distributed mainly in peripheral compartments including various tissues in the body. BRB-I-28 has a pK_a of 11.8 and an octanol:water partition coefficient of 2.82 at pH 7.3, which characterize this drug as being a relatively weak base. Thus, it is quite possible to expect that there will be greater binding of BRB-I-28 to α1-acid glycoprotein found to be increased in patients with myocardial infarction (Kremer et al. 1988).

The experimental results indicated that BRB-I-28 has special pharmacokinetic attributes, such as lower bioavailability, wider distribution, extensive metabolism, and rapid elimination in dogs. Considering the fact that BRB-I-28 is a relatively safe drug

(unpublished data), it is quite possible that one could use a fairly large dose at a more frequent dosage intervals as a novel antiarrhythmic agent in patients. However, the pharmacokinetic profiles, including the metabolism and plasma protein binding characteristics of BRB-I-28 in healthy human volunteers, have to be determined prior to any consideration for its clinical trial as a novel antiarrhythmic agent in human patients.

In conclusion, the limited elimination of parent BRB-I-28 and lower bioavailability suggest that this novel antiarrhythmic agent may undergo extensive metabolism in dogs. The short duration of antiarrhythmic effects of intravenous BRB-I-28 in dogs may be due to its extensive metabolism (Fazekas et al. 1993). BRB-I-28 has a peculiar pharmacokinetic attributes, such as 44.5% bioavailability and extensive metabolism. The longer mean residual time, less proarrhythmic effect, and the lack of cardiodepressant actions and low toxic effect make this antiarrhythmic agent worthy of further investigation.

References

Akaike H. A new look at the statistical model identification. Institute of Electrical and Electronics Engineers (IEEE) Transactions on Automatic Control 19: 716-723, 1973.

Bailey BR, Berlin KD, Holt EM, Scherlag BJ, Lazzara R, Brachmann J, Helm DVD, Powell DR, Pantaleo NS and Ruenitz PC. Synthesis, conformational analysis, and antiarrhythmic properties of 7-benzyl-3-thia-7-azabicyclo[3.3.1] nonan-9-one, 7-benzyl-3-azabicyclo [3.3.1]nonane hydroperchlorate, and 7-benzyl-9-thia-7-azabicyclo[3.3.1-]nonan-9-ol hydroperchlorates and derivatives: single-crystal x-ray diffraction analysis and evidence for chair-chair and chair-boat conformers in the solid state. Journal of Medicinal Chemistry 27: 758-767,1984.

Bourne DWA. Boomer, a simulation and modeling program for pharmacokinetic and pharmacodynamic data analysis. Computer Methods and Programs in Biomedicine 29: 191-195, 1989.

Chen CL, Sangiah S, Patterson E, Berlin KD, Garrison GL, Dunn W, Nan Y, Scherlag BJ and Lazzara R. Effects of BRB-I-28, a novel antiarrhythmic agent, and its derivatives on cardiac Na^+, K^+-ATPase, Mg^{2+}-ATPase activities and contractile force. Research Communication in Chemical Pathology and Pharmacology 78: 3-16, 1992.

Chen CL, Lesseley BA, Clarke CR, Roder JD, Sangiah S, Berlin KD, Garrison GL, Scherlag BJ, Lazzara R and Patterson E. High-performance liquid chromatographic determination of BRB-I-28, a novel antiarrhythmic agent, in dog plasma and urine. Journal of Chromatography-Biomedical Applications 583: 274-279, 1992.

Chen CL, Sangiah S, Roder JD, Chen H, Berlin KD, Garrison GL, Scherlag BJ, Lazzara R and Patterson E. High-performance liquid chromatographic determination of SAZ-VII-22, a novel antiarrhythmic agent, in dog plasma and urine. Analytical Sciences 9: 429-431, 1993a.

Chen CL, Sangiah S, Roder JD, Chen H, Berlin KD, Garrison GL, Scherlag BJ, Lazzara R and Patterson E. High-performance liquid chromatographic determination of SAZ-VII-23, a novel antiarrhythmic agent, in dog plasma and urine. Analytical Letters 26: 1125-1135, 1993b.

Fazekas T, Scherlag BJ, Mabo P, Berlin KD, Garrison GL, Chen CL, Sangiah S, Patterson E and Lazzara R. The comparative antiarrhythmic and proarrhythmic activity of a 3,7-diheterabicyclo[3.3.1]nonane, BRB-I-28, and lidocaine in the 1-4-day-old infarcted dog heart. Acta Physiologica Hungary. 81: 289-299, 1993.

Gibaldi M and Perrier D (Eds). Pharmacokinetics, 1st Ed. Vol. 1. Marcel Dekker,

Inc., New York, 1975.

Kremer JMH, Wilting J and Janssen LHM. Drug binding to human alpha-1-acid glycoprotein in health and disease. Pharmacological Review. 40: 1-47, 1988.

Mayer PR and Brazzell RK. Application of statistical moment theory to pharmacokinetics. The Journal of Clinic Pharmacology. 28: 481-483, 1988.

Patterson E, Scherlag BJ, Berlin KD and Lazzara R. Electrophysiological action of BRB-I-28 in canine myocardial tissues. Journal of Pharmacology and Experimental Therapy. 259: 558-565, 1991.

Patterson E, Scherlag BJ, Berlin KD and Lazzara R. Electrophysiologic actions of BRB-I-28 in ischemically-injured canine myocardium. Submitted for publication in Journal of Cardiovascular Pharmacology.

Scherlag BJ, Patterson E, Lazarra R, Bailey BR, Thompson, MD and Berlin KD. Comparative electrophysiological and hemodynamic actions of BRB-I-28 and lidocaine in the normal and infarcted dog heart. Journal of Electrophysiology 2: 461-477, 1988.

Smith GS, Thompson MD, Berlin KD, Holt EM, Scherlag BJ, Patterson E and Lazzara R. A study of the synthesis and antiarrhythmic properties of selected 3,7-diheterabicyclo[3.3.1]nonanes with substituents at the 2,4-positions and at 9-position. European Journal of Medicinal Chemistry 25: 1-8, 1990.

Yamaoka K, Nakagawa T and Uno T. Application of Akaike's information criterion (AIC) in the evaluation of linear pharmacokinetic equations. Journal of Pharmacokinetics and Biopharmaceutics 6: 165-175, 1978.

Zisman ST, Berlin KD, and Scherlag BJ. The preparation of amide derivatives of 3-azabicyclo[3.3.1]nonanes as new potential antiarrhythmic agents. Organic Preparations and Procedures Int. 22: 255-264, 1990.

TABLE 1

PHARMACOKINETIC PARAMETERS FOLLOWING I.V. BOLUS AND P.O. OF BRB-I-28 TO DOGS

Parameters	Value (mean±SD)
i.v. bolus (10 mg/kg)	(n = 5)
A1 (mg/L)	5.11 ± 0.96
A2 (mg/L)	1.10 ± 0.34
α (h^{-1})	5.430 ± 1.117
ß (h^{-1})	0.170 ± 0.066
K_{10} (h^{-1})	0.8308 ± 0.1710
K_{12} (h^{-1})	3.6666 ± 0.7950
K_{21} (h^{-1})	1.1075 ± 0.4114
$t_{1/2\alpha}$ (h)[a]	0.1164 (0.101-0.1632)
$t_{1/2ß}$ (h)[a]	4.645 (2.472-6.182)
V_c (L/kg)[a]	1.621 (1.392-2.109)
$V_{d(area)}$ (L/kg)[a]	7.849 (4.898-10.026)
$V_{d(ss)}$ (L/kg)[a]	8.759 (6.775-11.483)
Cl_B (ml/h/kg)[a]	1.289 (1.171-1.379)
AUC_{iv} (mg·h/L)	7.780 ± 0.513
$AUMC_{iv}$ (mg/L)	54.878 ± 13.083
MRT_{iv} (h)[a]	7.03 ± 1.52
Oral administration (20 mg/kg)	(n = 5)
K_{el} (h^{-1})[a]	0.4799 ± 0.1882
K_a (h^{-1})[a]	1.3421 ± 0.3086
$t_{1/2k\alpha}$ (h)	0.544 (0.413-0.799)
$t_{1/2kel}$ (h)	1.583 (0.895-2.038)
AUC_{po} (mg·h/L)	6.930 ± 0.292
$AUMC_{po}$ (mg/L)	27.408 ± 4.264
MRT_{po} (h)	3.94 ± 0.46
C_{max} (mg/L)	1.59 ± 0.13
t_{max} (h)	1.22 ± 0.04
F (%)	44.5

[a] presented as median (range).

Abbreviations: K_{10}-first-order elimination rate constant; K_{12}, K_{21} are the first-order rate constants describing distribution between central (plasma) and peripheral compartment (tissues); $t_{1/2\alpha}$ is distribution half-life after iv; $t_{1/2ß}$ is elimination half-life after iv; V_c = volume of the central compartment; $V_{d(area)}$ is apparent volume distribution calculated using AUC; Vd(ss) is apparent volume of distribution at stead state; Cl_B is body clearance of the drug.

TABLE 2

ELIMINATION OF FREE PARENT BRB-I-28 IN DOG URINE (N = 5)

Time (h)	Amount (% of dose)	
	iv (10 mg/kg)	po (20 mg/kg)
0-2	0.79 ± 0.34	1.74 ± 0.89
2-24	1.04 ± 0.86	0.80 ± 0.524
24-48	0.057 ± 0.025	0.024 ± 0.028
Total	1.85 ± 0.80	2.56 ± 1.17

TABLE 3

IN VITRO BINDING OF BRB-I-28 TO DOG PLASMA PROTEIN
(MEAN±SD, N = 5)

Initial Concentration[a] mg/L	C_p[b] mg/L	C_b[c] mg/L	Binding %
4	2.49 ± 0.10	1.75 ± 0.18	29.7 ± 7.4
8	4.76 ± 0.18	3.08 ± 0.46	35.1 ± 11.8
16	9.28 ± 0.61	7.35 ± 0.10	20.6 ± 5.5
Average			29.7 ± 10.3

[a] Total concentration at buffer side;
[b]C_p: Concentration on plasma side at equilibrium.
[c]C_b: Concentration on buffer side at equilibrium.

TABLE 4

EX VIVO BINDING OF BRB-I-28 TO DOG PLASMA PROTEIN
(MEAN±SD, N = 5)

Dog No.	C_p[a] mg/L	C_b[b] mg/L	Binding %
1	2.17	1.91	12.0
2	2.12	1.43	32.5
3	2.40	1.75	27.1
4	2.56	2.10	18.0
5	2.48	1.73	30.2
Average	2.35 ± 0.19	1.78 ± 0.25	24.0 ± 8.7

[a]C_p: Concentration on plasma side at equilibrium.
[b]C_b: Concentration on buffer side at equilibrium.

Figure Legends

Figure 1. Structure of BRB-I-28 (7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane·HCl). The drug has a pK_a of 11.8 and an octanol:water partition coefficient of 2.82 at pH 7.3. The molecular weight is 269.8.

Figure 2. Mean (±SD) plasma concentration-time curve of BRB-I-28 in dogs following i.v. bolus (10 mg/kg; n = 5) (●) and oral administration (20 mg/kg, n = 5) (○).

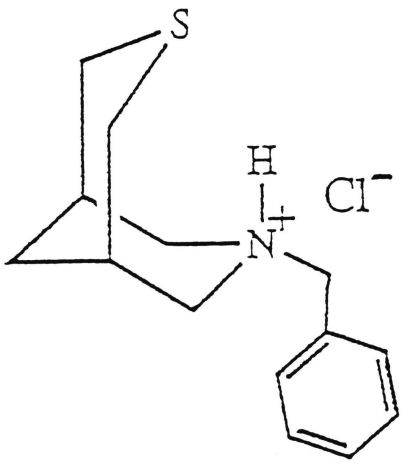


Figure 1

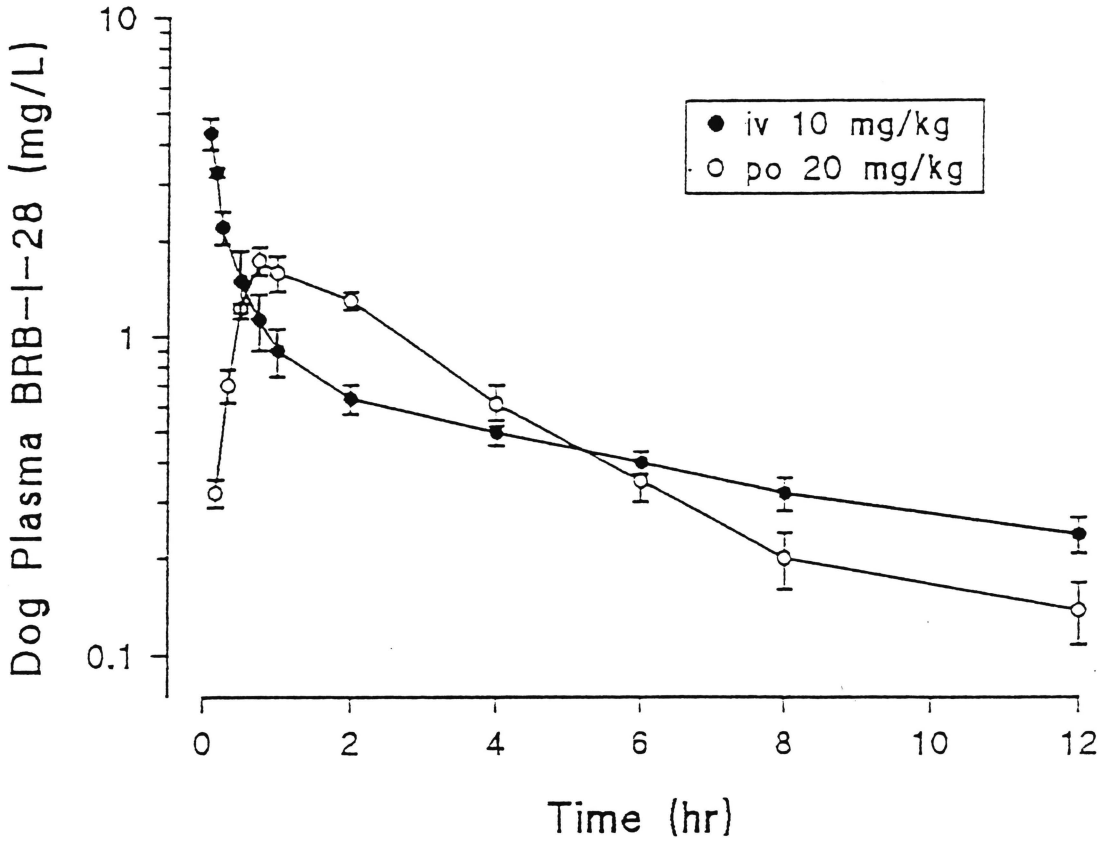

Dog Plasma BRB-I-28 (mg/L)
10
1
0.1
0
2
4
6
8
10
12
Time (hr)
iv 10 mg/kg
po 20 mg/kg

Figure 2

CHAPTER VI

IN VIVO AND *IN VITRO* METABOLISM STUDIES ON 7-BENZYL-3-THIA-7-AZABICYCLO[3.3.1]NONANE, AN ANTIARRHYTHMIC AGENT

Abstract

The metabolism of 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane (I) was studied *in vivo* in mongrel dogs and in rats and *in vitro* with rat liver microsomal preparations containing a NADPH-generating system. The major metabolites were isolated directly from hydrolyzed urine by HPLC, and their structures were determined by UV, MS, ^{1}H-NMR and ^{13}C-NMR spectroscopy and confirmed by comparison with authentic compounds. In dogs, I was found to be extensively metabolized to form II. About 95.6% of II was present as glucuronidate and/or sulfate conjugates, but no glucuronidate and/or sulfate conjugates of the parent drug I were detected. Following intravenous and oral administration of I, the plasma concentration curve of II could be best described by a 1-compartmental model. The plasma AUC of II was 20.1% (i.v.) and 179.4% (oral) of that of I in dogs, respectively, suggesting that I was metabolized significantly via a first pass effect following oral administration. There was 18.3% and 18.7% of the i.v. and oral dose of II excreted in the urine in 0-72 hr. In rats, I was metabolized via S-oxidation to form II, a major metabolite, and via the oxidation of the benzylic site to form III, a minor metabolite. The

metabolite II was also collected from *in vitro* hepatic microsomal oxidation system. Product II was produced via S-oxidation, presumably by the hepatic P-450 system. Metabolites II and III have reduced antiarrhythmic properties in comparison with the parent agent. Extensive metabolism of I probably accounts for its lower oral bioavalability and relatively short duration of pharmacological effects.

Introduction

Salt I·$HClO_4$ (Figure 1) has demonstrated more effective antiarrhythmic properties (1-3) and less proarrhythmic activity than lidocaine in dog models (4,5). Electrophysiological actions of this agent in myocardial tissue and ischemically-injured myocardium of canines showed that I·$HClO_4$ could be classified as a class Ib antiarrhythmic drug (6, 7). Compound I·$HClO_4$ was found to produce a significant elevation of systemic arterial blood pressure in dogs at the effective antiarrhythmic dose during sinus rhythm, in contrast to the hypotensive effect of lidocaine (1, 2, 4, 5). The basis of some electrophysiological effects of antiarrhythmic properties and moderate hypertensive response of I·$HClO_4$ could possibly be due to its inhibitory effects on myocardial Na^+,K^+-ATPase and Mg^{2+}-ATPase activities and its positive inotropic effects on atrial and papillary muscles (8). Minimal proarrhythmic effects and no cardiodepressant actions make this compound a very promising candidate as an antiarrhythmic agent.

The pharmacokinetic and tissue distribution profiles of I·$HClO_4$ in rats (9) and that of I·HCl in dogs (10) have been examined. Previous results have shown that I·HCl, which is slowly eliminated and has a bioavailability of only 44.5% in dogs, may undergo extensive metabolism. The purpose of the present study with I was to isolate and identify its major metabolite(s) *in vivo* from dogs and from rats, and also via an *in vitro* microsomal metabolism system using HPLC in combination with UV, MS and NMR spectroscopy. Moreover, it was also intended to characterize the pharmacokinetic profile of the major metabolite(s) and the mechanism of oxidation.

Materials and Methods

Chemicals

Salt I·$HClO_4$ was synthesized by an established method (1) and its soluble form, I·HCl, is reported herein. The ^{14}C-labelled I·$HClO_4$ with a specific activity of 0.64 μCi/mg, was synthesized and purified as previously described (11). All solvents used in this study were HPLC grade. All water used was purified through a Milli-Q™ water system (Millipore Corp., Marlborough, MA). Acetonitrile, methanol, chloroform, propanol, potassium phosphate monobasic (Fisher Chemicals, Fair Lawn, NJ), and triethylamine (Pierce Chemical Co. Rockford, IL) were used directly. ß-Glucuronidase from *Helix pomatia*, glucose-6-phosphate (G6P monosodium salt), NADP, and glucose-6-phosphate dehydrogenase (Type V. from Bakers Yeast) were purchased from Sigma Co. (St. Louis, MO). SKF 525-A was obtained from SK&F Drug Substance & Products Laboratory. 3-Benzoyl)-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane was synthesized via a Mannich-type reaction starting from *N*-benzyl-4-piperidinone (12) and used as internal standard (I.S.). Elemental analyses on all new compounds were performed by Galbraith Laboratories, Knoxville, TN 37921.

Sample Collection from Dogs

Ten adult, male mongrel dogs (15-20 kg) were preconditioned for 7 days and were housed in a controlled environment (12-hour light/12-hour dark photoperiod, 22±1 °C, 60±10% relative humidity). The dogs were allowed free access to food and water. Twenty-four hours before the experiments, food was withdrawn. The

dogs had free access to water before and during the experiments. All animals had polyethylene cannulas implanted in the right and left cephalic veins (for drug administration and collection of blood samples). All experiments were initiated between 0900 and 1100 h. Salt I·HCl was dissolved in water immediately before each experiment. Intravenous administration (10 mg/kg) was by bolus injection, and the cannulas were flushed immediately with 5 ml of sterile heparinized saline. Oral administration (20 mg/kg) was through gavage. Blood samples (5 ml) were collected via one of the cephalic vein catheters at 0, 5, 10, 30, and 45 min and at 1, 2, 4, 6, 8, and 12 hr after administration of I·HCl. After each sampling, the lost blood was replaced with an equal volume of sterile heparinized saline. Blood samples were heparinized, and centrifuged at 2000 x g for 10 min. Urine samples were collected from dogs housed in stainless steel metabolism cages at designated times, 0-2, 2-24, 24-48, and 48-72 hr, via a urinary catheter. Plasma and urine were stored at -20 °C. Aliquots of 0.25 ml of plasma and of 0.5 ml urine were used for the HPLC assay.

Sample Collection from Rats

Six male Sprague-Dawley rats, weighing approximately 200 g, were purchased from a commercial supplier (SASCO Inc., Omaha, Nebraska). The rats were preconditioned for 7 days and were housed in a controlled environment (12-hour light/12-hour dark photoperiod, 22±1 °C, 50±10% humidity). All rats were allowed free access to food and water. Twelve hours before initiation of the experiments, food was withdrawn, and the rats were transferred to stainless steel metabolism cages. The ^{14}C-labelled I·$HClO_4$ (10 mg/kg of 0.35% solution) (11) was dissolved in 40% dimethylsulfoxide/0.01 *N* HCl, and the solution was administered to 6 rats by

gavage. Pooled urine samples were collected for 8 hr after drug administration. Samples were then centrifuged, and aliquots of supernatants were frozen at -20 °C until analyzed.

Preparation of Microsomes

Unanesthetized, nonfasted, male, Sprague-Dawley rats (200-250) were killed by decapitation. The livers were removed immediately and washed with ice-cold phosphate buffer (0.01 *M* phosphate buffer containing 0.15 *M* KCl, pH 7.4) and homogenized in 4 volumes of the buffer using a polytron homogenizer (model PT 10/35, Brinkmann Instruments, Switzerland) for 45 seconds. The homogenate was centrifuged at 9000 x g for 10 min, the supernatant was centrifuged at 105,000 x g for 60 min, and the pellet was resuspended and centrifuged again at 105,000 x g for 60 min. The microsomes were suspended in 0.25 *M* sucrose and stored at -70 °C for future use. Because of the sensitivity of the microsomal flavin-containing monooxygenase to thermal inactivation, all procedures for isolation of microsomes were carried out at 0-4 °C. Protein determination was achieved using the bicinchoninic acid protein assay reagent with bovine serum albumin as standard (13).

***In Vitro* Incubation Conditions**

All incubation mixtures contained the following ingredients in final concentrations: Tri-HCl buffer (pH 7.4, 50 m*M*), microsome (1 mg/ml), glucose-6-phosphate (10 m*M*), glucose-6-phosphate dehydrogenase (1.2 units/ml), $MgCl_2$ (5 m*M*), $MnCl_2$ (5 μM) and I·HCl (27 μM). The mixture was preincubated at 37 °C

for 5 min. The metabolism reaction was initiated by the addition of NADP (final concentration was 1 m*M*). The contribution of flavin-containing monooxygenase to the metabolism of I·HCl was determined by a comparison of the metabolism results using fresh and preincubated (37 °C, 60 min) microsomes and by the use of cytochrome P-450 inhibitor (SKF 525-A, 2 m*M*). After incubation for specified times, the reaction was terminated by the addition of NaOH (final concentration was 0.2 m*M*).

Non-enzymatic oxidation of I·HCl was determined via the following methods: a) microsomes were not incubated in the incubation mixture, which eliminated the enzyme source; b) using the microsomal fraction inactivated by heating 10 min at 80°C and thus destroying the enzymes, and c) the NADPH-generating system was not added to the incubation mixture, thus eliminating the cofactor of the enzymes.

Isolation of Dog Urinary Metabolite(s)

Aliquots of 50 ml of dog urine, after alkalinization with NaOH to pH ±12, were extracted with 2-3 volumes of chloroform to obtain free metabolite(s). The aqueous supernatant was adjusted to pH 5 with acetic acid, 2 ml of ß-glucuronidase crude solution (214,400 units of ß-glucuronidase and 9000 units of sulfatase) was added, and then the solution was incubated at 37 °C for 24 hr. The hydrolysate solution was adjusted to pH ±12 with NaOH, and then it was extracted with chloroform to yield the glucuronide conjugated metabolite(s). Another aliquot of urine collected from a dog treated with I·HCl (p.o. 20 mg/kg, see above sample collection) was acid hydrolyzed to obtain total metabolites (free plus conjugated metabolites) by boiling with 3 *N* HCl for one hour. After cooling, the samples were

alkalinized to pH 12±0.5 via the addition of NaOH (2 *M*), and then the resulting mixtures were extracted with chloroform. Chloroform extracts were subjected to a stream of N_2. The solid residue was reconstituted in methanol, and 50 μl of this solution was utilized for HPLC separation.

Isolation of Rat Urinary Metabolite(s)

To 1 ml of urine sample was added 100 μl of 5 *N* NaOH to increase the pH of the sample to ≈12. The alkalinized urine sample was then extracted twice with 4 ml of chloroform:2-propanol (9:1). The organic phase was transferred to a clean test-tube and evaporated to dryness under N_2. The precipitate was redissolved in 200 μl of methyl alcohol. Aliquots (10 μl) of extracted samples and ^{14}C-labelled I·$HClO_4$ standard were applied to aluminum oxide TLC plates (Polygram Alox, Brinkmann Instruments, Inc., Wesbury, NY). After development in methyl alcohol, the elution tracks on the TLC plates were cut horizontally into 5 cm-wide bands, and the radioactivity in each band was measured by liquid scintillation counting. Areas of parallel TLC tracks, which corresponded to those with high relative radioactivity, were then scraped from the TLC plates, placed in methanol, and vortexed. These scrapings were centrifuged to remove suspended material and then subjected to MS analysis.

Isolation of *In Vitro* Metabolites

After incubation (100 ml of reaction solution) for 40 min, the reaction was stopped by the addition of 10 ml of 5 *M* NaOH. The remaining I·HCl and its metabolites in the mixture were extracted with chloroform, and the chloroform layer

was dried under a N_2 stream to a solid residue. Aliquots of 50 μl of a reconstituted methanol solution were prepared for HPLC separation.

HPLC Separation

Salt I·HCl and its metabolite(s) were separated with a Waters HPLC system (Waters, Millipore Co., Milford, MA) using a pump (model 501), a tunable absorption detector (Waters 484), and chromatographic software (Maxima 820). An Ultramex 5 C_6 column (5 μm, 250 x 4.6 mm) and a Ultramex 5 C_6 guard column (5 μm, 30 x 4.6 mm) purchased from Phenomenex (Torrance, CA) were used to separate parent I·HCl and metabolites. A mixture containing acetonitrile-methanol-37.5 m*M* phosphate buffer, pH 6.8 (28:28:44, v/v) containing 4.0 m*M* triethylamine, was used as the mobile phase. The column was eluted under isocratic or gradient conditions utilizing a flow rate of 1.2 ml/min at ambient temperature. The detection wavelength was 261 nm. Possible metabolites were collected from HPLC analysis and extracted with chloroform and then dried under a N_2 stream. The pellet was used for MS and NMR analysis.

UV Analysis

The elute of possible metabolites collected from the HPLC system was analyzed with a Shimadzu MPS 2000 UV spectrometer with the mobile phase as a reference, and data were compared with that from authentic compounds. The scanning wavelength ranged from 200-400 nm.

MS Analysis

MS data were recorded with a VG TS-250 mass spectrometer equipped with a computer system DEC-PDP11/73. EI mass spectra were recorded at 70 ev, accelerating voltage, 10 kV, and scan speed, 3 sec/decade. FAB-MS spectra were obtained on a model ZAB2-SE(HR) mass spectrometer. Samples were dissolved in dimethyl sulfoxide and loaded onto a glycerol-coated probe tip.

NMR Analysis

Both ^{1}H- and ^{13}C-NMR spectra were measured on a Model Varian XL-400 NMR spectrometer at 399.9 MHz and 100.6 MHz, respectively, employing a deuterium field-lock frequency in the normal manner. The samples were dissolved in $DCCl_3$.

Quantitation of the Amount of the Metabolite II by HPLC

Parent I·HCl and the major metabolite II present in plasma, urine, and the incubation mixture were measured using an HPLC assay previously reported (14, 15). Briefly, twenty-five μl of I.S. (10 μg/ml) were added to aliquots of 250 μl of biological fluids (plasma and urine) and the incubation solution. The samples were extracted with chloroform after alkalinization to pH $\approx$12. Chloroform was dried under a N_2 stream. The extracts were reconstituted with 50 μl of methanol, and 35 μl was utilized for HPLC analysis. The amount of the metabolite II formed was quantitated by a standard curve of peak-area ratios of various concentrations of I and II in plasma, in urine, and in the incubation solution with 0.04-10 μg/ml concentration.

Synthesis of Metabolites

Compound III was synthesized by established methods (12, 16), but I·HCl and II are reported herein. The products were judged to be pure by TLC analysis and were characterized by EI-MS, and ^{1}H- and ^{13}C-NMR analysis. Since compounds like II are very rare, a crystal of II was subjected to analysis by x-ray diffraction.

Preparation of 7-Benzyl-3-thia-7-azabicyclo[3.3.1]nonane Hydrochloride (I·HCl). Gaseous HCl was generated in a 250-ml collection flask containing solid NaCl. Then H_2SO_4 (~15 mL) was added dropwise, and the gas generated was passed through a $CaCl_2$ drying tube. Into a flask equipped with a magnetic stirrer and an ice bath was bubbled (4 bubbles/sec) $HCl_{(g)}$ to a chilled (5°C) solution of amine I (1) (5.00 g, 20.20 mmol) in ether (150 mL) over a 15 min period. The mixture was allowed to stir an additional 15 min at 0-5 °C. A white precipitate formed and was filtered and washed with cold ether (30 mL). The solid was recrystallized (H_3COH/ether, 1:1, 60 mL), and the white solid collected was washed with cold ether (25 mL) and dried (Abderhalden, 80°C/0.2 mm Hg, 12 h) to give 3.31 g (60.7%) of salt I; mp 246.0-247 °C. IR (KBr) 3040 (Ar-H), 2950 (C-H), 735, 700 (C-H out of plane, mono) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.84 [m, 2 H, H(9)], 2.38 [m, 2 H, H(1,5)], 2.71 [d, J = 11.9 Hz, 2 H, H(6,8)$_{ax}$], 3.13 [d, J = 11.9 Hz, 2 H, H(2,4)$_{ax}$], 3.36 [bs, 2 H, H(6,8)$_{eq}$], 3.60 [d, J = 10.3 Hz, 2 H, H(2,4)$_{eq}$], 4.29 (d, 2 H, CH_2Ph), 7.61-7.49 (Ar-H), 9.25 (bs, 1 H, N-H); ^{13}C NMR (DMSO-d_6) ppm 25.96 [C(1,5)], 28.65 [C(9)], 30.91 [C(2,4)], 56.41 [C(6,8)], 60.82 (CH_2Ph), 129.21, 129.82, 130.19, 130.43 (Ar-C); Mass spectral (EI) data calcd for $C_{14}H_{20}NSCl$ *m/z* (M+): 233.1238. Found: 233.1239. Anal. calcd for $C_{14}H_{20}NSCl$: C, 62.32; H, 7.47; N, 5.19. Found: C, 62.20; H, 7.38; N, 5.16.

Preparation of 7-Benzyl-3-thia-7-azabicyclo[3.3.1]nonane-3-oxide (II). A 200-mL, round-bottomed flask was equipped with a magnetic stirrer, an ice bath and a condenser with a N_2 inlet. To a stirred, chilled (5 °C) solution of the amine (I, 1.4 g, 6 mmol) in CH_3OH (60 mL) was added dropwise a solution of $NaIO_4$ (1.35 g, 6.3 mmol) in H_2O (15 mL) over 10 min. After stirring for 1 h, the suspension was filtered and washed with CH_3OH (50 mL); the filtrate was concentrated (rotary evaporator) to a residue which was partitioned between CH_2Cl_2 and H_2O (40 mL each). Additional extracts ($HCCl_3$, 3 x 40 mL) of the aqueous layer were combined with the initial extract, and the solution was dried (Na_2SO_4, overnight) and concentrated to afford an oil which solidified upon standing. Recrystallization ($HCCl_3$/pentane) of the solid gave 1.15 g (76.9%) of rhombic crystals of II; mp 140-141 °C. IR (KBr) cm^{-1} 3085, 3065, 3030 (Ar-H), 2955, 2920, 2895, 2815 (C-H), 1495, (C=C), 1020 (S=O), 740, 705 (C-H out of plane, mono); ^1H NMR ($DCCl_3$) δ 1.59 [bd, 1 H, H(9), J = 13.3 Hz], 1.86 [bd, 1 H, H(9), J = 13.2 Hz], 2.20 [d, 2 H, $H(2,4)_{ax}$, J = 11.7 Hz], 2.37 [bs, 2 H, H(1,5)], 2.62 [d, 2 H, $H(6,8)_{ax}$, J = 12.0 Hz], 2.78 [d, 2 H, $H(2,4)_{eq}$, J = 11.8 Hz], 3.51 [d, 2 H, $H(6,8)_{eq}$, J = 11.7 Hz], 3.55 (s, 2 H, $ArCH_2$), 7.25-7.39 (m, 5 H, Ar-H); ^{13}C NMR ($DCCl_3$) ppm 31.86 [t, C(9)], 32.59 [d, C(1,5)], 57.42 [t, C(2,4)], 58.59 [t, C(6,8)], 62.88 ($ArCH_2$), 127.20, 128.39, 129.12, 137.67 (Ar-C); ^{15}N NMR ($DCCl_3$) ppm 49.37 [N(7)]. Anal. calcd. for $C_{14}H_{19}NOS$: C, 67.43; H, 7.68. Found: C, 67.61; H, 7.73.

Preparation of 7-Benzyl-3-thia-7-azabicyclo[3.3.1]nonane-3-oxide Hydroperchlorate (II·$HClO_4$). A 50-mL Erlenmeyer flask was equipped with a magnetic stirrer and an ice bath. To a stirred, chiled (5 °C) solution of the sulfoxide (II, 0.47, 1.88 mmol) in ether (20 mL) and $(H_3C)_2CHOH$ (3 mL) over 10 min.

Filtering the precipitate, washing the latter with ether (~50 mL), and then recrystallizing (95% EtOH) the solid, gave 0.51 g (78.1%) of crystalline salt II·$HClO_4$; mp 137-138 °C. IR (KBr) cm^{-1} 3090 (Ar C-H), 2970, 2950 (C-H), 1465 (C=C), 1095 (Cl-O), 745, 705 (C-H out of plane, mono); ^1H NMR (D_3COD) δ 1.70 [bd, 1 H H(9), J = 14.0], 2.01 [bd, 1 H, H(9), J = 13.9 Hz], 2.61 [bd, 2 H, $H(2,4)_{ax}$, J = 11.8 Hz], 2.69 [bs, 2 H, H(1,5)], 3.06 [bd, 2 H, $H(2,4)_{eq}$, J = 11.8 Hz], 3.36 [bd, 2 H, $H(6,8)_{ax}$, J = 13.1 Hz], 3.94 (s, 2 H, $ArCH_2$), 4.19 [bd, 2 H, $H(6,8)_{eq}$, J = 12.9 Hz], 7.35-7.42 (m, 5 H, Ar-H); ^{13}C NMR (D_3COD) ppm 30.80 [C(9)], 36.27 [C(1,5)], 53.94 [C(2,4), 58.56 [C(6,8)], 61.07 ($ArCH_2$), 129.43, 129.78, 131.49, 135.29 (Ar-C); ^{15}N NMR (DMSO-d_6) ppm 56.45 [N(7)]. Anal. Calcd. for $C_{14}H_{20}ClNSO_5$: C, 48.06; H, 5.76. Found: C, 47.84; H, 5.74.

Pharmacokinetic Analysis

Data fitting and pharmacokinetic parameter calculations were performed using the Boomer program (17). An appropriate pharmacokinetic model was chosen on the basis of lowest weighted squared residuals, lowest Akaike's information criterion (AIC) value, R-squared, and the correlation coefficiency (18, 19). The area under the curve (AUC) was calculated by the trapezoidal rule (20). Renal clearance (CL_r) was calculated by dividing the amount of compound excreted in the urine by the AUC. Mean residence time (MRT) was calculated by dividing the area under the first moment curve (AUMC) by AUC (21). The time (t_{max}) taken to achieve peak concentration (C_{max}) was calculated using differential calculus (20). The PharmK program (22) was used to calculated t_{max}, C_{max} and the elimination phase half-life. The results are presented as means and standard deviations.

Evaluation of Antiarrhythmic Effects of Metabolites

Antiarrhythmic properties of the metabolites II and III have already been assessed with dogs which were examined 24 h after ligation of the left anterior descending coronary artery (1, 3, 23). The metabolites were administered intravenously in doses of 3 and 6 mg/kg, the same doses as with the parent drug. The metabolites were dissolved in 50% ethyl alcohol. Previous tests with 50% alcohol alone revealed there were no significant effects of the injected ethyl alcohol on various electrophysiological properties nor on blood pressure (2).

X-Ray Experimental

A crystal of size 0.33 x 0.26 x 0.14 mm was selected for all crystallographic measurements of II. Cell dimensions were obtained by least-squares fit to $\pm 2\theta$ values of 48 reflections measured at 138K using MoKα_1 radiation. All X-ray measurements were carried out on an Enraf-Nonius CAD-4 diffractometer equipped with a liquid N_2 low-temperature device. Crystal data: $C_{14}H_{19}ONS$, M.W. = 249.4, monoclinic, $p2_1/c$, a = 13.084 (3), b = 6.527 (1), c = 15.397 (3) Å, β = 104.66 (2)°. V = 1272.1 Å^3, Z = 4, D_x = 1.302 Mg m^{-1}, F(000) = 536, λ(MoKα) = 0.71069Å, μ (Mokα) = 1.9 cm^{-1}. The intensity of all the unique reflections within 2θ range 0-53 °C were collected at 138±2K using Mokα radiation (graphite monochromator) and employing the θ-2θ scan technique with a variable scan width of 0.80 + 0.34tanθ) and a variable horizontal aperture width of (3.0 + 0.86tanθ)mm. Maximum scan time for a reflection was 90s. Three standard reflections were monitored every 2 hours of exposure, and the data showed maximum variation of less than 3%. The crystal orientation was checked regulaly by three control reflections. A total of 2618

unique reflections were recorded of which 2073 were considered 'observed' on the basis, $I > 2\sigma(I)$. The intensities were corrected for Lorentz and polarization factors, but no absorption correction was made. The structure was solved by direct methods and the use of program MULTAN80 (24) and refined by a full-matrix least-squares routine SHELX (25) in which the quantity, $\Sigma\omega(F_o - F_c)^2$ was minimized, where $\omega = 1/\sigma^2(F_o)$. The hydrogen atoms were located from a difference Fourier map and hydrogen parameters were refined isotropically. In the final stages of refinement, non-hydrogen atoms were given anisotropic thermal parameters. The refinement converged to a final $R = 0.042$, $R_w = 0.042$ for 2073 observed reflections and 230 variables, $s = 2.0$, $(\Delta/\sigma)_{max} = 0.03$. The maximum and minimum electron density in the final difference map, $\pm 0.2 e/Å^3$.

Results

Metabolism in Dogs

The HPLC chromatogram of the urinary extract obtained without hydrolysis showed no significant peaks for metabolite(s), but I·HCl appeared at about 17.5 min. A large peak appeared at 5.1±0.3 min from the extract obtained from glucuronidase/sulfatase-hydrolyzed dog urine (Figure 2). The possible metabolites M-1 [retention time (RT) = 5.4 min] and parent I·HCl (RT ≈ 17.5 min) were collected for UV, MS and NMR analysis.

The EI-MS spectrum of M-1 is given in Figure 3a. The mass spectrum of M-1 shows a molecular ion at *m/z* 249 (M^+) and fragment ion peaks at *m/z* 232, 106 and 91 (base peak), which does not match perfectly with that of synthetic II (Figure 3b).

It is apparently contaminated to a small extent. The chemical shifts obtained from ^{1}H-NMR analysis are illustrated in Figure 4a. The same situation is observed in the ^{1}H NMR of the sample (Figure 4b). Comparisons were also made of the retention times in HPLC, along with UV, and ^{13}C-NMR analysis with authentic samples (data are not shown). Metabolite M-1 is thus identified as 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane-3-oxide (II). The compound at RT ≈ 17.5 min was determined as parent I·HCl (data are not shown).

Urinary Excretion of the Metabolite II from Dogs

Table 1 shows the data for percentages of II over the dose excreted from dog urine after intravenous bolus dose of 10 mg/kg and oral administration of 20 mg/kg of I·HCl. The results demonstrate that most of II is present in the form of glucuronide/sulfate conjugates.

Metabolism in Rats

The same major metabolite, i.e. II, was determined in rat urine to be that in dog urine (data are not presented). In addition, another minor metabolite from rat urine was isolated. The mass spectrum of this minor metabolite was presented in Figure 5a. The molecular ion was at *m/z* 247 (67%) with major fragment ion peaks at *m/z* 214 (6%), 199 (12%), 186 (11%), 148 (19%), 142 (64%), 134 (8%), 105 (100%, base peak) and 77 (39%). This fragmentation pattern and relative intensities of the major components in the urine match quite well with the synthesized III (see discussion). Some obvious impurities are also present.

Identification of the Metabolites *In Vitro*

Fig. 6 shows a typical HPLC profile of the extract from incubates of I·HCl with hepatic microsomal system of rats. Metabolite M-2 (RT = 5.1±0.3 min) and parent I·HCl were collected for MS and NMR spectroscopy. The results of MS and NMR analyses showed that M-2 was II, and data for compound at RT ≈ 17.5 min matched well that for I·HCl.

Kinetics of Microsomal I·HCl Metabolism

Using the HPLC method described above, the concentrations of I·HCl and II, the major metabolite in rat liver microsomes, were determined at various times during the reaction. These results are presented in Figure 7. The results indicate that the concentration of I·HCl decreased rapidly within the first 10 min, while the concentration of II increased steadily up to 40 min and then reached steady state.

Effect of Incubation Conditions on Metabolism of I·HCl

The contribution of flavin monooxygenases to the metabolism of I·HCl was determined by comparison of the metabolism in fresh and preincubated microsomes (60 minutes at 37 °C) (26, 27) and by the use of SKF 525-A, a cytochrome P-450 inhibitor. The results shown in Table 2 demonstrate that I·HCl is predominantly metabolized by the P-450 system (94.4%). Involvement of flavin-containing monooxygenase in the metabolism of I·HCl was very limited (10.3%).

Pharmacokinetics of II in Dog Plasma

Figure 8 shows the mean blood concentration-time curve of II, a major

metabolite of I·HCl, after intravenous (10 mg/kg) and oral administration (10 mg/kg) of I·HCl. Some pharmacokinetic parameters of II, after intravenous and oral administration of I·HCl, are presented in Table 3.

Pharmacological Activity of II and III

The antiarrhythmic activities of sulfoxide II and benzamide III have already been assessed in anesthetized dogs with ligation of the anterior descending artery (1, 3, 23). The results indicate that metabolites II and III have much less antiarrhythmic activity than that of parent I·$HClO_4$, although these two metabolites still induce some reduction in the rate of sustained ventricular tachycardias (16).

X-Ray Diffraction Results for II

In view of the scarcity of models for compounds I, II and III, a single crystal of II was obtained and subjected to x-ray diffraction analysis. The final atomic parameters of the non-hydrogen atoms are listed in Table 4. A stereoview of an ORTEP plot of the molecule is shown in Figure 14. Bond distance, bond angles and selected torsions angles are shown in Figure 15. The bicyclo[3.3.1]nonane system assumes a chair-chair (CC) conformation as seen in 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane·$HClO_4$ (I·$HClO_4$) and other related structures (1, 45). The interesting difference in the present structure is the disposition of the phenyl ring which assumes a nearly symmetrical position giving the overall molecule a pseudo-mirror symmetry. In all related compounds reported, the phenyl ring takes an asymmetrical position with respect to the bicyclic ring system.

In Table 5, selected geometrical features of the present structure II are

compared with those of a few known related structures (IV, V, VI and VII) (11, 44, 45). In all the five structures, the bicyclo[3.3.1]nonane system, consisting of a thiane ring and a piperidine ring, assumes a CC conformation. In three of the structures (IV, V and VI), the N atom is protonated, while compounds II and V have a free amine. Compound V has a third ring fused to the nonane system. The following conclusions can be drawn from a comparison of the geometries of these structures: (i) The C-S distances range between 1.817 and 1.831 Å and are virtually unaffected by the various substitution patterns. Even in the present structure II where the S atom is double-bonded to an O atom, the two C-S distances (1.821 and 1.820 Å) are comparable to 1.817(5) Å given by Sutton (46) as the mean distance for paraffinic C-S bonds. The C-S-C angle in II (96.9°) is significantly smaller than that in others, where it ranges between 99.3 and 100.9°. (ii) The average C-N distances in the free amine structures II and V are significantly shorter (by about 0.04 Å) than those in the three N-protonated structures (IV, VI, VII). The changes in the C-N-C angles are not significant. (iii) The S...N contact distance in compound II (2.86 Å) is about 0.15 Å shorter than that observed in the three N-protonated structures in each of which an intramolecular N-H...S hydrogen bond is formed. The longer S...N contact in compound V is probably due to the fusion of a third ring and subsequent flattening of the nonane system. (iv) The torsion angles around the S atom (48° and 47°) in compound II are comparable to those in the other compounds. These angles indicate flattening of the thiane ring at the sulfur end. The flattening is least in compound II and most in compound V. (v) In the present structure II, the nitrogen end of the piperidine ring is decidedly puckered, the torsion angles around N atom (62° and 62°) being about 6° higher than the ideal value of 56° for a chair conformation. In all of

the other structures, the nitrogen end of the ring experiences shows minor flattening (torsion angles range between 52° and 57°). Puckering at the nitrogen end with torsion angle of 63° has been observed in 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane-9-one (1), a bicyclo[3.3.1]nonane system which has a boat-chair (BC) conformation. (vi) The angle between the various planes in the fused ring system display major strain in all compounds. The angle between the two 4-membered planes (P1 and P2) is the smallest (112°) in the present structure II. The puckering at the nitrogen end of the piperidine ring is indicated by the smaller angle (124°) between the planes P2 and P5. In all other compounds, this angle ranges between 130° and 132°. (vii) Torsion angles C(8)-N(7)-C(10)-C(11) and C(6)-N(7)-C(10)-C(11) show a distinctly different phenyl ring orientation in compound II (a symmetrical one) compared to the others. The S=O bond makes an inclination angle (α) of 36° with the plane of the thiane ring. A substituent is considered in an equatorial position when $0<\alpha\leq30°$, in an inclinal position when $30<\alpha\leq60°$, and in axial position when $60<\alpha\leq90°$. Both the S=O distance (1.513 Å) and the C-S-C angle (96.4°) are comparable to those (1.504 Å and 95.1°) found in 4-acetyl-*trans*-3-methylcarbamoyl-1,4-thiazinane-1-oxide (47), where the S=O group takes an inclinal position with α = 44°. An axial S=O group (α = 85°) in the *cis* isomer of the compound possesses a shorter S=O distance and a smaller C-S-C angle (1.495 Å and 94.6°) (48). The dependence of the C-S bond length and C-S-C angle on the orientation of the S=O bond with respect to the thiane ring has been observed in some thiane sulfoxides (44, 45).

Discussion

The present study was designed to establish the structures of the major phase I urinary metabolite(s) of 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane (I) in dogs and in rats. Thus, metabolite(s) were first deconjugated by hydrolyzing the urine with ß-glucuronidase/sulfase or acid. A large proportion of the metabolites was excreted in the urine within the first 24 hr. Therefore, pooled urine from this time period was used for the isolation and identification of the metabolite. The structures were identified with UV, MS, ^{1}H-NMR and ^{13}C-NMR analyses and were confirmed by co-elution on HPLC with synthetic standards. Several possible metabolite peaks were visible in the HPLC chromatogram. Based on the molecular weight determination via MS and the corresponding fragmentation properties observed, as well as the NMR and UV analyses, the major metabolite in dogs was identified as 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane-3-oxide (II). No benzylic oxidation was found in dogs, probably because of a very limited metabolic rate for I in dogs. The major metabolite in rats remained II, although a minor metabolite, 7-benzoyl-3-thia-7-azabicyclo[3.3.1]nonane (III), was also identified. Confirmation of the assignments was made by chromatographic and spectroscopic comparison with data from synthetic standards. The results indicate that there is a species difference in the metabolism of I.

To be sure, S-oxidation is a very common metabolic pathway for sulfur-containing compounds (28, 29). The metabolic route for benzylic oxidation may involve a two-step oxidation, first by the P-450 system to produce an alcohol, and then by alcohol dehydrogenase. However, the exact mechanism for benzylic

oxidation remains unknown.

To confirm the identification of sulfoxide II and benzamide III, it is necessary to understand the fragmentation pattern of I in the MS. It is interesting that the molecular ion for I·HCl was not seen in the EI-MS but only the parent with *m/z* 233. This is common in this family of heterocycles (30). Major peaks in the MS (EI, 29 mv) of I·HCl occur at *m/z* 233, 232, 142, 134, and 91 (100%) which we suggest are due to the fragments shown in Figure 9a. The mass at *m/z* 91 is, of course, the tropylium ion. Some of the suggested fragments are reminiscent of those found in the MS of piperidine (31-33) and 4-hydroxymethyl-*N*-benzylpiperidine (33, 34) (see Figures 9b, 9c). Again the tropylium ion predominated in these models. In the case of I·HCl, the mass spectral pattern appears to be the result of fragmentation of the two rings although the loss of the benzyl group to give the tropylium ion may also give an ion from which a majority of the remaining fragments may originate. However, there is a peak at *m/z* 200 in the MS of I·HCl, which conceivably could result from a loss of SH from $I{\cdot}HCl^{+\cdot}$ as suggested from the fragmentation of thiane (33, 35).

The MS-EI pattern for thiane-1-oxide is reported (33, 35, 36) to produce the fragments shown in Figure 10 with most peaks unidentified. In our examples, namely $I{\cdot}HClO_4$ and I·HCl, the major fragment was the tropylium ion (*m/z* 91) with other peaks of much lower intensities at *m/z* 232, 158, 106, and 65. On the basis of comparison of the MS pattern of thiane-1-oxide, we suggest structures as shown (Figure 11) for certain fragments from II including the $M^{+\cdot}$ at 249 and additional fragments as illustrated. In the identification of metabolites from $I{\cdot}HClO_4$ and/or

C-14 labelled I·$HClO_4$ (11), TLC analysis indicated the presence of II·$HClO_4$ (the *m/z* 349 shown in Figure 11) while the MS analysis indicated the presence of II (*m/z* 249) in the urine of dogs. The existence of III in rat urine could not be confirmed by TLC analysis, but MS analysis (Figure 5) strongly suggested its presence in low concentration although the fragmentation pattern was slightly contaminated with impurities.

In rats, the minor product III was tentatively identified via mass spectra analysis (EI-313 mv) with major peaks at *m/z* 247 ($M^{+\cdot}$), 214, 199, 186, 142, 132, 105 (100%), and 77. The signals may arise from the fragments shown in Figure 12. There is also a peak at *m/e* 279 which suggests the structure shown, namely the amide-sulfone. Due to the lack of mass spectra data on closely related model systems, the assignments for fragments from III must be tentative. However, the percentages for the intensities of the masses from the mass spectrum of the extract are 67 (247), 6 (214), 12 (199), 11 (186), 64 (142), 8 (134), 100 (105), and 39 (7) which match fairly well those percentages for peaks obtained from an authentic sample of III, the values for the latter being 63, 6, 8, 9, 54, 5, 100, and 56, respectively. The extract likely contains more than one specimen which could account for the slight differences in the intensities. However, there is no other intuitively expected signal at *m/z* 105, for example, than that for the benzoyl ion shown. This peak supports the presence of III in the extract. It is tentatively assumed that the amide-sulfone is the structure for the *m/z* 279, and it is an impurity in the mass spectrum of the extract. The mass spectrum of the simple model system thiane 1,1-dioxide has been reported (37, 38) with major peaks at *m/z* 70 [$C_5H_{10}+\cdot$;

8% from loss of SO_2], 69 [$C_5H_9^{+\cdot}$; 43% from loss of $\cdot SO_2H$], 55[$C_4H_7^{+\cdot}$; 43%], 42 [$C_3H_6^{+\cdot}$; 100%], and 41 [$C_3H_5^{+\cdot}$; 70%]. Surprisingly, the loss of SO_2 apparently did not occur to an appreciable extent. In our proposed system with *m/z* 279, a predicable fragmentation pattern is not obvious since both rings can participate. Sulfone formation is not unreasonable from the metabolism, however. That the compound with *m/z* 279 is suggested to be a chair-boat form (which could complicate the mass spectral pattern) has some foundation in view of X-ray diffraction studies of similar bicyclo[3.3.1]nonane systems (39, 40).

It is interesting to note that sparteine is reported to be primarily if not solely metabolized via *N*-oxidation (41). Sparteine is structurally related to I. In our hands (30), attempted oxidations of I and relatives thereof by a number of reagents, including hydrogen peroxide and *m*-$ClC_6H_4CO_3H$, have not produced any *N*-oxides, the indication being the nitrogen atom is shielded by the other heteroatom at the 3- or 7-position. Dealkylation of nitrogen via metabolism is common among biologically active amines (42). This may well occur in our systems since the metabolic process appears to be more complex for members I·$HClO_4$ and I·HCl than for sparteine. It is also noteworthy that certain six-membered sulfur-containing heterocycles undergo S-oxidation by bacterial oxygenases (43) with sulfoxide formation the major result. Consequently, the metabolites detected from our work seem to parallel to some degree that reported in simpler but related systems.

Incubation of I·HCl with a microsomal fraction from rats resulted in the formation of a large amount of II. Its formation was not observed with denaturated microsomes, which have neither the NADPH-generating system nor the microsomes in an incubation solution. Therefore, the metabolism was clearly enzyme-catalyzed,

not chemically catalyzed. The formation of II was dependent upon the presence of microsomes, NADPH, and oxygen. The two enzyme systems, P-450 dependent mixed function oxidase system (MFO) and the flavin-containing monooxygenase (FMO), are known to oxidize nitrogen and sulfur-containing compounds (28, 29, 42). Because FMO in microsomes is destroyed by temperature inactivation (37 °C for 60 minutes) in the absence of an NADPH-generating system (26, 27), MFO can be quantitated in the heat inactivated microsomal incubation. The difference in the metabolism between heat inactivated and untreated microsomal incubation is considered as an FMO-dependent metabolism (28). The amount of sulfoxide II formation was very small in heat (37 °C for 60 min) inactivated microsome. In addition, the metabolism of I·HCl to form II was markedly inhibited by SKF 525-A. Therefore, the P-450-dependent MFO is primarily responsible for the metabolism of I·HCl via sulfoxidation. The HPLC peak at 3.0 min (Fig. 6) was further separated by HPLC using a more polar mobile phase. The benzamide III, from benzylic oxidation of I, was observed in the MS, but it has not been possible to obtain adequate sample for proper identification by TLC. Sulfoxidation appears to be the major metabolic route for I·HCl *in vitro*. Sulfoxidation is also the major metabolic pathway of I *in vivo* in dogs and in rats. Although I·$HClO_4$ and I·HCl contain nitrogen in the molecule, hitherto, we have been unable to confirm the presence of metabolite(s) formed from the *N*-oxidation. Possibly steric hindrance around the N atom retards the rate of *N*-oxidation as discussed previously. In addition, the method described may be not sufficient to quantify this type of metabolite in the urine.

Both the enzymatic digestion and the acid hydrolysate of urine from dogs

treated with I·HCl show similar results in that II is the major metabolite of I. Most of II was found to be in the conjugated state, especially with glucuronide/sulfate conjugate (95.6%). Only a small amount of II was in free state (about 4.4%). One possible link of glucuronide/sulfate to the molecule may be at the nitrogen position of I, but this has not been confirmed. After comparing the chromatographic profiles of enzymatic and acidic hydrolysate, several possible metabolite peaks were found in the acidic hydrolysate, suggesting that there are possibly additional metabolite(s) which may be conjugated with intermediates other than glucuronidate/sulfate. Characterization of new minor metabolite(s) of I·$HClO_4$ and I·HCl besides sulfoxide and benzylic oxidation is in progress.

We previously demonstrated that when I·HCl (I) was administrated orally to dogs at 20 mg/kg, there were observed low plasma levels with 44.5% bioavailability (10). Less than 3% of parent I·HCl was excreted via the urine (10). Low plasma levels could be caused by either poor absorption from the gastrointestinal tract or rapid clearance of the compound. However, 80% absorption was found in rats after oral administration (9). Our results show that 18.3% and 18.7% of sulfoxide II, with respect to the dose of administration, was recovered from urine within 0-72 hr. All of these results suggest that I·HCl undergoes significant metabolism. This may be one reason for the low recovery rate of I·HCl from urine. Excretion of the parent I·HCl and its major metabolite via bile has not been examined. Therefore, the mechanism for excretion of metabolite(s) and parent I via the biliary and or/gastrointestinal tract remains unknown.

The apparent volume distribution of I·HCl and I·$HClO_4$ appears to be somewhat large in dogs and in rats (9, 10). The possible reason for this may be the

lower percentage of the parent I·HCl bound to the plasma proteins, particularly albumin, which facilitate the escape of most of the drug from the vascular compartments to other extravascular tissues such as heart, liver, kidney (9, 10). Large accumulation in the liver may account for the rapid metabolism of I in animals, which may create a lower concentration of I·HCl and I·$HClO_4$ in plasma leading to higher volume of distribution in dogs. The relatively rapid metabolism and elimination of I·HCl is consistent with the relatively short duration of action of I in suppressing the induction of sustained ventricular tachycardia in dogs (2, 4, 5). On the other hand, antiarrhythmic properties of II and III were much less than those of the parent compound (1, 3, 23). Therefore, extensive metabolism of I·HCl and reduced antiarrhythmic effect of II and III may be the reason for short antiarrhythmic effect of I·HCl.

Disposition of the metabolite II can adequately be delineated via a 1-compartmental model. The plasma concentration data for II could be described by following equation.

$$Cp = Be^{-\beta t} - Be^{kt}$$

where: Cp=plasma concentration at time t after administration (mg/L); B=concentration at time zero extrapolated from the elimination phase (mg/L); ß=terminal slop (h^{-1}); and k is the first-order metabolite formation rate constant (k_f) (h^{-1}). The pharmacokinetic models for describing the disposition of II after intravenous and oral administration of I are shown in Figure 13.

About 30 min after intravenous and oral administration of I, the concentration of II maximized. Because of higher polarity, the volume of distributions of II and MRT were much less than that of I. The value of AUCII/AUCI after p.o. of I

(179.4%) was significantly large than that after i.v of I (20.1%), indicating that I undergoes an extensive first pass effect after oral administration. Compound II was found to be eliminated rapidly via the kidney (kmu 7.085 h-1 and 17.14 h-1 for intravenous and oral administration of I·HCl, respectively). Renal clearance of the parent compound I was 0.0239 L/h/kg for i.v. and 0.074 L/h/kg for p.o., respectively. However, II had renal clearance of 1.42 L/h/kg for i.v. and 0.285 L/h/kg for p.o., suggesting that II has a high elimination via the kidney, especially after intravenous administration of I·HCl.

In summary, I is extensively metabolized *in vivo* in dogs and in rats and *in vitro* in an hepatic microsomal system with the formation of 7-benzyl-3-thiabicyclo[3.3.1]-nonane-3-oxide (II), a major metabolite, via S-oxidation. In rats, there is the additional formation of 7-benzoyl-3-thia-7-bicyclo[3.3.1]nonane (III), a minor metabolite, via benzylic oxidation (Figure 1). The cytochrome P-450 dependent mixed-function oxidase system is predominantly involved in the metabolism to the sulfoxide II. The results of this study support previously obtained pharmacodynamic and pharmacokinetic data.

References

1. a) B. R. Bailey, K. D. Berlin, E. M. Holt, B. J. Scherlag, R. Lazarra, J. Brachmann, D. van der Helm, D. R. Powell, N. S. Pantaleo and P. C. Ruenitz: Synthesis, conformational analysis, and antiarrhythmic properties of 7-benzyl-3-thia-7-azabicyclo[3.3.1] nonan-9-one, 7-benzyl-3-azabicyclo [3.3.1]nonane hydroperchlorate, and 7-benzyl-9-thia-7-azabicyclo[3.3.1]nonan-9-ol hydroperchlorates and derivatives: single-crystal x-ray diffraction analysis and evidence for chair-chair and chair-boat conformers in the solid state. J. Med. Chem. **27**, 758-767 (1984). b) K. D. Berlin B. J. Scherlag, C. R. Clarke, S. R. Otia, S. A. Zisman, S. Sangiah and S. V. Mulekar: Salts of 3-azabicyclo [3.3.1] nonanes as antiarrhythmic agents and precursors thereof. U. S. Patent 5, 084, 572. c) M. K. Pugsley, M. J. A. Walker, G. L. Garrison, P. G. Howard, R. Lazzara, E. Patterson, W. P. Penz, B.J. Scherlag and K. D. Berlin: The cardiovascular and antiarrhythmic properties of a series of novel sparteine analogs. Proc. West. Pharmacol. Soc. **35**, 87-91 (1992).

2. B. J. Scherlag, E. Patterson, R. Lazarra, B. R. Bailey, M. D. Thompson, and K. D. Berlin: Comparative electrophysiological and hemodynamic actions of BRB-I-28 and lidocaine in the normal and infarcted dog heart. J. Electrophysiol. **2**, 461-477 (1988).

3. G. S. Smith, M. D. Thompson, K. D. Berlin, E. M. Holt, B. J. Scherlag, E. Patterson and R. Lazzara: A study of the synthesis and antiarrhythmic properties of selected 3,7-diheterobicyclo[3.3.1]nonanes with substituents at the 2,4-positions and at 9-position. Eur. J. Med. Chem. **25**, 1-8 (1990).

4. T. Fazekas, B. J. Scherlag, P. Mabo, K. D. Berlin, G. L. Garrison, C. L. Chen, S. Sangiah, E. Patterson and R. Lazzara: The comparative antiarrhythmic and proarrhythmic activity of a 3,7-diheterobicyclo[3.3.1]nonane, BRB-I-28, and lidocaine in the 1-4-day-old infarcted dog heart. Acta Physiologica Hungary. **81**, 297-307 (1993).

5. T. Fazekas, P. Mabo, K. D. Berlin, B. J. Scherlag, R. Lazzara: Comparative electrophysiological effects of a new chemical class of antiarrhythmic agents, 3,7-diheterobicyclononanes, in the 1-4-day-old infarcted dog heart. The Physiologists, **35**, 221 (1992).

6. E. Patterson, B. J. Scherlag, K. D. Berlin and R. Lazzara: Electrophysiological action of BRB-I-28 in canine myocardial tissues. J. Pharmacol. Exp. Ther. **259**, 558-565 (1991).

7. E. Patterson, B. J. Scherlag, K. D. Berlin and R. Lazzara: Electrophysiologic actions of BRB-I-28 in ischemically-injured canine myocardium. J. Cardiovasc. Pharmacol. **21**, 637-646 (1993).

8. C. L. Chen, S. Sangiah, E. Patterson, K. D. Berlin, G. L. Garrison, W. Dunn, Y. Nan, B. J. Scherlag and R. Lazzara: Effects of BRB-I-28, a novel antiarrhythmic agent, and its derivatives on cardiac Na^+, K^+-ATPase, Mg^{2+}-ATPase activities and contractile force. Res. Commun. Chem. Pathol. Pharmacol. **78**, 3-16 (1992).

9. F. K. Alavi, C. R. Clarke, S. Sangiah, K. D. Berlin, S. A. Zisman, C. L. Chen, G. L. Garrison, B. J. Scherlag, and R. Lazzara: Disposition of BRB-I-28 (7-benzyl-7-aza-3-thiabicyclo[3.3.1]nonane hydroperchlorate), a novel antiarrhythmic agent. Drug Invest. **3**, 317-323 (1991).

10. C. L. Chen, S. Sangiah, J. D. Roder, H. Chen, K. D. Berlin, G. L. Garrison, B. J. Scherlag, R. Lazzara and E. Patterson: Pharmacokinetics and plasma protein binding of BRB-I-28, a novel antiarrhythmic agent, in dog. Drug Invest. **6**, 237-243 (1993).

11. S. A. Zisman, K. D. Berlin, F. K. Alavi, S. Sangiah, C. R. Clarke and B. J. Scherlag: Synthesis of 7-benzyl-7-aza-3-thiabicyclo[3.3.1]nonane hydroperchlorate-*6,8,10*-$^{14}C_3$. J. Labelled Compd. Radiopharmaceut. **27**, 885-888 (1989).

12. S. A. Zisman, K. D. Berlin and B. J. Scherlag: The preparation of amide derivatives of 3-azabicyclo[3.3.1]nonanes as new potential antiarrhythmic agents. Org. Prep. Proced. Intern. **22**, 255-264 (1990).

13. P. K. Smith, R. I. Krohn, G. T. Hermanson, A. K. Mallia: Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**, 76-85 (1985).

14. C. L. Chen, B. A. Lesseley, C. R. Clarke, J. D. Roder, S. Sangiah, K. D. Berlin, G. L. Garrison, B. J. Scherlag, R. Lazzara and E. Patterson: High-performance liquid chromatographic determination of BRB-I-28, a novel antiarrhythmic agent, in dog plasma and urine. J. Chromatogr. (Biomedical Applications) **583**, 274-279 (1992).

15. C. L. Chen, S. Sangiah, J. D. Roder, H. Chen, K. D. Berlin, G. L. Garrison, B. J. Scherlag and R. Lazzara: Simultaneous determination of a novel antiarrhythmic agent, BRB-I-28, and its sulfoxidation metabolite in plasma and urine by high-performance liquid chromatograpgy. J. Liquid Chromatogr. in press (1994).

16. S. A. Zisman: A study of stereochemical and substituent effect on antiarrhythmic activity of selected 3-azabicyclo[3.3.1]nonanes and derivatives. Ph.D. Thesis. Oklahoma State University (1989).

17. D. W. A. Bourne: Boomer, a simulation and modeling program for pharmacokinetic and pharmacodynamic data analysis. Computer Methods and Programs in Biomedicine **29**, 191-195 (1989).

18. H. A. Akaike: A new look at the statistical model identification. Institute of Electrical and Electronics Engineers (IEEE) Transactions on Automatic Control **19**, 716-723 (1973).

19. H. G. Boxenbaum, S. Kiegman, R. M. Elashoff: Statistical estimations in pharmacokinetics. J. Pharmacokinet. Biopharmaceut. **2**, 123-148 (1974).

20. M. Gibaldi and D. Perrier (Eds). Pharmacokinetics, 1st Ed. Vol. 1. Marcel Dekker, Inc., New York, 1975.

21. P. B. Mayer and R. K. Brazzell: Application of statistical moment theory to pharmacokinetics. J. Clinic Pharmacol. **28**, 481-483 (1988).

22. D. R. Lu and F. Mao: An interactive program for pharmacokinetic modeling. J. Pharm. Sci. **82**, 537-542 (1993).

23. B. J. Scherlag, E. Patterson, S. Sangiah, and K. D. Berlin: unpublished results.

24. P. Main, S. J. Fiske, S. E. Hull, L. Lessinger, G. Germain, J.-P. Declerq and M. M. Woolfson: "MULTAN80". A system of computer program for the automatic solution of crystal structures from x-ray diffraction data. University of york, England, 1980.

25. G. M. Sheldrick: "SHELX76". Program for crystal structure determination. University of Cambridge, England, 1986.

26. M. Dyroff and R. A. Neal: Studies of the mechanism of metabolism of thioacetamide S-oxide by rat liver microsomes. Mol. Pharmacol. **23**, 219-227 (1983).

27. M. O. Howard-Martin, J. F. Newton, D. J. Keohane, L. P. Yodis, C. M. Saverino, C. W. Qualls, Jr. and L. W. Scwartz: *In vitro* metabolism of the novel antiinflammatory agent 6-(4'-fluorophenyl)-5-(4'-pyridyl)-2,3-dihydroimidazo-(2,1-*b*)-thiazole (SK&F 86002). Drug. Metab. Dispos. **18**, 607-612 (1990).

28. D. M. Ziegler: Microsomal flavin-containing monooxygenase: Oxygenation of nucleophilic nitrogen and sulfur compounds. In "Enzymatic Basis of Detoxication" (W. B. Jacoby, ed), pp. 201-227. Academic Press, New York, 1980.

29. D. M. Zieger: Flavin-containing monooxygenase. Drug Metab. Rev. **19**, 2-32 (1988).

30. K. D. Berlin, B. R. Bailey, G. S. Smith, M. D. Thompson, S. A. Zisman, G. L. Garrison, S. Sangiah, C. L. Chen, C. R. Clarke and F. K. Alavi. Unpublished results, 1990-present.

31. A. M. Duffield, H. Budzikiewicz, D. H. Williams and C. Djerrasi. Mass spectrometry in structural and stereochemical problems. LXIV. A study of the fragmentation processes of some cyclic amines. J. Am. Chem. Soc. **87**, 810-816 (1965).

32. R. A. Saunders and A. E. Williams: The mass spectra of cyclic amines. In "Advances in Mass Spectrometry", (W.L. Mead, ed) Vol 3, pp. 681-699. The Institute of Petroleum, London, 1966.

33. Q. N. Porter: Saturated heterocycles with one nitrogen atom in the ring. In "Mass Spectrometry of Heterocyclic Compounds" (Q. N. Porter, ed.), pp. 472-532. 1985.

34. F. D. Popp and R. F. Watts: The synthesis and ring opening of *N*-substituted-2-oxo-6-azaspiro[2.5]octanes. J. Heterocycl. Chem. **15**, 675-676 (1978).

35. R. Smarkman and Th. J. De Boer: The mass spectra of some cyclic sulphides. In "Advances in Mass Spectrometry", (K. Kendrick, ed). Vol. **4**, 357-367. The Institute of Petroleum, London, 1968.

36. S. Tamagaki and S. Oae: Effect of ring size on the mass spectral fragmentation of cyclic sulfoxide. Bull. Chem. Soc. Jap. **45**, 1767-1772 (1972).

37. Q. W. Porter and G. H. Upstill: Unpublished observations cited in: "Mass Spectrometry of Heterocyclic Compounds", (Q. N. Porter, ed), pp 372 (reference 22), Wiley-Interscience, New York, 1985.

38. R. Smakman and Th. J. De Boer: The mass spectra of some aliphatic and alicyclic sulphoxides and sulphones. Org. Mass Spectrum. **3**, 1561-1588 (1970).

39. O. I. Levina, K. A. Potekhin, E. N. Kurkutova, Yu. T. Struchkov, V. A. Palyulin, S. I. Kozhushkov, and N. S. Zefirov: Molecular and crystal structure of 3,3,7,7-tetraoxo-9-oxa-3,7-dithiobicyclo[3.3.1]nonane. Dokl. Akad. Nauk. SSSR, **185**, 118-120 (1985).

40. N. S. Zefirov and V. A. Palyulin: Conformational analysis of bicyclo[3.3.1]nonanes and their hetero analogs. In "Topics in Stereochemistry" (E. L. Eliel and S. H. Wilen, ed), pp 171-230, Wiley-Interscience, New York, 1991.

41. M. Eichelbaum: Polymorphism of drug oxidation in man: novel findings. Trends in Pharmacological Sci. **2**, 31-34 (1981).

42. P. G. Wislocki, G. T. Miwa, and A. Y. H. Lu: Reaction catalyzed by the cytochrome P-450 system. In "Enzymatic Basis of Detoxication" (W. B. Jacoby, ed), pp. 135-182. Academic Press, New York, 1980.

43. J. Latham and C. Walsh: Bacterial cyclohexanone oxygenase. A versatile flavoprotein oxygen transfer catalyst. In "International Symposium on Bioorganic Chemistry", (R. Breslow, ed) pp. 208-216. Annals of the New York Academic of Sciences, 1986.

44. B. R. Bailey, K. D. Berlin, E. M. Holt: Isolation and single-crystal x-ray diffraction analysis of *N*-benzyl-3-thia-7-azabicyclo[3.3.1]nonan-9,9-diol perchlorate, a novel hydrate formed from reaction of *N*-benzyl-3-thia-7-azabicyclo[3.3.1]nonan-9-one with perchloric acid. Phosphorus & Sulfur. **20**, 131-137 (1984).

45. G. S. Smith, K. D. Berlin, S. A. Zisman, E. M. Holt, V. A. Green and D. v. d. Helm: The mannich condensation with 1-hetera-4-cyclohexanones. The novel formation of 7-benzyl-3-hetera-7-azabicyclo[3.3.1]nonan-9-ones, 3,6-dibenzylhexyahydro-8a-methoxy 5*H*-4a,8-(methanoheteramethano)-2*H*-pyrido[3,4-e]-1,3-oxazines and 2,4,10,12-tetrabenzyl-2,4,10,12-tetraaza-15-heteradispiro[5.1.5.3]hexadecan-7-ones. Single crystal x-ray diffraction analysis of 3,6-dibenzylhexlhexahydro-8a-methoxy-5*H*-4a,8-(methanothiomethano)-2*H*-pyrido[3,4-*e*]-1,3-oxazine and 2,4,10,12-tetrabenzyl-2,4, 10,12-tetraaza-15-thiadispiro[5.1.5.3]hexadecan-7-one. Phosphorus & Sulfur. **39**, 91 (1988).

46. L. E. Sutton, M. A. D. Phil, F. R. I. C., F. R. S (eds): "Tables of Interatomic Distances and Configuration in Molecules and Ion", The Chemical Society; London, 1965.

47. F. Vanhouteghem, A. T. H. Lenstra, H. J. Giese, C. van der Auwera and M. J. Anteunis: 4-Acetyl-*trans*-3-methylcarbamoyl-1,4-thiazinane 1-oxide. Acta Cryst. **C41**, 1818-1821 (1985).

48. S. B. Sanni, M. De Wolf, A. T. H. Lenstra, C. Van der Auwera and M. J. Anteunis. (3R)-*cis*-4-acetyl-*cis*-3-methylcarbamoyl-1,4-thiazinano 1-oxide, $C_8H_{14}N_2O_3S$. Acta Cryst. **C40**, 1624-1626 (1984).

49. F. Robert: The crystal and molecular structure of 2-chloro-4-*tert*-butylthiacyclohexane sulphoxide. Acta Cryst. **B33**, 3480-3484 (1977).

50. F. Robert, S. Gauchotte: The crystal and molecular structure of 2-chloro-4-*tert*-butyl-6-methylthiacyclohexane sulphoxide. Acta Cryst. **B33**, 3484-3487 (1977).

TABLE 1

EXCRETION OF II FROM DOGS' URINE FOLLOWING I.V. AND P.O. ADMINISTRATION OF I. VALUES ARE MEAN±SD (N = 5)

Time (hr)	% dose excreted			
	i.v. 10 mg/kg		p.o. 20 mg/kg	
	free	conjugated	free	conjugated
0-2	0.10±0.08	1.23±0.47	0.05±0.05	1.44±0.18
2-24	0.16±0.14	11.2±7.66	0.05±0.04	10.4±7.64
24-48	0.23±0.35	4.07±0.91	0.08±0.07	5.89±5.78
48-72	0.11±0.08	1.23±0.30	0.02±0.02	0.77±0.63
Total	0.59±0.35	17.7±6.58	0.20±0.08	18.5±13.4

TABLE 2

EFFECTS OF INCUBATION CONDITIONS AND INHIBITOR OF CYTOCHROME P-450 ON THE FORMATION OF SULFOXIDE

Incubation Conditions[a]	Sufoxide II Formation (nmol/mg protein/min)
Complete activating system[b]	139.8±10.1
Preincubation at 37 °C for 60 min[c]	125.4±9.13
Incubation with SKF 525A	7.82±2.47
No microsome[d]	ND[e]
Incubation with denaturated microsome[f]	ND
No NADPH generating system	ND

[a]The incubation conditions and quantitation methods are described under Materials and Methods. Control conditions consisted of a microsomal protein concentration of 1 mg/ml with incubation time of 40 min. The inhibitor was incubated with microsomes for 5 minutes before addition of I·HCl (27 μM); [b]Activity was (MFO + FMO); [c]Activity was only MFO; [d]Incubation mixture do not contain microsome; [e]Below limit of quantitation; [f]I·HCl was added to the incubation mixture containing inactivated microsome (80 °C for 10 min).

TABLE 3

SOME PHARMACOKINETIC PARAMETERS OF II AFTER INTRAVENOUS AND ORAL ADMINISTRATION OF I TO DOGS (N = 5)

Parameters	i.v. 10 mg/kg	p.o. 20 mg/kg
k_f, $h,^{-1}$	0.071±0.060	1.079±0.505
k_{el}, h^{-1}	0.441±0.417	2.225±1.130
k_{mu}, h^{-1}	7.085±0.805	17.14±15.14
V, L/kg	1.218±0.993	0.684±0.798
$t_{1/2}$, h	0.51±0.30	1.59±2.07
t_{max}, h	0.49±0.22	0.68±0.29
C_{max}, mg/L	0.93±0.21	5.16±2.04
CL_r, L/h/kg	1.42±0.70	0.28±0.09
AUC, mg·h/kg	1.57±0.72	12.43±3.18
AUMC, mg·h2/kg	2.26±1.55	27.00±14.7
MRT, h	1.39±0.45	2.01±0.84
AUC_{II}/AUC_I, %	20.1±9.32	179.4±45.9

Abbreviations: k_f = first-order formation rate; k_{el} = first-order elimination rate via nonrenal pathway; k_{mu} = first-order elimination rate via renal pathway; V = volume of distribution at central compartemnt; $t_{1/2}$ = elimination half-life; C_{max} = peak plasma concentration; t_{max} = time to reach C C_{max}; CL_r = clearance via kidney; AUC = area under the concentration-time curve; AUMC = area under the first moment curve.

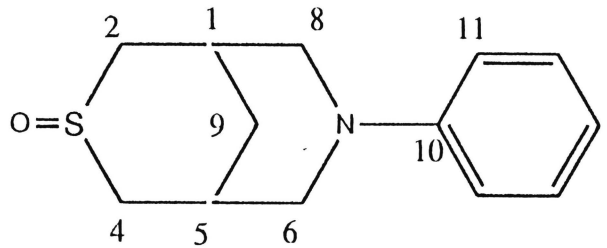
TABLE 4

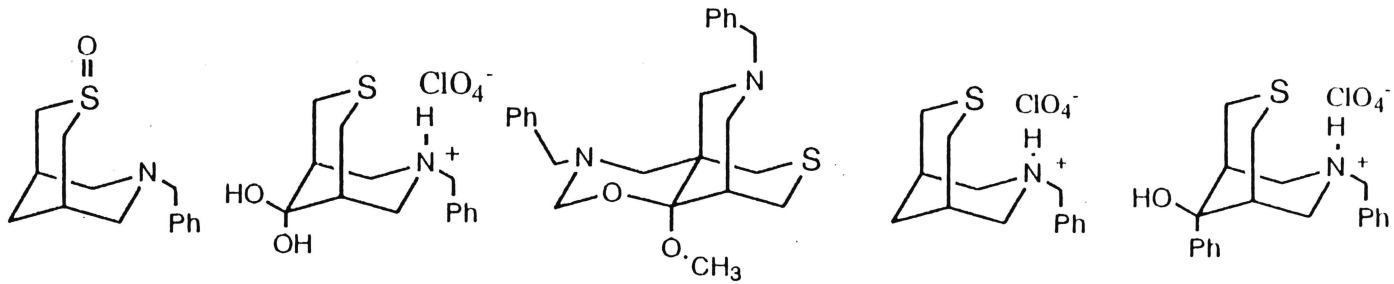
Positional and equivalent isotropic thermal parameters of non-hydrogen atoms. E. s. d.'s are within parentheses.

ATOM	x	y	z	U_{eq}
C (1)	0.2781 (2)	0.7209 (4)	0.0768 (1)	0.0260 (7)
C (2)	0.3943 (2)	0.7604 (4)	0.0802 (2)	0.0292 (8)
S (3)	0.48947 (4)	0.58973 (9)	0.15183 (4)	0.0246 (2)
O (1)	0.5844 (1)	0.5950 (2)	0.1126 (1)	0.0325 (6)
C (4)	0.4221 (2)	0.3508 (3)	0.1116 (1)	0.0230 (7)
C (5)	0.3036 (2)	0.3456 (3)	0.1048 (1)	0.0234 (7)
C (6)	0.2784 (2)	0.3768 (3)	0.1956 (1)	0.0230 (7)
N (7)	0.3121 (1)	0.5825 (3)	0.2289 (1)	0.0208 (6)
C (8)	0.2551 (2)	0.7411 (4)	0.1687 (1)	0.0253 (7)
C (9)	0.2458 (2)	0.5075 (4)	0.0388 (2)	0.0274 (8)
C (10)	0.3099 (2)	0.6135 (4)	0.3234 (1)	0.0245 (7)
C (11)	0.2025 (2)	0.5980 (4)	0.3429 (1)	0.0235 (7)
C (12)	0.1682 (2)	0.4156 (4)	0.3732 (1)	0.0286 (8)
C (13)	0.0710 (2)	0.4032 (4)	0.3924 (2)	0.0364 (9)
C (14)	0.0049 (2)	0.5728 (4)	0.3811 (2)	0.0371 (9)
C (15)	0.0374 (2)	0.7545 (4)	0.3501 (2)	0.0341 (8)
C (16)	0.1352 (2)	0.7666 (4)	0.3314 (1)	0.0268 (8)

TABLE 5

Geometrical parameters of some related bicyclo[3.3.1]nonane structures.





Distances (A)	II	IV	V	VI	VII
S(3)-C(2)	1.820	1.825	1.824	1.831	1.817
S(3)-C(4)	1.821	1.820	1.819	1.830	1.825
N(7)-C(6)	1.465	1.510	1.459	1.506	1.502
N(7)-C(8)	1.462	1.503	1.460	1.512	1.510
N(7)-C(10)	1.476	1.506	1.450	1.516	1.521
N(7) . .S(3)	2.86	2.98	3.10	3.04	3.05

TABLE 5 (Con't)

Angles (°)					
C(2)-S(3)-C(4)	96.9	99.6	100.9	99.3	99.8
C(6)-N(7)-C(8)	111.5	111.2	110.7	112.6	110.9
C(6)-N(7)-C(10)	113.1	112.9	109.9	111.9	112.6
C(8)-N(7)-C(10)	113.2	110.6	111.6	110.0	110.6
< C-N-C >	112.6	111.6	110.7	111.5	111.3
Torsion Angles (°)					
C(1)-C(2)-S(3)-C(4)	48	45	39	46	45
C(2)-S(3)-C(4)-C(5)	-47	-47	-40	-45	-44
C(5)-C(6)-N(7)-C(8)	62	55	57	53	55
C(1)-C(8)-N(7)-C(6)	-62	-55	-56	-53	-52
C(8)-N(7)-C(10)-C(11)	65	-172	-165	170	-170
C(6)-N(7)-C(10)-C(11)	-63	63	71	-64	65

Figure Legends

Fig. 1. *Proposed biotransformation pathways of 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane (I). II = 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane-3-oxide, III = 7-benzoyl-3-thia-7-azabicyclo[3.3.1]nonane.*

Fig. 2. *Typical chromatographic profiles of pooled dog urine [0-24 hr from dogs dosed with I (20 mg/kg, po)]. a) blank urine; b) urine extracted directly with chloroform; c) urine from b was hydrolyzed with glucuronidase.*

Fig. 3. *a) EI-MS spectrum of the metabolite (RT = 5.1±0.3 min) from dogs; b) the spectrum of authentic standard II.*

Fig. 4. *^{1}H-NMR of metabolite (RT = 5.1±0.3 min) from dogs. a) the spectrum of the metabolite isolated from dog urine; b) the spectrum of authentic standard II.*

Fig. 5. *MS spectrum of a metabolite from rats. a) the spectrum of the metabolite isolated from rat urine; b) the spectrum of authentic standard III.*

Fig. 6. *HPLC of the extract obtained from incubation of I·HCl with rat liver microsomes.*

Fig. 7. *Formation of the major metabolite of I·HCl after incubation with liver microsomal suspension of rats.*

Fig. 8. *Mean plasma concentration (±SD)-time curve of II in dogs following intravenous administration of 10 mg/kg (n = 5) and Oral administration of 20 mg/kg of I·HCl (n = 5).*

Fig. 9. *The fragmentation of I·HCl (a); piperidine (b); and 4-hydroxymethyl-N-benzylpiperidine (c) in EI-MS.*

Fig. 10. *The fragmentation of thiane-1-oxide in EI-MS.*

Fig. 11. *The fragmentation of II in EI-MS.*

Fig. 12. *The fragmentation of III in EI-MS.*

Fig. 13. *The pharmacokinetic models for the disposition of I and II.*

Fig. 14. *A stereoview of compound II with 50% probability ellipsoids for the non-hydrogen atoms.*

Fig. 15. *Bond distances, bond angles and selected torsion angles in II. Estimated standard deviations range: 0.002-0.003 Å (distances), 0.2-0.3° (angles) and 0.1-0.2° (torsion angles).*

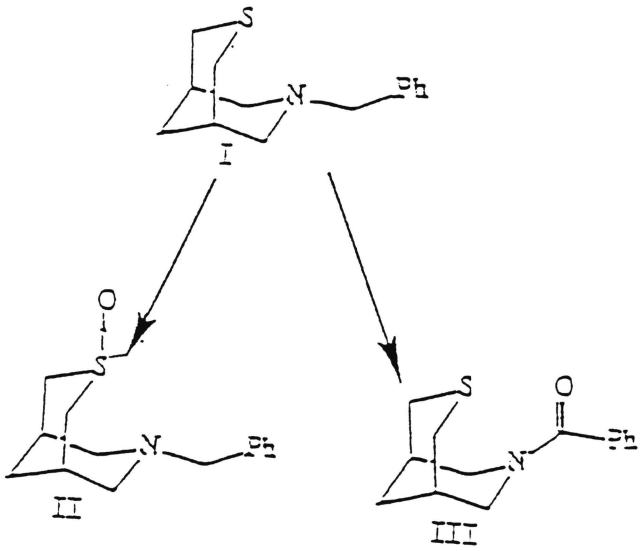


Figure 1

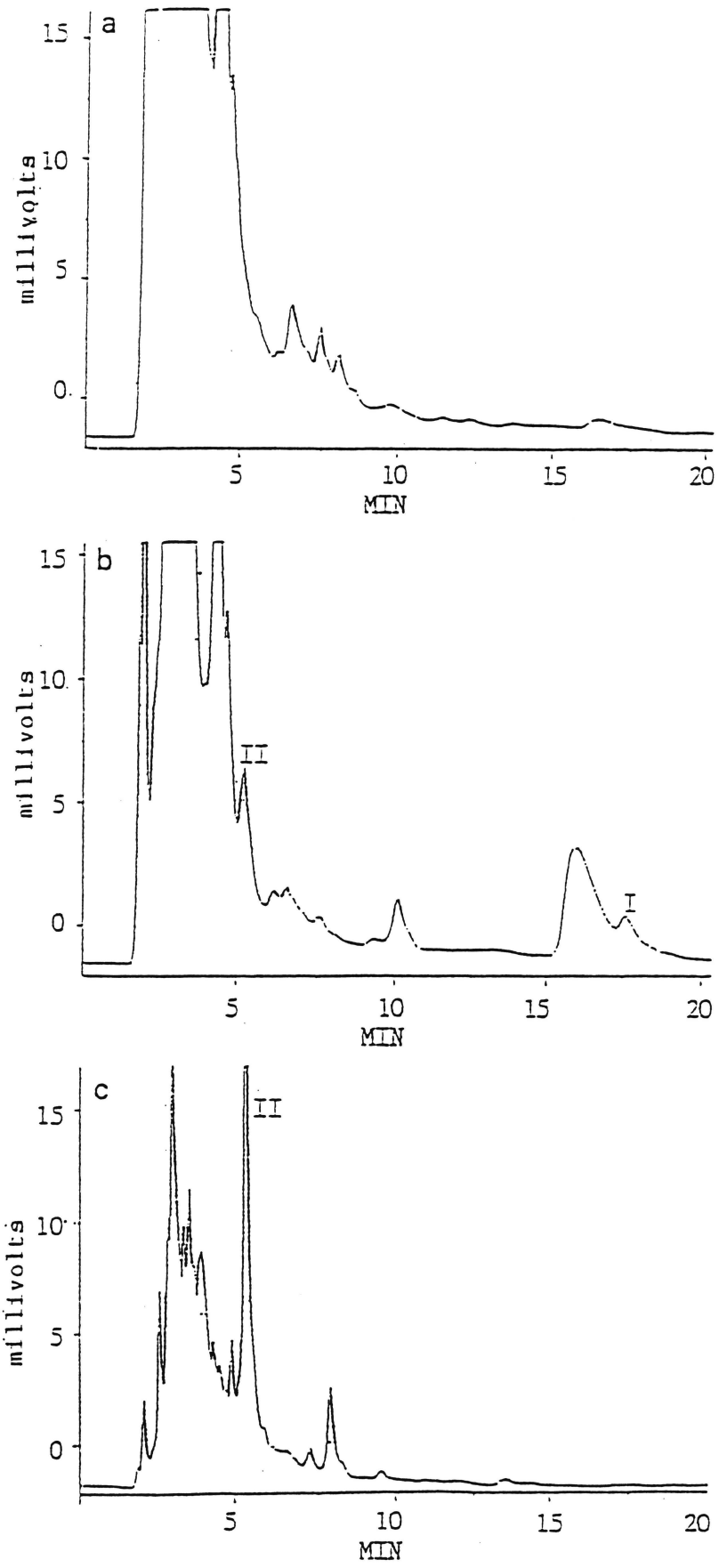


Figure 2

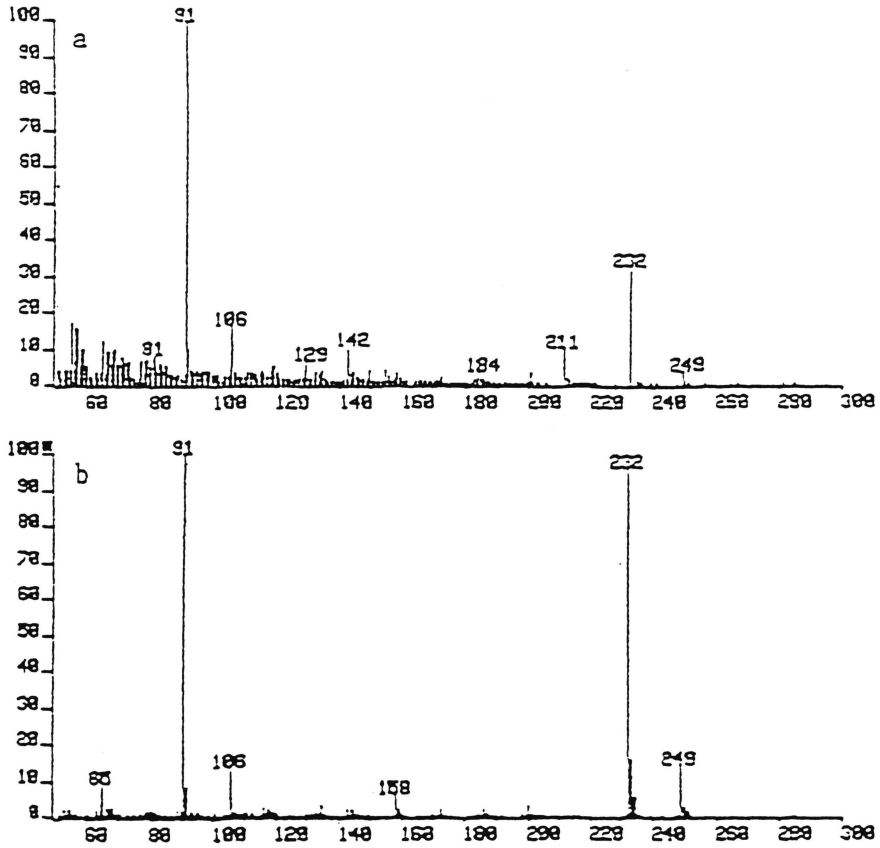


Figure 3

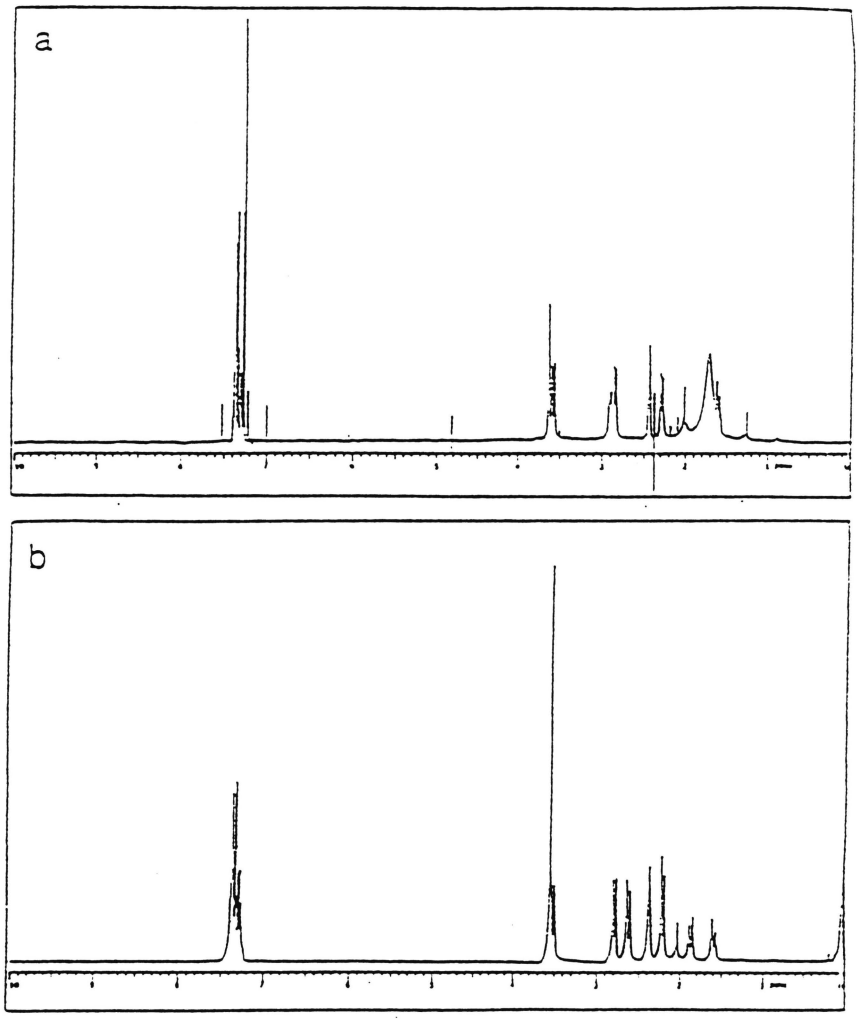


Figure 4

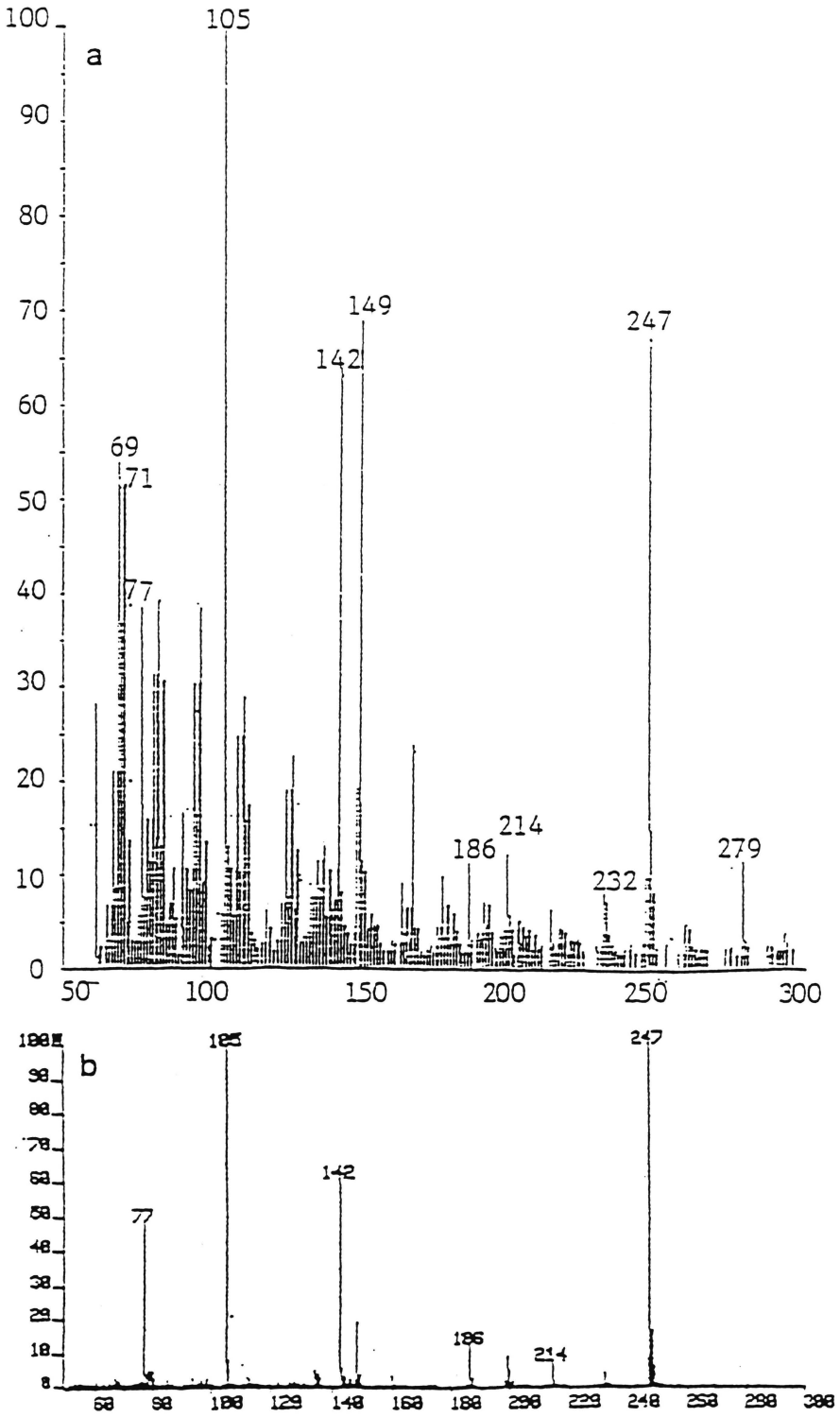


Figure 5

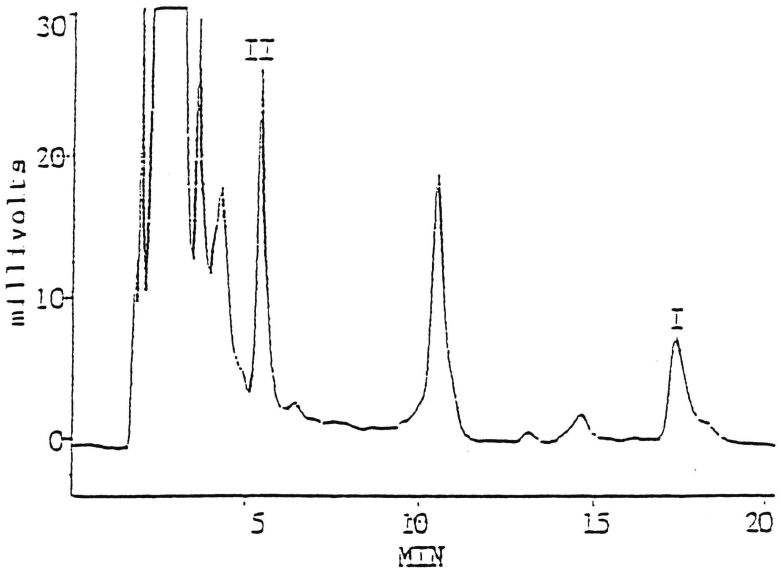


Figure 6

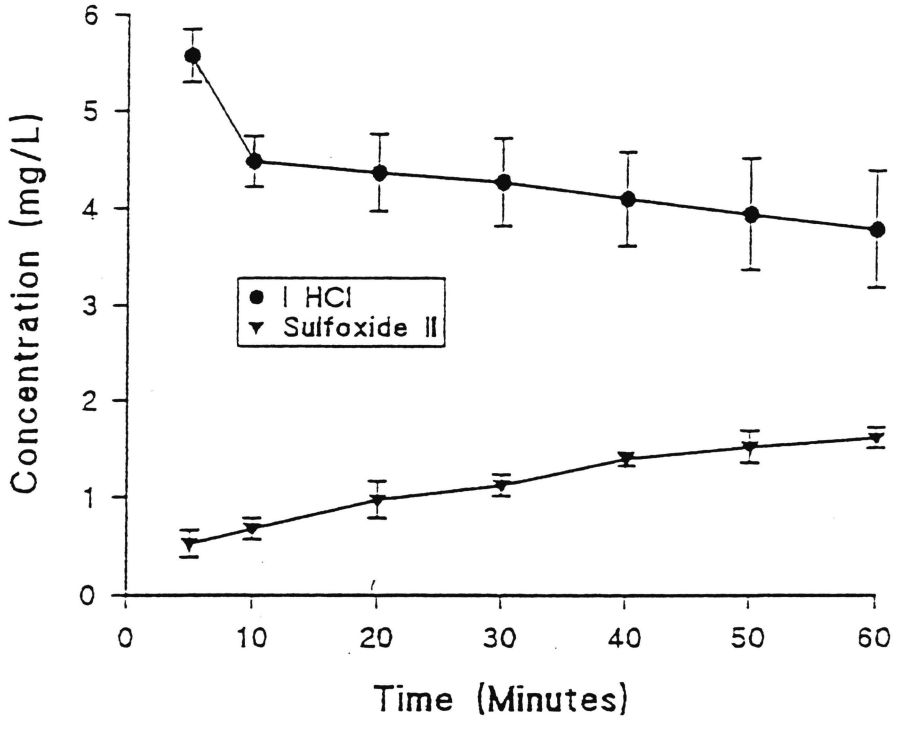

Concentration (mg/L)
6
5
4
3
2
1
0
● I HCl
▼ Sulfoxide II
0
10
20
30
40
50
60
Time (Minutes)

Figure 7

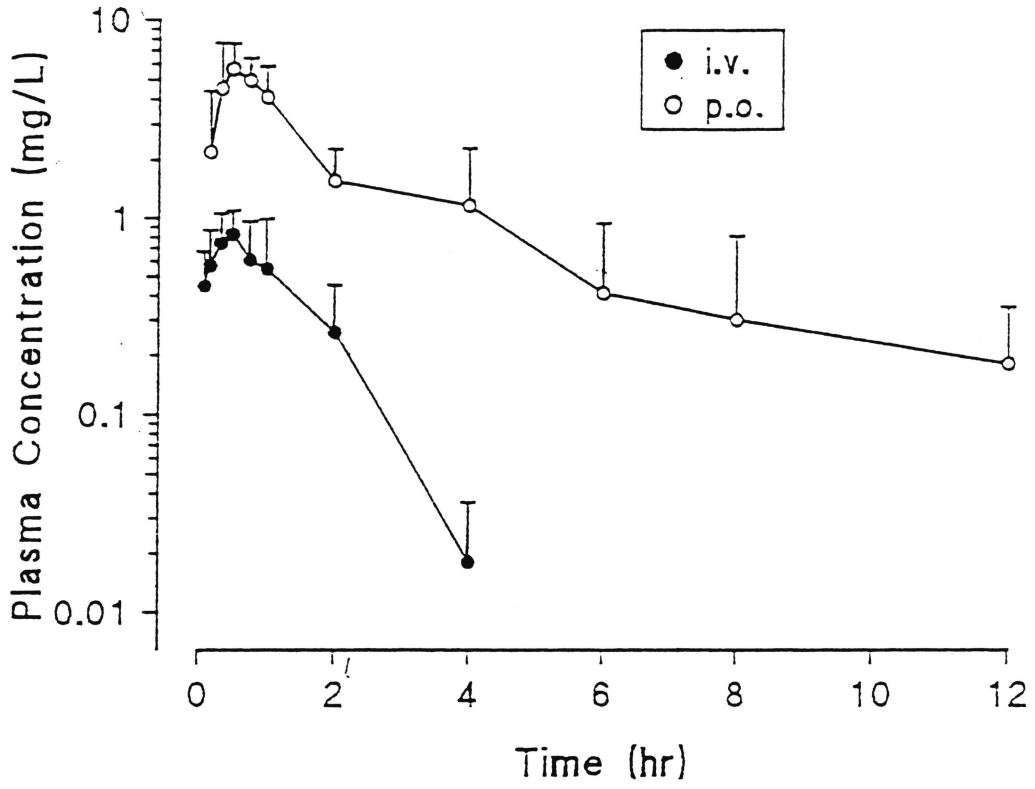
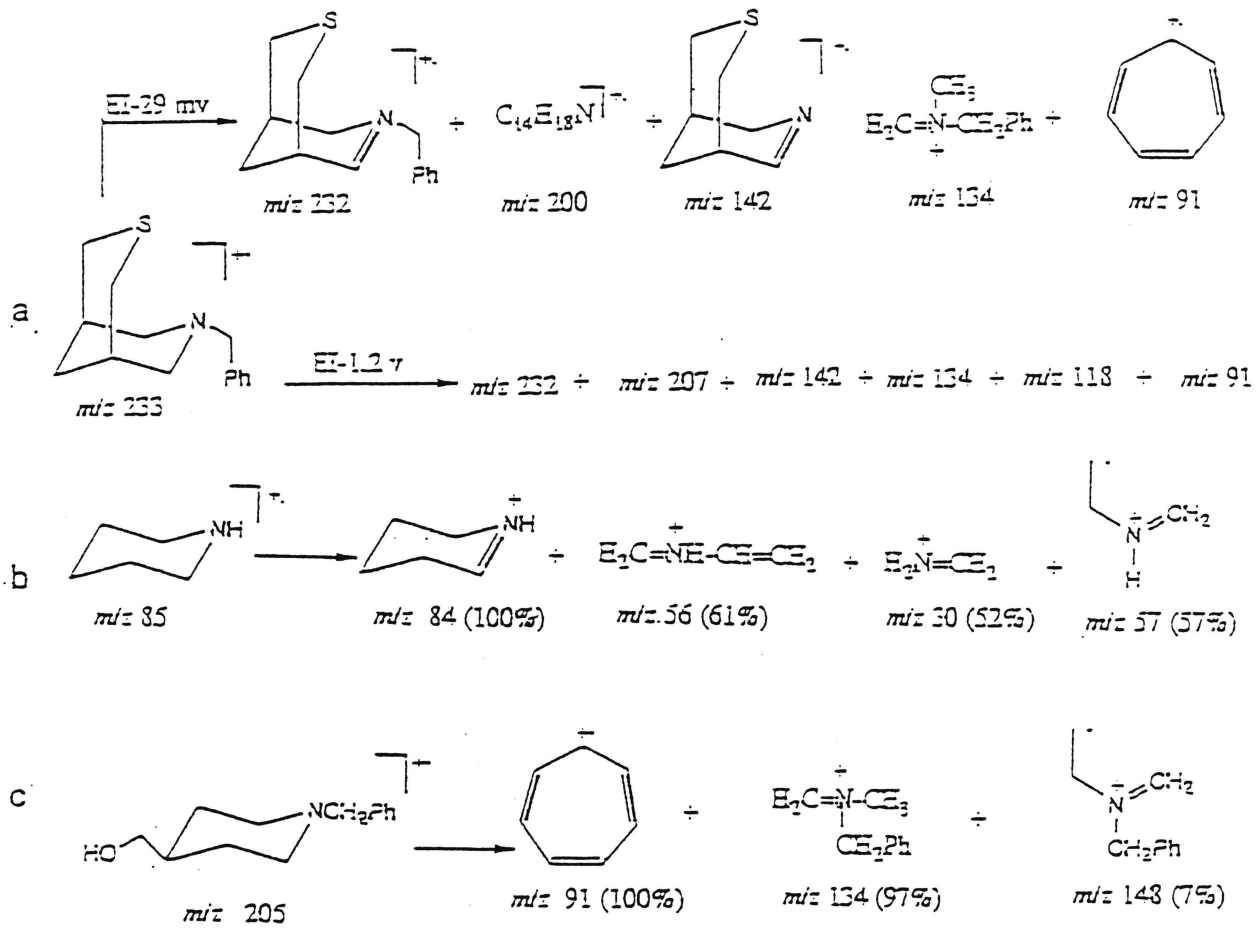


Figure 8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m/z 232
m/z 200
m/z 142
m/z 134
m/z 91
a
m/z 233
EI-1.2 v
m/z 232 + m/z 207 + m/z 142 + m/z 134 + m/z 118 + m/z 91
b
m/z 85
m/z 84 (100%)
m/z 56 (61%)
m/z 30 (52%)
m/z 57 (57%)
c
m/z 205
m/z 91 (100%)
m/z 134 (97%)
m/z 148 (7%)

Figure 9

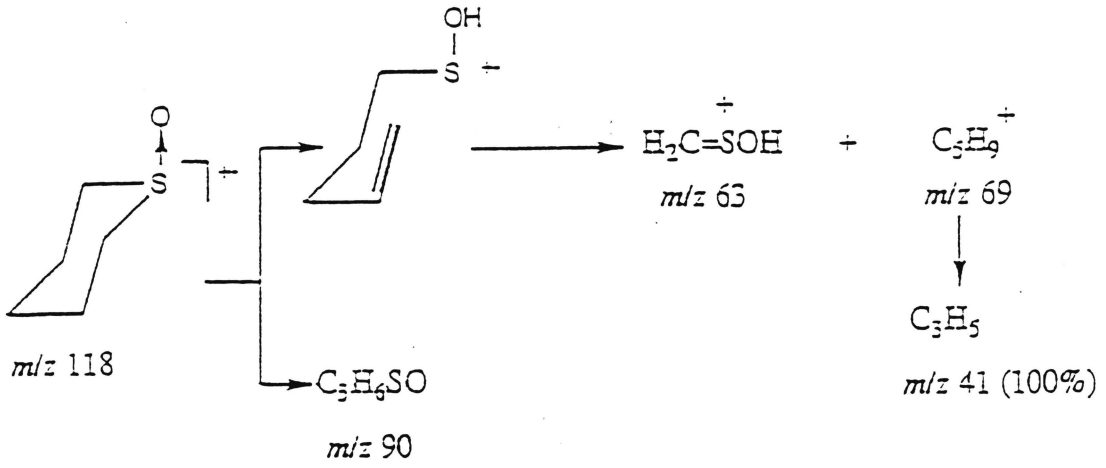


Figure 10

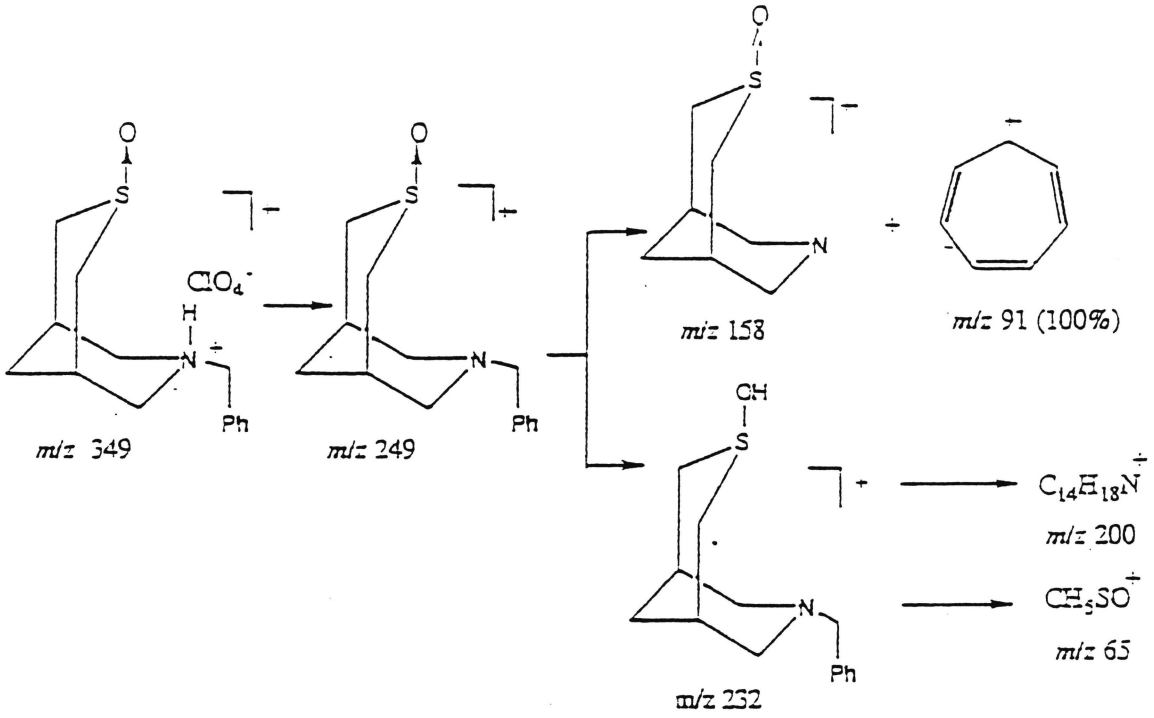


Figure 11

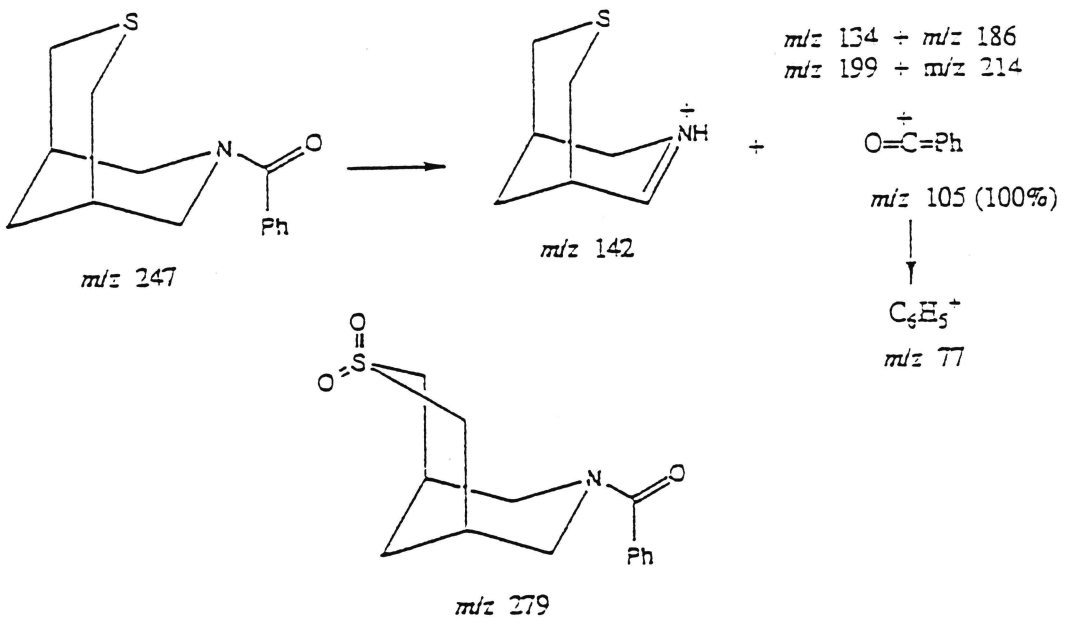

S
N
O
Ph
m/z 247
S
NH
m/z 142
+
m/z 134 + m/z 186
m/z 199 + m/z 214
O=C=Ph
m/z 105 (100%)
C6H5+
m/z 77
O
O=S
N
O
Ph
m/z 279

Figure 12

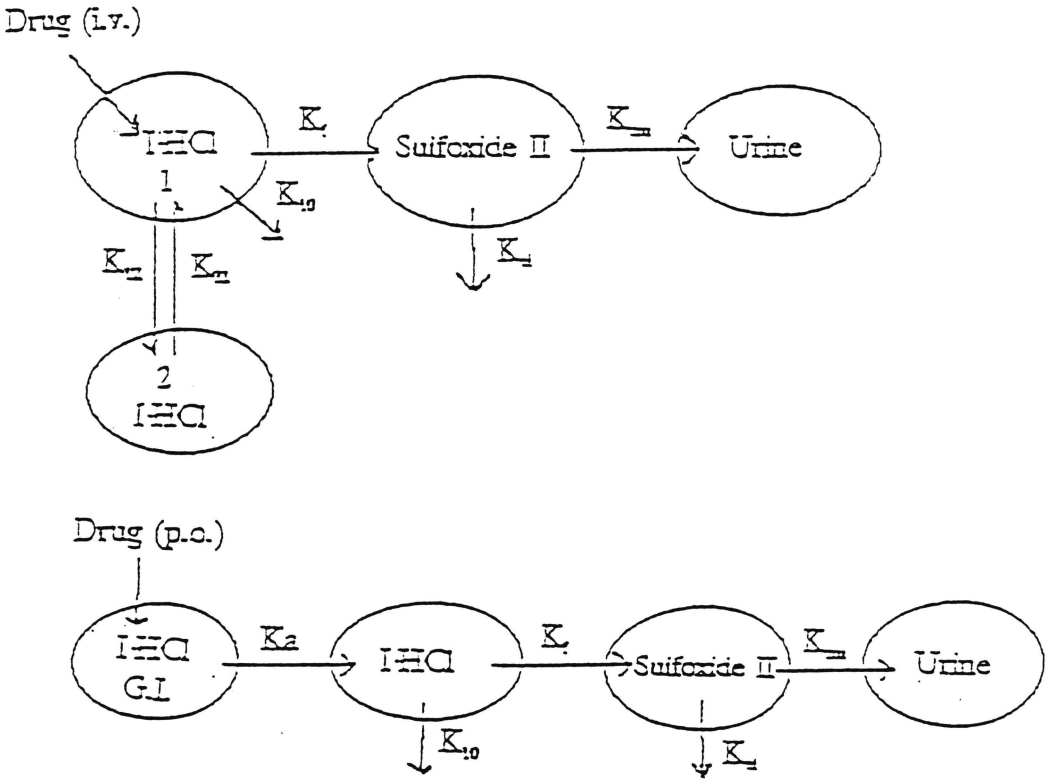

Drug (i.v.)
IHC
1
Sulfoxide II
Urine
2
IHC
Drug (p.o.)
IHC
GI
IHC
Sulfoxide II
Urine

Figure 13

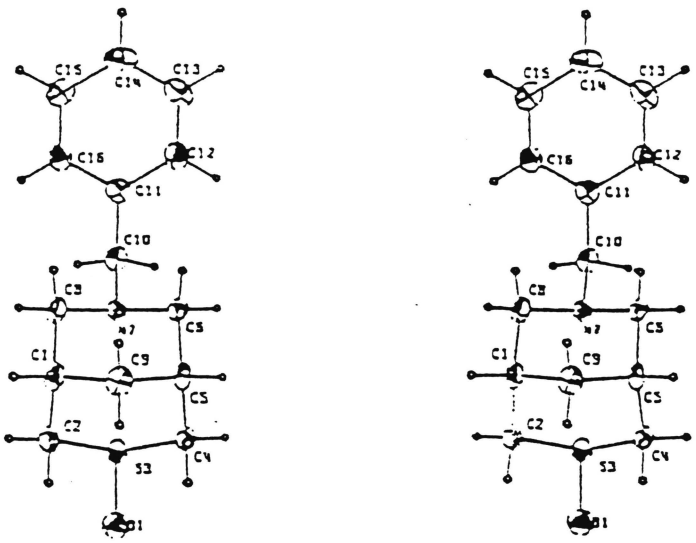
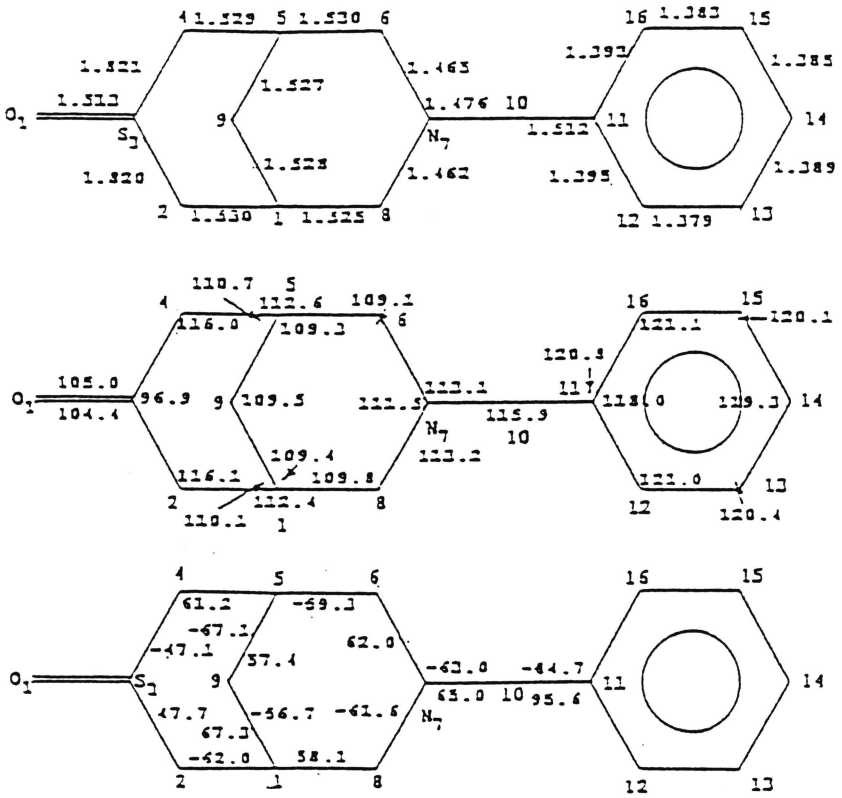

C15
C14
C13
C16
C12
C11
C10
C8
N7
C6
C1
C9
C5
C2
S3
C4
O1
C15
C14
C13
C16
C12
C11
C10
C8
N7
C6
C1
C9
C5
C2
S3
C4
O1

Figure 14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16 1.383 15
1.521
1.513
1.527
1.465
1.393
1.385
O1
9
1.476 10
1.512 11
14
S3
N7
1.523
1.462
1.395
1.389
1.520
2 1.530 1 1.525 8
12 1.379 13
110.7 5
4
112.6 109.1
16
15
116.0
109.3
6
121.1
120.1
120.8
105.0
96.9 9
109.5
111.1
117
118.0
119.3
14
O1
104.4
115.9
10
N7
123.2
109.4
116.1
109.8
121.0
13
2
112.4
8
12
120.4
110.1
1
4
5
6
16
15
61.2
-69.3
-67.1
-47.1
62.0
57.4
-63.0
-44.7
O1
S3
9
11
14
47.7
65.0 10 95.6
-56.7
-61.6
N7
67.3
-62.0
58.1
2
1
8
12
13

Figure 15

CHAPTER VII

ORAL, INTRAVENOUS PHARMACOKINETICS AND PLASMA PROTEIN BINDING OF GLG-V-13, A COMBINED CLASS IB AND CLASS III ANTIARRHYTHMIC AGENT

Abstract

GLG-V-13 {3-[4-(1*H*-imidazol-1-yl)benzoyl]-7-isopropyl-3,7-diazabicyclo[3.3.1]-nonane dihydroperchlorate} has been shown to be a combined class Ib and class III antiarrhythmic agent. The oral, intravenous pharmacokinetics and plasma protein binding of GLG-V-13 in dogs and in rabbits have now been investigated. Plasma GLG-V-13 concentration-time profiles, following an i.v. bolus dose of 6 mg/kg, were fitted to a 2-compartment model. The volume of distribution at steady state ($V_{d(ss)}$), the total systemic clearance (Cl_B), the elimination half-life ($t_{1/2\beta}$) and the MRT were 4.441 L/kg, 1.113 L/h/kg, 2.485 h and 3.879 h in dogs and 3.723 L/kg, 1.548 L/h/kg, 1.401 h, and 2.26 h in rabbits. The MRT in dogs is significantly longer than that in rabbits. Following i.v. dosing, approximately 9.38% of the parent compound was excreted in dogs urine (0-72 h). Changes in plasma GLG-V-13 concentrations, after oral administration of GLG-V-13 (6 mg/kg), were best described by the 1-compartment pharmacokinetic model. The T_{max} and C_{max} were 1.694 h, 0.54 mg/L in dogs and 1.44 h, 0.35 mg/L in rabbits. On oral administration, GLG-V-13 was moderately eliminated ($t_{1/2kel}$, 1.867 h^{-1} in dogs and 3.961 h^{-1} in rabbits, respectively).

Oral bioavailability was estimated to be 53.2%±11.3% in dogs and 66.7%±7.7% in rabbits. The volume of distribution in the central compartment in rabbits was significantly larger than that in the dogs after oral administration of GLG-V-13. About 8.74% of the oral dose (6 mg/kg) was excreted via the dog urine (0-72 h). Protein binding was performed *in vitro* and *ex vivo* by equilibrium dialysis at 37 °C. *In vitro* binding of GLG-V-13 to dog plasma protein was 29.4±9.90% (from 0.5 to 4 mg/L). *Ex vivo* binding of GLG-V-13 to dog plasma protein was 10.4±7.20%. Thus, there is a significant difference between *in vitro* and *ex vivo* binding of GLG-V-13 to plasma protein. Reasonably good oral bioavailability, prolonged antiarrhythmic properties, and the lack of proarrhythmic effects make this agent worthy of further study.

Introduction

Most currently available antiarrhythmic drugs are less than ideal for therapeutic management of clinical cases of various arrhythmias (Nygaard et al., 1986; Woosley, 1991). Many class I agents have proarrhythmic and negative inotropic effects (Woosley, 1991). Since the Cardiac Arrhythmias Suppression Trial (CAST) (Rogers et al., 1989; Echt, 1991; Rogers et al., 1991), the use of class I antiarrhythmic agents in patients has been limited because such agents tested were found to increase mortality. Class II agents are limited by their depressant effects on nodal function (Woosley, 1991; Teo et al., 1993). Some class IV agents have not been very effective against ventricular arrhythmias (Woosley, 1990; 1991). Several trials have demonstrated that class III agents provide better therapeutic results than those of other class agents (Mason et al., 1989; Burkurt et al., 1990; Cairns, 1991; Campbell, 1993; Greene et al., 1993). However, current class III agents, such as sotalol and amiodarone, are no longer considered as pure class III agents, since they possess several other actions and serious side effects typical of class III agents. Therefore, seeking new antiarrhythmic agents remains a serious challenge to cardiovascular scientists.

3,7-Diheterobicyclo[3.3.1]nonane (DHBCN) derivatives have demonstrated antiarrhythmic properties (Jeyaraman and Avila, 1981; Bailey et al., 1984; Hartenstein and Wager, 1986; Thompson et al, 1987; Smith et al., 1990). Several DHBCN derivatives exhibited antiarrhythmic activity in animal models and therefore are viable candidates for the treatment of life-threatening disorders in humans who experience sudden heart attacks or major infarctions of the heart (Zisman, 1989; Beatch et al.,

1991; Berlin et al., 1992; Garrison, 1993). One of the DHBCN derivatives, GLG-V-13, 3-[4-(1H-imidazol-1-yl)benzoyl]-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane dihydroperchlorate (Figure 1), has been demonstrated to prevent sustained monomorphic ventricular tachycardia in 5/6 dogs with proarrhythmic action seen only in one dog (Fazekas et al., 1993a). The drug significantly increases the refractory period of the right ventricle and prolongs AH, HV, and the QT intervals (Fazekas, 1992: 1993a; 1993b; 1994). GLG-V-13 appears to have some class Ib activity and most significant class III antiarrhythmic activity without hemodynamic depressant effect (Fazekas et al., 1992; 1993a; 1993b; 1994). Additional characterization of electrophysiological effects of GLG-V-13 in normal and ischemically-injured myocardial cells is in progress (Patterson et al., 1994).

The long duration of pharmacological effects, low proarrhythmic activity, apparent lack of cardiodepressent effects or reverse use-dependency, and combined class Ib and class III antiarrhythmic actions of GLG-V-13 make this compound a most promising candidate as an antiarrhythmic agent. Thus, there is merit in characterizing the pharmacokinetics of GLG-V-13 in animals. The purpose of these studies was to characterize disposition and protein binding of GLG-V-13 using a reversed-phase HPLC technique reported previously (Chen et al., 1994).

Materials and Methods

Chemicals

All the reagents used in this study were HPLC grade. Deionized water filtered through a Milli-Q™ water system (Millipore Corp., Marlborough, MA) was used.

Acetonitrile, methanol, chloroform, and potassium phosphate monobasic (Fisher Chemicals, Fair Lawn, NJ) and triethylamine (Pierce Chemical Co. Rockford, IL) were employed in this study as purchased. GLG-V-13 was synthesized by the established method (Garrison et al., 1993). The SAZ-VII-22 {(3-(4-chlorobenzyl)-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane} (Figure 1), was synthesized via a Mannich reaction starting from 4-thianone (Zisman et al. 1990) and was used as an internal standard.

Animals

Five adult, male mongrel dogs (20±1.2 kg) and ten New Zealand White rabbits (2.9±0.3 kg) were preconditioned for 7 days and were housed in a controlled environment (12-hour light/12-hour dark photoperiod, 22±1 °C, 60±10% relative humidity). The dogs and rabbits were allowed free access to food and water. Twenty-four hours before the experiments, food was withdrawn. The dogs and rabbits had free access to water before and during the experiments.

Sample Collection from Dogs

The dogs had polyethylene cannulas implanted in the right and left cephalic veins (for drug administration and collection of blood samples). All experiments were initiated between 0900 and 1100 h. GLG-V-13 was dissolved in water immediately before each experiment. Intravenous administration of GLG-V-13 (6 mg/kg) was done by bolus injection, and the cannulas were flushed immediately with 5 ml of sterile heparinized saline. Following two weeks after intravenous administration of GLG-V-13, the five dogs were considered ready for use in the oral pharmacokinetic

studies. Following administration of an oral dose of 6 mg/kg, blood samples (5 ml) were collected via one of the cephalic vein catheters at 0, 5, 10, 30, and 45 min and at 1, 2, 4, 6, 8, and 12 hr periods. After each sampling, the lost blood was replaced with an equal volume of sterile heparinized saline. Blood samples were heparinized, and centrifuged at 2000 x g for 10 min; plasma samples were collected and stored at -20 °C until analysis. Urine samples were collected before administration of the drug to serve as control and at 12, 24, 48, and 72 h after administration of the drug via an urinary catheter. After measuring the volume, the urine samples were stored at -20 °C until analysis. Aliquots of 0.25 ml of plasma and of 0.1 ml urine were used for HPLC assay.

Sample Collection from Rabbits

The procedures for collecting blood samples from rabbits were the same as from dogs except drug administration and sample collection (Ca. 0.5 ml) utilized an ear vein. Aliquots of 0.2 ml of plasma were used for HPLC analysis.

Sample Extraction

Twenty-five μl of the concentration of 12 μg/ml of an internal standard was added to 250 μl of dog plasma samples or buffer (for rabbit, 200 μl of plasma). After alkalinization with 100 μl of 5 *M* NaOH, 6 ml of chloroform was added, and the mixture was vortexed for 3 min. Following centrifugation (1000 x g, 10 min), the organic phase was transferred to a clean test tube. The chloroform extracts were evaporated to dryness under a stream of N_2. The residue was reconstituted in 50 μl of methanol, and 35 μl of this solution was injected for HPLC analysis. Extraction

of GLG-V-13 from urine was similar to that from plasma, except aliquots of 0.1 ml of dog urine were used.

HPLC Determination of GLG-V-13

GLG-V-13 concentrations in plasma, urine and buffer from equilibrium dialysis were determined using a recently developed reversed-phase high-performance liquid chromatographic method (Chen et al. 1994). The Waters HPLC system (Waters, Millipore Co., Milford, MA) was comprised of a pump (model 501), a tunable absorption detector (Waters 484) and chromatographic software (Maxima 820). A Ultramex 5 C_6 column (5 μm, 250 x 4.6 mm) and Ultramex 5 C_6 guard column (5 μm, 30 x 4.6 mm) purchased from Phenomenex (Torrance, CA) were used to resolve GLG-V-13 and SAZ-VII-22 (internal standard). The mobile phase was acetonitrile-methanol-37.5 m*M* phosphate buffer, pH 6.8 (27:27:46 v/v) containing 3.6 m*M* triethylamine. The column was eluted under isocratic conditions utilizing a flow rate of 1.2 ml/min at ambient temperature. The detection wavelength was 256 nm for GLG-V-13. Standard curves were prepared daily by comparing peak area ratios of GLG-V-13 concentrations in plasma, urine and buffer to those of the internal standard.

Pharmacokinetic Analysis

Data fitting and pharmacokinetic parameter calculations were carried out initially by estimation using the Boomer program (Bourne, et al. 1989) and finally by analysis using PharmK program (Lu and Mao, 1993). An appropriate pharmacokinetic model was chosen on the basis of lowest weighted squared residuals,

lowest Akaike's information criterion (AIC) value, R-squared, and correlation coefficiency (Akaike 1973; Boxenbaum et al. 1974; Yamaoka et al. 1978). The area under the curve (AUC) was calculated by the trapezoidal rule between first (0 hours) and last sampling times plus C/k, where C is the concentration of last sampling and k is the elimination rate constant (Gibaldi et al. 1975). Systemic clearance (Cl_B) was determined by dividing the dose by the AUC. Mean residence time (MRT) was calculated by dividing the area under the first moment curve (AUMC) by AUC (Mayer et al. 1988). The volume of distribution at steady state (V_{dss}) was calculated as the product of Cl_B and MRT. The time (T_{max}) taken to achieve peak concentration (C_{max}) was calculated using differential calculus (Gibaldi et al. 1975). Bioavailability (F) was estimated by the following equation:

$$F\ (\%) = AUC_{po} \times Dose_{iv}/AUC_{iv} \times Dose_{po}$$

The results are presented as means and standard deviations, except for pharmacokinetic parameters which are not normally distributed and are presented as medians (ranges).

Binding to Plasma Protein *In Vitro* and *Ex Vivo*

Plasma protein binding was determined as previously described (Chen et al., 1993). An equilibrium dialysis technique was performed at 37 °C using a 5-cell model of a Spectrum Equilibrium Dialyzer (Spectrum Medical Industries, Inc., Los Angles, CA). The cells were separated by a semipermeable membrane (Spectrum Medical Industries, Inc., Los Angles, CA), with a molecular weight cutoff of 3,500, which was rinsed in the above isotonic buffer for at least 24 hrs. For *in vitro* protein binding, one milliliter of dog plasma was dialyzed against 1 ml of an isotonic buffer solution

of 0.05 *M* phosphate and 0.07 *M* NaCl, pH 7.4. The various concentrations of 0.5, 1, 2 and 4 μg/ml of GLG-V-13 were added to the buffer side. The cells were incubated at 37 °C for 8 hrs with gentle shaking (12 rpm). For *ex vivo* protein binding, 0.5 ml of plasma (5 min after intravenous administration of 6 mg/kg of GLG-V-13) was dialyzed against the same volume of the above isotonic buffer solution. In preliminary studies, it was found that equilibrium was achieved within 4.5 hr. GLG-V-13 was stable under these conditions. There was 93.6±6.0% of total recovery of GLG-V-13 from both sides of the dialysis chamber, suggesting that no significant binding to the membrane occurs. After 8 hrs, it was presumed that the system was at equilibrium, and 250 μl aliquot were taken from both chambers. The HPLC method described above was used to determine the concentrations of GLG-V-13 both in the plasma side and the buffer side. Standard curves were prepared daily using peak-area ratios of various GLG-V-13 concentrations in plasma or buffer prepared as above. A blank plasma sample was dialyzed in each binding experiment to assess the presence of endogenous or exogenous compounds that might interfere with the GLG-V-13 measurements. The percentage of binding was calculated as a ratio of (C_p-C_b) over (C_p).

Results

Intravenous Pharmacokinetic Profiles

The plasma GLG-V-13 concentration-time curves in dogs and in rabbits are presented in Figures 2 and 3, respectively. The concentration range of GLG-V-13 was 0.08-2.46 mg/L in dogs, and 0.07-2.69 mg/L in rabbits following iv of 6 mg/kg (n

= 5), respectively. The plasma concentration data of GLG-V-13 in dogs and rabbits, after intravenous administration of 6 mg/kg to both dogs and rabbits, were best fitted to the exponential equation shown below.

$$C(t) = A_1e^{-\alpha t} + A_2e^{-\beta t}$$

The C(t) is the plasma GLG-V-13 concentration at time t; A_1, A_2, B_1 and B_2 represent the mathematic coefficients; α and ß represent the hybrid rate constants for the distribution phase and the terminal elimination phase, respectively.

Pharmacokinetic parameters of intravenous administration of GLG-V-13 are presented in Table 1. Following intravenous administration, disposition of GLG-V-13 was best described by a two-compartment open model with a moderate rate of elimination $t_{1/2\beta}$ (2.485 h in dogs and 1.401 h in rabbits) and extensive distribution ($V_{d(ss)}$ = 4.441 L/kg in dogs and 3.723 L/kg in rabbits).

Oral Pharmacokinetic Profiles

The concentration range of GLG-V-13 was 0.02-0.53 mg/L in dogs (n = 4), and 0.15-0.32 mg/L in rabbits (n = 5) following oral administration of 6 mg/kg, respectively. The plasma concentration data of GLG-V-13, after intravenous administration of 6 mg/kg to both dogs and rabbits, were best fitted to the exponential equation shown below.

$$C(t) = A(e^{-kelt} - e^{-kat})$$

The C(t) is the plasma GLG-V-13 concentration at time t, A represents the mathematic coefficient, and k_a and k_{el} represent the first order elimination absorption and elimination rates, respectively.

The pharmacokinetic parameters of oral administration of GLG-V-13 are

presented in Table 2. Following oral administration, the change in concentration of GLG-V-13 in plasma with time was fitted to an one-compartment open model with relatively slow absorption (T_{max} was 1.694 h in dogs and 1.44 h in rabbits) and intermediate elimination rates ($t_{1/2kel}$ was 1.867 h in dogs and 3.961h in rabbits). The oral bioavailability was estimated to be 53.2±11.3% for dogs and 66.7±7.7% for rabbits.

Dog Urinary Excretion

About 9.38% of the intravenous and 8.74% of the oral dose of 6 mg/kg was eliminated as free parent compound in urine (0-72 h). Most of the parent GLG-V-13 was eliminated before 12 hr (Table 3).

Protein Binding

The plasma protein binding of GLG-V-13 *in vitro* remained relatively constant over a concentration of 0.5-4 mg/L with the mean binding percentage of 29.4±9.9% (Table 4). The mean plasma protein binding percentage of GLG-V-13 *ex vivo* was 10.4±7.2% (Table 5). There was a significant difference between *in vitro* and *ex vivo* plasma protein binding.

Discussion

The present results demonstrated that GLG-V-13 has reasonably good absorption with 53.2% and 66.7% oral bioavailability of the parent GLG-V-13 in dogs and rabbits, respectively. In experiments with dogs, repeated attempts to gavage one

dog failed. Therefore, calculation of oral bioavailability was based on data from four other dogs. It appears that there is a delay in absorption of GLG-V-13 from the gastrointestinal tract with t_{max} of 1.69 h in dogs and 1.44 h in rabbits. There is no significant difference in the oral absorption rate constants between dogs and rabbits, but the half-life of absorption is markedly shorter in rabbits than that in dogs, suggesting that there is a relatively rapid absorption in rabbits in comparison with that of dogs. However, the time required to reach the maximum plasma concentration in dogs and in rabbits was the same.

The apparent volume of distribution of GLG-V-13 in dogs and in rabbits was relatively large. It seems that a wide distribution is characteristic of DHBCN derivatives, such as BRB-I-28 (Alavi et al. 1991; Chen et al, 1993a), SAZ-VII-22 (Chen et al., 1993b), and SAZ-VII-23 (Chen et al, 1993c). However, GLG-V-13 has a relatively smaller volume of distribution than that of other DHBCN derivatives tested. This could be attributed to the fact that GLG-V-13 is more hydrophilic than other DHBCN derivatives. The lower percentage of the parent GLG-V-13 bound to the plasma proteins, which may facilitate escape of most of the drug from the vascular compartments to other extravascular tissues, and relatively rapid elimination from the body may have lowered the concentration of GLG-V-13 in plasma, leading to an increased volume of distribution in dogs, but not as great as with BRB-I-28. There is no significant difference in the volume(s) of distribution(s) after intravenous administration of GLG-V-13 in dogs and in rabbits. However, the volume of distribution of GLG-V-13, after oral administration, in the central compartment is significantly higher in rabbit than that in dogs.

GLG-V-13 has relatively rapid elimination rates in dogs and in rabbits.

Different elimination patterns were found in dogs and in rabbits with different routes of administration. After intravenous administration, the rabbits and dogs had similar elimination half-lives, but the mean residence time (MRT) in dogs was much longer than that in rabbits. Following oral administration, the elimination half-life in rabbits was much longer than that in dogs, but the MRT was similar in both species. These results suggest that GLG-V-13 has different pharmacokinetic patterns in different species.

It was interesting to observe that there was a significant difference between *ex vivo* and *in vitro* binding of GLG-V-13 to plasma protein. The reason for this is unknown.

GLG-V-13 was excreted in the free state in dog urine with most of parent compound being eliminated in the first 12 hr. A conjugated state of GLG-V-13 was not detected after urine samples were hydrolyzed with a crude solution of ß-glucuronidase. Minor elimination of parent GLG-V-13 (<10%) suggested that GLG-V-13 may have undergone extensive metabolism. A representative chromatogram with several peaks (Figure 4) of urine collected from a dog treated with intravenous administration of GLG-V-13 suggested the presence of various potential metabolites of GLG-V-13. Characterization of these possible metabolite(s) is in progress.

In summary, the data presented in this study indicate that GLG-V-13 has good oral bioavailability, wide distribution, and relatively rapid elimination rates in dogs and in rabbits. Considering that GLG-V-13 has a long duration of pharmacological effects, has very low toxicity profiles (unpublished data), has little proarrhythmic effects and lacks cardiodepressant actions compared to other DHBCN derivatives, the compound is worthy of continuing investigation as an antiarrhythmic agent.

References

Akaike, H. A new look at the statistical model identification. Institute of Electrical and Electronics Engineers (IEEE) Transactions on Automatic Control 19: 716-723, 1973.

Bailey, B. R., Berlin, K. D., Holt, E. M., Scherlag, B. J., Lazarra, R., Brachmann, J., Helm, D. V. D., Powell, D. R., Pantaleo, N. S., and Ruenitz, P. C., Synthesis, conformational analysis, and antiarrhythmic properties of 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonan-9-one, 7-benzyl-3-azabicyclo[3.3.1]nonane hydroperchlorate, and 7-benzyl-9-thia-7-azabicyclo[3.3.1]nonan-9-ol hydroperchlorates and derivatives: single-crystal x-ray diffraction analysis and evidence for chair-chair and chair-boat conformers in the solid state. Journal of Medicinal Chemistry 27: 758-767,1984.

Beatch, G. N., Abreham, S., Mac Lead, B. A., Yoshida, N. R., Walker, M. J. A. Antiarrhythmic properties of tedisamil (KC8857). A putative transient outward K^+ current blocker. Eur. J. Pharmacol. 102: 13-18, 1991.

Berlin, K. D., Scherlag, B. J., Clarke, C. R., Otiv, S. R., Zisman, S. A., Sangiah, S., Mulekar, S. V., Salts of 3-azabicyclo[3.3.1]nonanes as potential antiarrhythmic agents, and precursors thereof. U.S. Patent 5,084,572, 1992; Chem. Abstr. 115: 114550c, 1991.

Bourne, D. W. A. Boomer, a simulation and modeling program for pharmacokinetic and pharmacodynamic data analysis. Computer Methods and Programs in Biomedicine 29: 191-195, 1989.

Boxenbaum, H. G., Riegman, S., Elashoff, R. M. Statistical estimations in pharmacokinetics. J. Pharmacokinet. Biopharmacokinet. 2: 123-148, 1974.

Burkart, F., Pfisterer, M., Kiowski, W. Effects of antiarrhythmic therapy on mortality in survivors of myocardial infarction with asymptomatic complex ventricular arrhythmias: Basel antiarrhythmic study of infarct survival (BASIS). J. Am. Coll. Cardiol. 16: 1711-1718, 1990.

Cairns, J. A. Post-myocardial infarction mortality in patients with ventricular premature depolarizations: Canadian amiodarone myocardial infarction arrhythmia trial pilot study. Circulation. 84: 550-557, 1991.

Campbell, T. J. Class III antiarrhythmic action: the way forward? the Medical Journal of Australia. 158: 732-733, 1993.

Chen, C. L., Sangiah, S., Roder, J. D., Chen, H., Berlin, K. D., Garrison, G. L., Scherlag, B. J., Lazzara, R., and Patterson, E. Pharmacokinetics and plasma protein binding of BRB-I-28, a novel antiarrhythmic agent, in dog. Drug Invest. 6: 237-243, 1993a.

Chen, C. L., Sangiah, S., Roder, J. D., Chen, H., Berlin, K. D., Garrison, G. L., Scherlag, B. J., Lazzara, R., and Patterson, E. High-performance liquid chromatographic determination of SAZ-VII-22, a novel antiarrhythmic agent, in dog plasma and urine. Analytical Sciences 9: 429-431, 1993b.

Chen, C. L., Sangiah, S., Roder, J. D., Chen, H., Berlin, K. D., Garrison, G. L., Scherlag, B. J., Lazzara, R., and Patterson, E. High-performance liquid chromatographic determination of SAZ-VII-23, a novel antiarrhythmic agent, in dog plasma and urine. Analytical Letters. 26: 1125-1135, 1993c.

Chen, C. L., Sangiah, S., Roder, J. D., Chen, H., Berlin, K. D., Garrison, G. L., Scherlag, B. J., and Lazzara, R. Determination of GLG-V-13, a novel antiarrhythmic agent, in plasma and urine by high-performance liquid chromatography. J. Liquid Chromatogr. 17(4): 913-927, 1994.

Echt, D, S., Liebson, P. R., Mitchell, L. B., Peters, R. W., Obias-Manno, D., Barker, A. H., Arensburg, D., Baker, A., Friedman, L., Greene, H. L., Huther, M. L., Richardson, D. W., and the CAST Investigators. Mortality and morbidity in patients receiving encainide, flecainide, or placebo. The cardiac arrhythmia suppression trial. N. Engl. J. Med. 324: 781-788, 1991.

Fazekas, T., Mabo, P., Berlin, K. D., Scherlag, B. J., and lazzara, R. Comparative electrophysiological effects of a new chemical agents, 3,7-diheterabicyclo[3.3.1]nonanes, in the 1-4-day old infarcted dog heart. The Physiologists. 35, 221 (Abstract), 1992.

Fazekas, T., Scherlag, B. J., Mabo, P., Patterson, E., Berlin, K. D., Garrison, G. L. and Lazzara, R. Egy uj refrakter periodust megnyujto antiarimias vegyulet, a GLG-V-13 szivelektrofiziologiai jellemzese. Cardiologic Hungarica. 22: 9-15, 1993a.

Fazekas, T., Scherlag, B. J., Mabo, P., Berlin, K. D., Garrison, G. L., Chen, C. L., Sangiah, S., Patterson, E., and Lazzara, R. The comparative antiarrhythmic and proarrhythmic activity of a 3,7-diheterobicyclo[3.3.1]nonane, BRB-I-28, and lidocaine in the 1-4-day-old infarcted dog heart. Acta Physiologica Hungary. 81: 289-299, 1993b.

Fazekas, T., Scherlag, B. J., Mabo, P., Patterson, E., Berlin, K. D., Garrison, G. L., and Lazzara, R. Electrophysiological characterization of a new heart rate-lowering and refractoriness prolonging antiarrhythmic agent, GLG-V-13, in the 1-4 day-old infarcted dog heart. Acta Physiologica Hungarica. Submitted, 1994.

Garrison, G. L. Selected derivatives of 3,7-diheterobicyclo[3.3.1]nonanes which possess multi-class antiarrhythmic activity. Ph.D. Dissertation. Oklahoma State University, 1993.

Gibaldi, M., and Perrier, D., (Eds). Pharmacokinetics, 1st Ed., Vol. 1., Marcel Dekker, Inc., New York, 1975.

Greene, H. L., and The CASCADE Investigators. Randomized antiarrhythmic drug therapy in survivors of cardiac arrest (The CASCADE Study). Am. J. Cardiol. 72: 280-287, 1993.

Hartenstein, J., and Wager, B. Antiarrhythmic agents, in "Cardiovascular Drugs", Bristol, J. A., ed., John Wiley & Sons, New York, 1986, pp. 305-415.

Jeyaraman, R., and Avila, S. Chemistry of 3-azabicyclo[3.3.1]nonanes. Chemical Reviews. 81: 149-174 (1981).

Lu, D. R., and Mao, F. An interactive programs for pharmacokinetic modeling. J. Pharm. Sci. 82: 537-542, 1993.

Mason, J. W., and The ESVEM Investigators. The ESVEM Trial. Circulation. 79: 1354-1360, 1989.

Mayer, P. R., and Brazzell, R. K., Application of statistical moment theory to pharmacokinetics. The Journal of Clinic Pharmacology. 28: 481-483, 1988.

Nygaard, T. W., Sellers, T. D., Cook, T. S., and DiMarco, J. P. Adverse reactions to antiarrhythmic drugs during therapy for ventricular arrhythmias. J. Am. Med. Assoc. 256: 82-84, 1986.

Patterson, E., Scherlag, B. J., Berlin, K. D., Garrison, G. L., Sangiah, S., Chen, C. L., and Lazzara, R. Electrophysiological action of GLG-V-13 in canine myocardial tissues and in ischemically-injured canine myocardium. Unpublished data, 1994.

Rogers, W. J., and The CAST Investigators. Preliminary report: effect of encainide and flecainide on mortality in a randomized trial of arrhythmia suppression after myocardial infarction. N. Engl. J. Med. 321: 406-412, 1989.

Rogers, W. J., and The CAST Investigators. Mortality and morbidity in patients receiving encainide, flecainide, or placebo. N. Engl. J. Med. 324: 781-788, 1991.

Smith, G. S., Thompson, M. D., Berlin, K. D., Holt, E. M., Scherlag, B. J., Patterson, E., and Lazzara, R. A study of the synthesis and antiarrhythmic properties of selected 3,7-diheterobicyclo[3.3.1]nonanes with substituents at the 2,4-positions and at the 9-position. European Journal of Medicinal Chemistry 25: 1-8, 1990.

Teo, K. K., Yusuf, S., and Furberg, C. D. Effects of prophylactic antiarrhythmic drug therapy in acute myocardial infarction. An overview of results from randomized controlled trials. J. Am. Med. Assoc. 270: 1589-1595, 1993.

Thompson, M. D., Smith, G. S., Berlin, K. D., Holt, E. M., Scherlag, B. J., Helm, D.V.D., Muchmore, S. W., Fidelis, K. A. Synthesis and antiarrhythmic properties of novel 3-selena-7-benzyl-3-selena-7-azabicyclo[3.3.1]nonane-9-one and 7-benzyl-3-selena-7-azabicyclo[3.3.1]nonane hydroperchlorate. J. Med. Chem. 30: 780-788, 1987.

Woosley, R. L. Antiarrhythmic drugs. Annu. Rev. Pharmacol. Toxicol. 31: 427-455, 1991.

Woosley, R. L., and Roden, D. M. Pharmacological causes of arrhythmogenic actions of antiarrhythmic drugs. Am. J. Cardiol. 59: 19E-25E, 1987.

Yamaoka, K., Nakagawa, T., and Uno, T. Application of Akaike's information criterion (AIC) in the evaluation of linear pharmacokinetic equations. J. Pharmacokinet. Biopharmaceut. 6: 165-175, 1978.

Zisman, S. T., Berlin, K. D., and Scherlag, B. J. The preparation of amide derivatives of 3-azabicyclo[3.3.1]nonanes as new potential antiarrhythmic agents. Organic Preparations and Procedures Int. 22: 255-264, 1990.

Zisman, S. A. A study of stereochemical and substituent effects on antiarrhythmic activity of selected 3-azabicyclo[3.3.1]nonanes and derivatives. Ph.D. Dissertation. Oklahoma State University, 1989.

TABLE 1

PHARMACOKINETIC PARAMETERS FOLLOWING INTRAVENOUS BOLUS OF 6 MG/KG OF GLG-V-13 TO DOGS AND RABBITS

Parameters	Value (mean±SD)	
	Dogs (n = 5)	Rabbits (n = 5)
A1 (mg/L)	2.03 ± 1.21	2.09 ± 0.778
A2 (mg/L)	1.52 ± 0.38	1.32 ± 0.77
α (h^{-1})	8.079 ± 4.632	5.132 ± 3.305
ß (h^{-1})	0.301 ± 0.101	0.453 ± 0.214
K_{10} (h^{-1})	0.6880 ± 0.3796	0.9622 ± 0.400
K_{12} (h^{-1})	3.9773 ± 2.8720	2.1898 ± 1.6065
K_{21} (h^{-1})	3.7145 ± 2.2027	2.6129 ± 1.5777
$t_{1/2\alpha}$ (h)[a]	0.073 (0.052-0.483)	0.121 (0.079-0.767)
$t_{1/2ß}$ (h)[a]	2.485 (1.456-2.985)	1.401 (1.007-5.226)
V_c (L/kg)[a]	1.843 (1.094-3.155)	1.721 (1.133-2.677)
$V_{d(area)}$ (L/kg)[a]	3.598 (2.882-4.775)	3.477 (2.496-11.59)
$V_{d(ss)}$ (L/kg)[a]	4.441 (2.659-5.718)	3.723 (3.166-3.979)
Cl_B (ml/h/kg)[a]	1.113 (0.781-1.713)	1.548 (1.424-1.720)
AUC_{iv} (mg·h/L)	5.672 ± 1.787	3.761 ± 0.361
$AUMC_{iv}$ (mg·h^2/L)	22.316 ± 9.1408	8.567 ± 1.838*
MRT_{iv} (h)[a]	3.87 ± 0.62	2.26 ± 0.33**

[a] presented as median (range); *$p<0.05$; **$p<0.01$.

Abbreviations: K_{10}-first-order elimination rate constant; K_{12}, K_{21} are the first-order rate constants describing distribution between central (plasma) and peripheral compartment (tissues); $t_{1/2\alpha}$ is distribution half-life after iv; $t_{1/2ß}$ is elimination half-life after iv; V_c = volume of the central compartment; $V_{d(area)}$ is apparent volume distribution calculated using AUC; $Vd_{(ss)}$ is apparent volume of distribution at steady state; Cl_B is body clearance of the drug.

TABLE 2

PHARMACOKINETIC PARAMETERS FOLLOWING ORAL ADMINISTRATION OF 6 MG/KG OF GLG-V-13 TO DOGS AND RABBITS

Parameters	Value (mean±SD)	
	Dogs (n = 4)	Rabbits (n = 5)
A	2.1831 ± 1.3167	0.4899 ± 0.1296
K_{el} (h^{-1})[a]	0.3955 ± 0.0810	0.1886 ± 0.0647**
K_a (h^{-1})[a]	0.8802 ± 0.1917	2.7403 ± 2.3738
$t_{1/2k\alpha}$ (h)[a]	0.750 (0.671-1.113)	0.439 (0.103-0.730)*
$t_{1/2kel}$ (h)[a]	1.867 (1.353-2.121)	3.961 (2.545-6.736)*
Cl_B[a]	2.403 (1.412-3.486)	2.557 (2.064-2.799)
V_c[a]	2.828 (2.177-5.241)	10.14 (6.595-13.377)**
AUC_{po} (mg·h/L)	2.755 ± 1.088	2.508 ± 0.288
$AUMC_{po}$ (mg·h^2/L)	12.81 ± 4.520	17.47 ± 7.587
MRT_{po} (h)	4.70 ± 0.36	6.83 ± 2.23
C_{max} (mg/L)	0.54 ± 0.14	0.35 ± 0.12
t_{max} (h)	1.69 ± 0.28	1.44 ± 0.72
F (%)	53.2 ± 11.3	66.7 ± 7.7%

[a] presented as median (range); *$p<0.05$; **$p<0.01$.

Abbreviations: K_{el} is first-order elimination rate constant; K_a is the first-order absorption rate constant; $t_{1/2k\alpha}$ is absorption half-life after p.o.; $t_{1/2kel}$ is elimination half-life after p.o.; V_c = volume of the central compartment; Cl_B is body clearance of the drug; t_{max} is the time to reach the maximum concentration in plasma; F is oral bioavailability.

TABLE 3

ELIMINATION OF FREE PARENT GLG-V-13 IN DOG URINE

Time (h)	Amount (% of dose)	
	iv (6 mg/kg) (n = 5)	po (6 mg/kg) (n = 4)
0-12	8.42 ± 9.56	7.13 ± 2.24
12-24	0.59 ± 0.21	1.03 ± 0.32
24-48	0.28 ± 0.09	0.43 ± 0.31
48-72	0.10 ± 0.06	0.15 ± 0.07
Total	9.38 ± 9.59	8.74 ± 2.15

TABLE 4

IN VITRO BINDING OF GLG-V-13 TO DOG PLASMA PROTEIN
(MEAN±SD, N = 5)

Initial Concentration[a] mg/L	C_p[b] mg/L	C_b[c] mg/L	Binding %
0.5	0.27 ± 0.02	0.18 ± 0.03	33.8 ± 13.1
1	0.53 ± 0.03	0.36 ± 0.04	32.7 ± 9.40
2	0.94 ± 0.05	0.73 ± 0.07	22.6 ± 7.70
4	1.97 ± 0.10	1.40 ± 0.14	28.6 ± 6.80
Average			29.4 ± 9.90

[a] Total concentration at buffer side;
[b]C_p: Concentration on plasma side at equilibrium.
[c]C_b: Concentration on buffer side at equilibrium.

TABLE 5

EX VIVO BINDING OF GLG-V-13 TO DOG PLASMA PROTEIN
(MEAN±SD, N = 5)

Dog No.	C_p[a] mg/L	C_b[b] mg/L	Binding %
1	0.55	0.63	13.3
2	0.90	1.13	20.4
3	0.53	0.55	3.60
4	0.86	0.89	3.40
5	0.31	0.35	11.4
Average	0.63 ± 0.25	0.71 ± 0.30	10.4 ± 7.2

[a]C_p: Concentration on plasma side at equilibrium.
[b]C_b: Concentration on buffer side at equilibrium.

Figure Legends

Figure 1. Structure of GLG-V-13 {3-[4-(1*H*-imidazol-1-yl)benzoyl]-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane dihydroperchlorate} (A). The molecular weight is 539.37. B is the SAZ-VII-22, the internal standard.

Figure 2. Mean (±SD) plasma concentration-time curves of GLG-V-13 in dogs following i.v. bolus (6 mg/kg; n = 5) (●) and oral administration (6 mg/kg; n = 4) (o).

Figure 3. Mean (±SD) plasma concentration-time curves of GLG-V-13 in rabbits following i.v. bolus (6 mg/kg; n = 5) (●) and oral administration (6 mg/kg; n = 5) (o).

Figure 4. The representative HPLC chromatograms from dogs treated with GLG-V-13. (a) 0 hr blank urine; (b) 0-12 hr urine; (c) 12-24 hr urine; (d) 24-48 hr urine; (e) 48-72 urine. Pearks 1-4 are possible metabolites. Peak 5 is the parent compound, GLG-V-13.

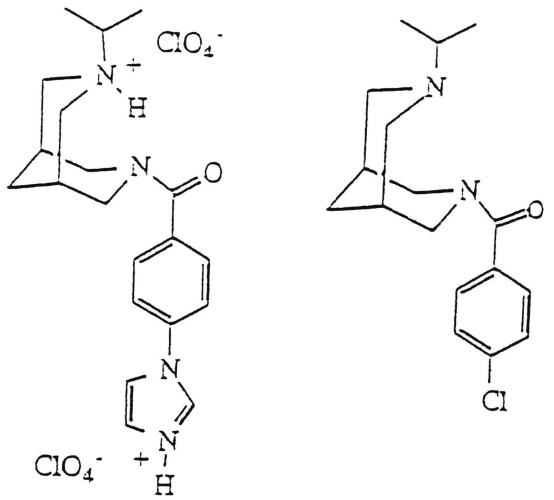


Figure 1

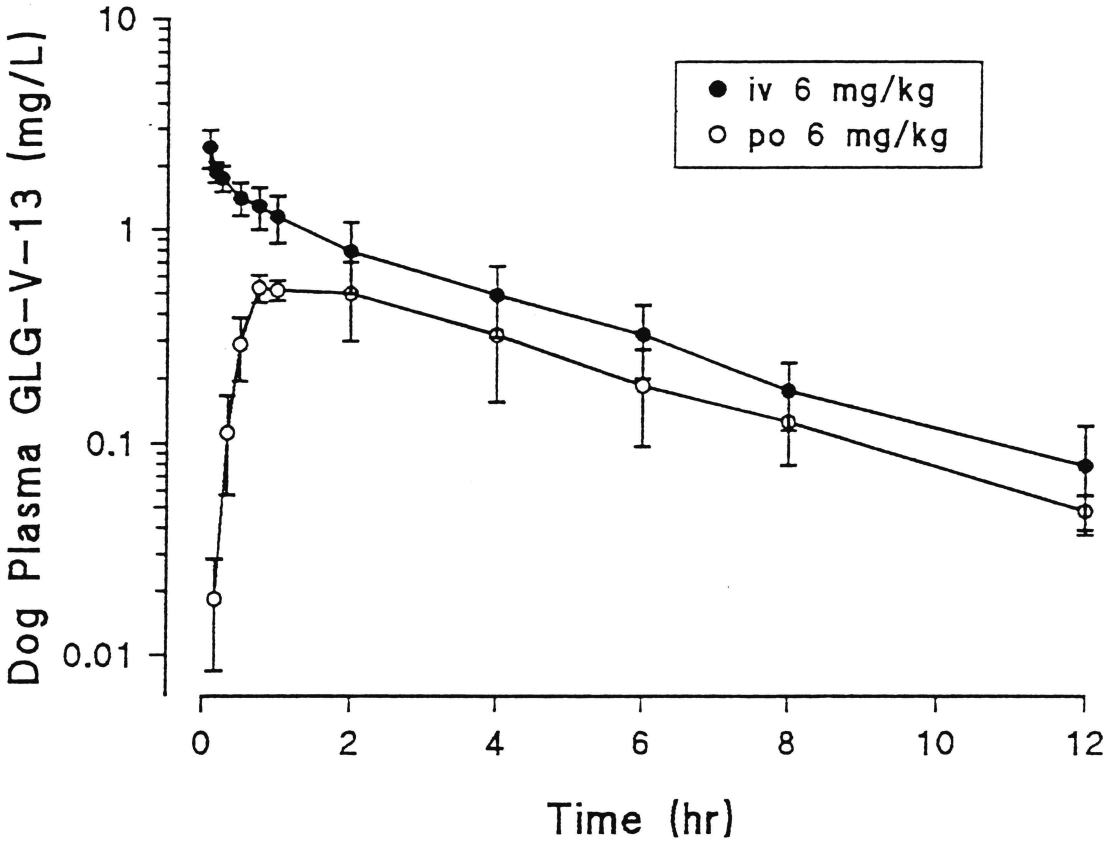
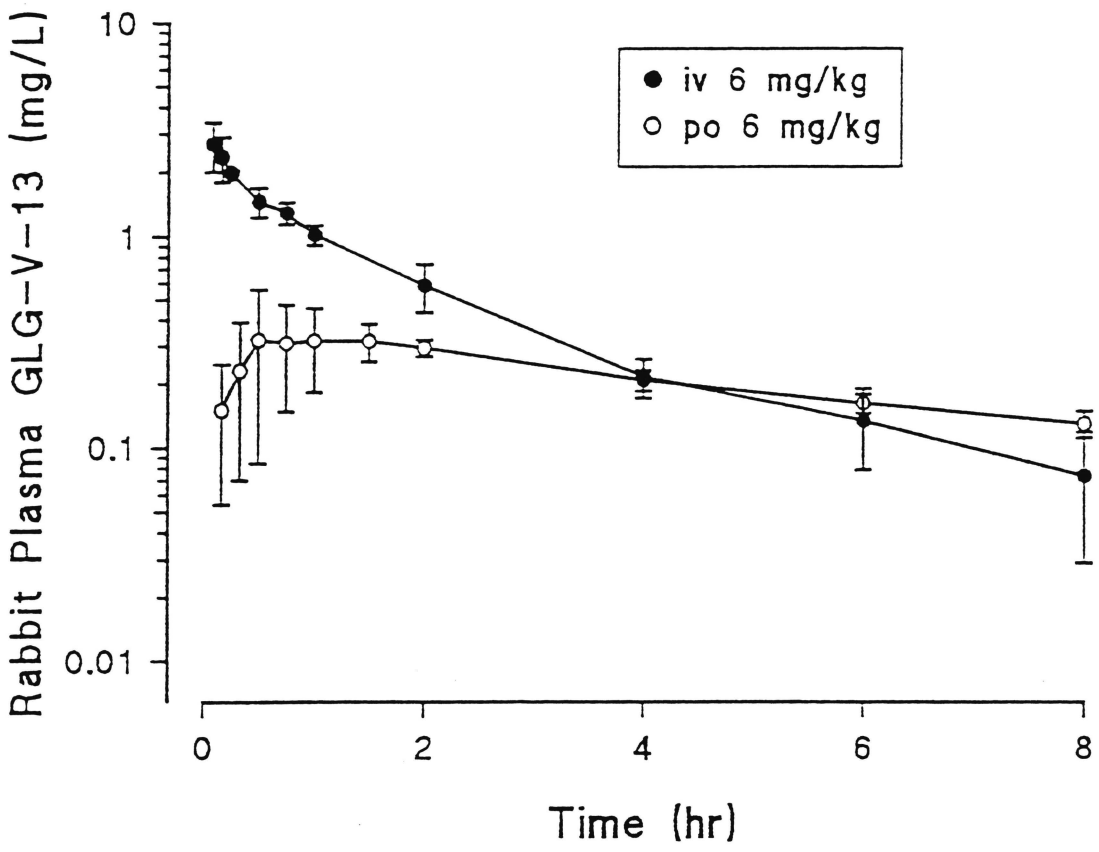

Dog Plasma GLG-V-13 (mg/L)
10
1
0.1
0.01
0
2
4
6
8
10
12
Time (hr)
iv 6 mg/kg
po 6 mg/kg

Figure 2


Rabbit Plasma GLG-V-13 (mg/L)
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1
0.1
0.01
iv 6 mg/kg
po 6 mg/kg
0
2
4
6
8
Time (hr)

Figure 3

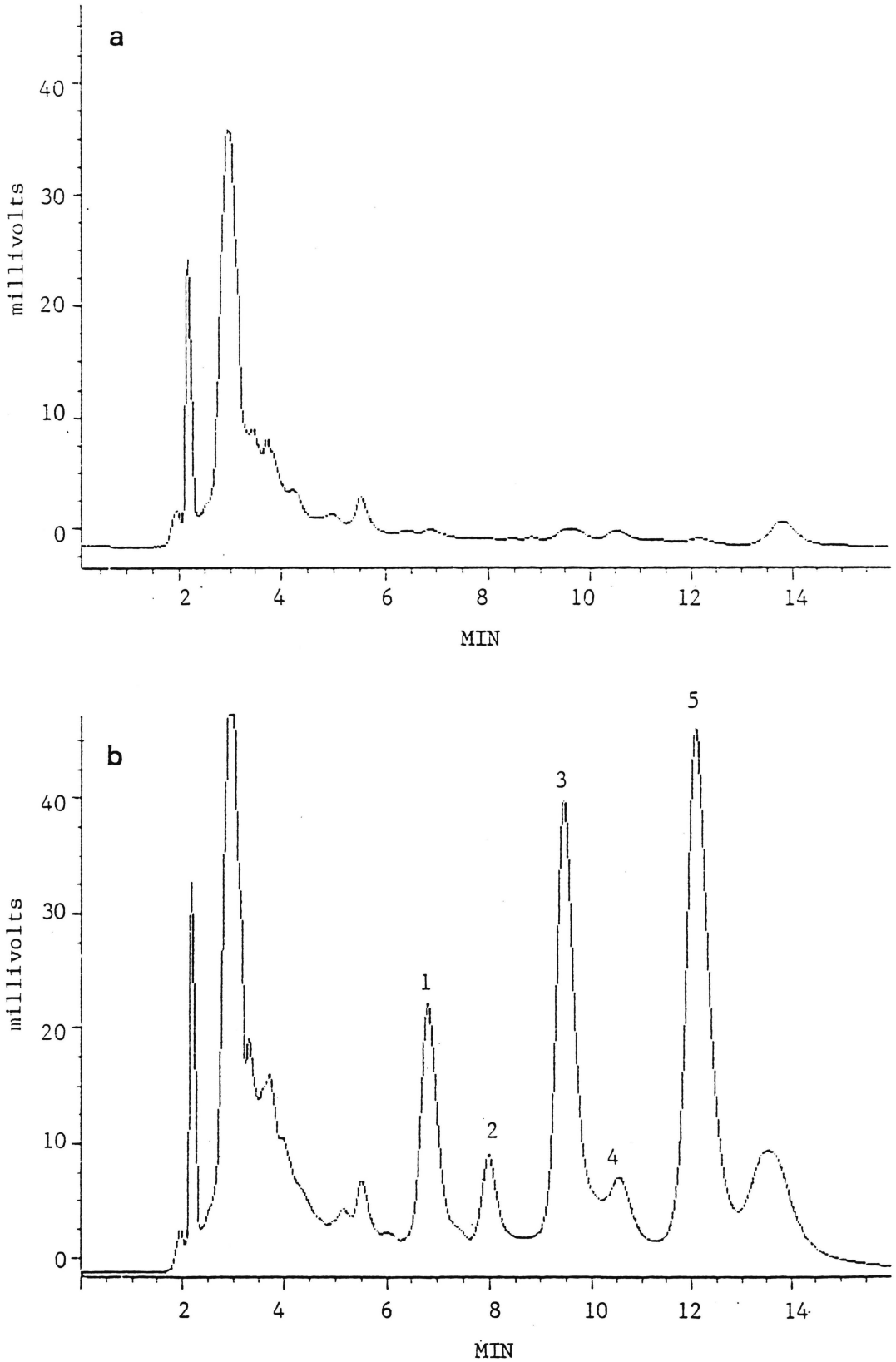
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Figure 4

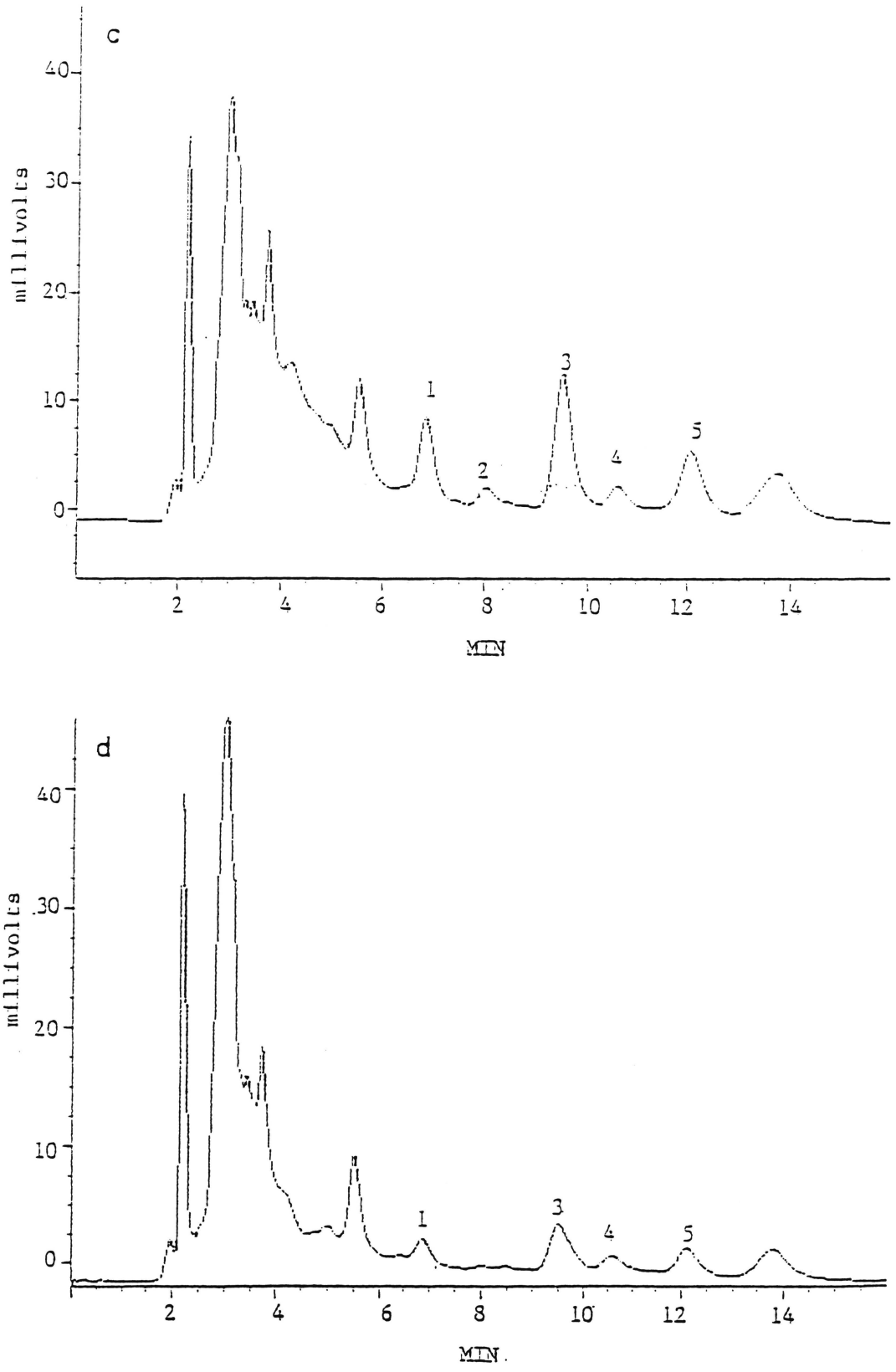
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Figure 4

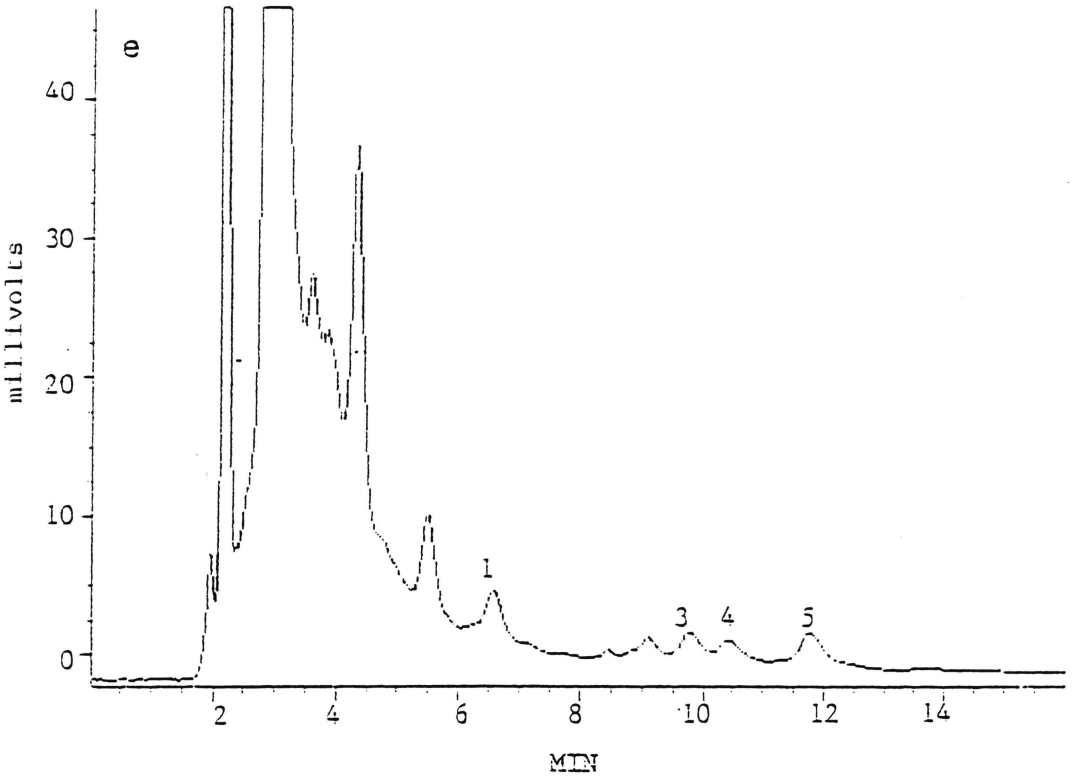


Figure 4

CHAPTER VIII

EFFECTS OF BRB-I-28, A NOVEL ANTIARRHYTHMIC AGENT, AND ITS DERIVATIVES ON CARDIAC Na^+,K^+-ATPASE, Mg^{2+}-ATPASE ACTIVITIES AND CONTRACTILE FORCE

Abstract

The effects of BRB-I-28, SAZ-VII-22 and SAZ-VII-23, a novel class of antiarrhythmic agents, and other 3,7-diheterobicyclo[3.3.1]nonane (DHBCN) derivatives on guinea pig myocardial Na^+,K^+-ATPase and Mg^{2+}-activated ATPase activities were investigated in comparison with those of tedisamil, lidocaine and ouabain. BRB-I-28, SAZ-VII-22, SAZ-VII-23, tedisamil and their derivatives produced concentration-dependent inhibition on both Na^+,K^+-ATPase and Mg^{2+}-activated ATPase. Ouabain had no effect on the Mg^{2+}-activated ATPase activity, and GLG-IV-44 had no significant inhibition on Na^+,K^+-ATPase. Molar refractivity, retention time in reverse-phase HPLC, and partition coefficients were determined, and the influence of these three parameters on the inhibitory effects of DHBCN on ATPase was examined. It seems that inhibitory effects of DHBCN derivatives on Na^+,K^+-ATPase and Mg^{2+}-activated ATPase increase with an increase in lipophilicity, while hydrophilic groups of the drugs may not be important for interaction between drugs and ATPases. The effects of BRB-I-28 on contractile force development in rabbit atrial and papillary muscles were studied. At paced rates of 0.5 and 1.0 Hz in

atrial muscle, BRB-I-28 produced an apparent positive inotropic effect in isolated rabbit atrial muscle, which is consistent with its inhibitory effects on Na^+,K^+-ATPase and Mg^{2+}-ATPase activities. Inhibitory effects on myocardial Na^+,K^+-ATPase and Mg^{2+}-activated ATPase activities may be the basis of some electrophysiological effects of antiarrhythmic properties of BRB-I-28, SAZ-VII-22, SAZ-VII-23, and tedisamil.

Introduction

3,7-Diheterobicyclo[3.3.1]nonane (DHBCN) derivatives are known to possess potential antiarrhythmic properties (Jeyaraman et al.,1981). BRB-I-28 (7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane hydroperchlorate) and its derivatives, such as SAZ-VII-22 and SAZ-VII-23, are recently developed novel antiarrhythmic agents belonging to the family of DHBCN (Figure 1).

Preliminary studies in dogs established that BRB-I-28 is more effective than lidocaine in inhibiting induced ventricular tachycardia (Bailey et al., 1984; Scherlage et al., 1988). BRB-I-28 and its derivatives were reported to have electrophysiological properties of class I antiarrhythmic drugs (Patterson et al., 1991). It was recently suggested that some of the antiarrhythmic properties of class I agents could possibly be mediated by inhibition of myocardial Na^+,K^+-ATPase activity (Almotrefi and Dzimiri, 1990,1991). It has been reported that both chemical structure and lipophilicity strongly influence inhibitory effect on Na^+,K^+-ATPase (Fricke et al., 1981; Dzimiri et al., 1987). The purpose of this study was to determine the effects of BRB-I-28, SAZ-VII-22, SAZ-VII-23 and some of their derivatives on guinea pig myocardial Na^+,K^+-ATPase and Mg^{2+}-activated ATPase in comparison with those of tedisamil, a newer class III antiarrhythmic agent (Oexcle et al., 1987), lidocaine, a class IB antiarrhythmic agent, and ouabain, a specific inhibitor of Na^+K^+-ATPase. An attempt was made to examine the relationship between lipophilic properties and molar refractivities of DHBCN derivatives and their inhibitory effects on myocardial Na^+,K^+-ATPase and Mg^{2+}-activated ATPase. In addition, the effects of BRB-I-28 on the contractile force of isolated rabbit atrial and papillary muscle were

determined.

Materials and Methods

Preparation of Myocardial ATPase Enzymes

Adult, male, albino, guinea pigs weighing 650±80 g were sacrificed by a blow on the head and exsanguinated. Myocardial Na^+,K^+-ATPase was prepared following a modified procedure of Akera (Akera et al., 1969). The hearts were rapidly removed and the ventricular muscles were washed several times with ice-cooled isotonic solution containing 0.25 *M* sucrose, 5.0 *mM* L-histidine, 5.0 m*M* ethylene diamine tetraacetate (EDTA·Na_2) and 0.15% sodium deoxycholate, pH 6.8 with Trizma® base. About 15 g of muscle was minced and homogenized in a polytron homogenizer (Polytron® Model#PT 10/35, Brinkman Instruments, Switzerland) three times with 20 seconds duration and with 6 volumes of above ice-cooled isotonic sucrose solution. The homogenate was centrifuged for 30 minutes at 12,000 x g. Following centrifugation of the supernatant for 60 minutes at 100,000 x g, the sediment was suspended in 30 ml of a suspension solution containing 0.25 *M* sucrose, 5.0 m*M* L-Histidine, and 1 m*M* ethylene diamine tetraacetic acid, with pH 7.0 with Trizma® base. The suspension was centrifuged again at the same speed for the same time. The pellet was resuspended in 20 ml of the above suspension solution. The same volume of LiBr solution (2.0 *M*) was added, and the new solution was stirred gently for 1 hour. The mixture was centrifuged for 60 minutes at 100,000 x g. The sediment was resuspended in the same suspension solution and recentrifuged. A final suspension was filtered through four-layers of gauze. All above procedures were

carried out at 2 °C.

Determination of Enzyme Activity

ATPase activity was determined by using slightly modified procedures previously reported (Broekhuysen et al., 1972; Akera et al., 1971). A final volume of 1ml reaction medium containing 50 m*M* Tris HCl buffer (pH 7.5) with 5 m*M* $MgCl_2$, 15 m*M* KCl, 100 m*M* NaCl, and 0.1 ml of enzyme (60 μg of protein) preparation was used in all experiments. BRB-I-28, SAZ-VII-22, SAZ-VII-23 and other DHBCN derivatives were dissolved in 30% or 50% methanol or dimethyl sulfoxide (DMSO). Tedisamil (*N,N'*-bis(cyclopropylmethyl)-3,7-diazabicyclo[3.3.1]nonane·2HCl, ouabain·HCl and lidocaine·HCl (Sigma Chemical Co., St. Louis) were dissolved in water. Various concentrations of the above drugs in 0.1 ml volume were added to the reaction medium. Methanol or DMSO (30% or 50%) and water served as vehicle controls. The reaction medium was preincubated with drugs or vehicles at 37 °C for 5 minutes. The reaction was started with adding 2 m*M* of Tris·ATP (Sigma Chemical Co., St. Louis) and was allowed to proceed for 20 minutes at 37 °C and then terminated with 1 ml of 15% trichloroacetic acid. After centrifugation, the inorganic phosphate liberated in a 1.0 ml aliquot of supernatant was measured by simply adding 3 ml of 6 *N* H_2SO_4:water:2.5% ammonium molybdate:10% L-ascorbic acid (1:2:1:1) via the modified method of Fiske and SubbaRow (1925). Absorbance was measured at 660 nm after 20 minutes at 37 °C. The Mg^{2+}-dependent ATPase activity was assessed in the absence of K^+ and Na^+. The Na^+,K^+-ATPase activity was calculated by substracting the Mg^{2+}-activated

ATPase activity from total ATPase activity. Protein content of the enzyme preparations was determined by bicinchoninic acid protein assay (Smith et al., 1985) with bovine serum albumin as the standard. The concentrations necessary to inhibit 50% of the maximal enzyme activity for individual drug was calculated using selected computer software (Tallarida, 1986).

Determination of Retention Time

The HPLC system (Millipore, Milford, MA) with the chromatographic column Ultramex 5 C_6(250 x 4.6 mm) and guard column-Ultramex 5 C_6 (30 x 4.6 mm) purchased from Phenimenex was used. The mixture of acetonitrile:methanol: 37.5 m*M* phosphate buffer, pH 6.8:triethylamine (50:50:75:0.1) was used as a mobile phase. The flow rate was 1.2 ml/min under isocratic condition and at ambient temperature. The wavelength of detection was 261 nm. Then 25 μl of the individual compound in solution was injected, and the retention time was determined using a computer software.

Determination of Molar Refractivity and Log P

Log P and molar refractivity values for the compounds in Table 3 were computed with the CLOGP® program (MedChem Software Manual, Release 3.54, 1989, Daylight Chemical Information Systems, Inc., Irvine, CA.). The log P calculations are based on the fragment constant method [Rekker, R., The Hydrophobic Fragmental Constant, Elsevier, Amsterdam (1977)], in a 1-octanol/water partitioning system. CLOGP® is a computer-assisted method in which the structure of the solute is input via its SMILES line notation. The structure is decomposed into

defined substructural fragments to which hydrophobic concentrations are assigned from a data base of fragment contributions derived from experimental log P measurements. The log P is computed with the ReKKer equation below which gives the log P for the neutral form of the solute. The terms, a_i and f_i, are the number of occurrences of fragment i in the solute and the hydrophobic contribution of the fragment, respectively.

$$\log P = \Sigma\, a_i f_i$$

The molar refractivity is calculated in an analogous manner from atom and bond refractivities with a model calibrated with the Lorenz-Lorenz equation with measured densities and refractive indices.

Effects of BRB-I-28 on Contractile Force Development in Atrial and Papillary Muscles

Adult, albino, New Zealand rabbits of both sexes were anesthetized with inhalational ether and killed using blunt head trauma. The heart was excised and washed in modified Tyrode's solution equilibrated with 100% oxygen. (The modified Tyrode's solution contained in m*M*: NaCl, 140; KCl, 5; $MgCl_2$, 2.5; Glucose, 10; HEPES, 5; $CaCl_2$, 2.5; Na_2PO_4, 1.0; and aspartic acid, 2.0; pH 7.4). Atrial appendages and right ventricular papillary muscles were removed and placed in glass tissue baths (50 ml) containing modified Tyrode's solution at 37 °C. The muscle bath was continuously aerated with oxygen. The muscles were attached to Grass isometric force transducers via 4-0 suture and were stimulated at a basic cycle length of 1000 msec (1 Hz) using 4 msec duration stimuli from a Grass S-48 stimulator and SIU-5 or SIU-7 stimulus isolation unit. The resting tension was adjusted to produce

maximal force development. Force development was recorded continuously on a Grass polygraphy. A minimum equilibration period of 1 hour was allowed before control measurements were obtained. Compound of BRB-I-28, dissolved in a modified Tyrode's solution to a final concentration of 6 mg/ml, was added in 0.5 log unit increments (from 0.32 to 100 μg/ml) to the bath to achieve the listed cumulative drug concentration. A period of 15 min was allowed between studies at each drug concentration. Control measurements were redetermined 15 min after replacement of the muscle bath solution with modified Tyrode's solution containing no BRB-I-28.

Results

Effects on Na^+,K^+-ATPase Activity

BRB-I-28, SAZ-VII-22, SAZ-VII-23, tedisamil and lidocaine produced a concentration-dependent inhibition on Na^+,K^+-ATPase activity(Table 1). The concentrations necessary for 50% inhibition (IC_{50}) of the maximal enzyme activity of Na^+,K^+-ATPase were: 0.789±0.047 m*M* for BRB-I-28, 0.766±0.036 m*M* for SAZ-VII-22, 1.219±0.125 m*M* for SAZ-VII-23, 0.792±0.06 m*M* for tedisamil, 13.03±0.20 m*M* for lidocaine and 2.24±0.68 μM for ouabain. The inhibitory effects of BRB-I-28, SAZ-VII-22, SAZ-VII-23 and tedisamil are greater than that of lidocaine but much less than that of ouabain.

Effects on Mg^{2+}-activated ATPase Activity

BRB-I-28, SAZ-VII-22, SAZ-VII-23, tedisamil and lidocaine produced a concentration-dependent inhibition of Mg^{2+}-activated ATPase activity (Table 2).

However, ouabain failed to produce any inhibitory effect on the enzyme up to 0.1 m*M* concentration. The IC_{50} values for the Mg^{2+}-activated ATPase activity were: 0.324±0.04 m*M* for BRB-I-28, 0.501±0.076 m*M* for SAZ-VII-22, 1.217±0.103 m*M* for SAZ-VII-23, 0.378±0.04 m*M* for tedisamil and 17.8±0.19 m*M* for lidocaine, with BRB-I-28 being the most potent.

Influence of Molar Refractivity, Lipophilicity and HPLC Retention Time

The results on molar refractivity (MR), log P, retention time (RT) and IC_{50} values for the DHBCN derivatives on Na^+,K^+-ATPase and Mg^{2+}-activated ATPase activities are presented in Table 3. It appears that there is a positive correlation between the lipophilicity of these compounds and their abilities to inhibit both Na^+,K^+-ATPase and Mg^{2+}-activated ATPase activities. [Log IC_{50} for Na^+,K^+-ATPase vs log RT, r = 0.77; log IC_{50} for Mg^{2+}-ATPase vs log RT, r = 0.60; log IC_{50} for Na^+,K^+-ATPase vs log MR, r = 0.39; log IC_{50} for Mg^{2+}-ATPase vs log MR, r = 0.14; log IC_{50} for Na^+,K^+-ATPase vs log P, r = 0.073; and log IC_{50} for Mg^{2+}-ATPase vs log MR, r = 0.75].

Effects on Atrial and Papillary Muscles

At concentrations of 0.32-10 μg/ml and at paced rates of 0.5-3.0 Hz, BRB-I-28 failed to significantly alter atrial force development (Figure 2). Maximal force development with paired pacing was not altered. At higher concentrations, a biphasic response was observed with increased ($p<0.05$) contractility observed at a stimulation rates of 0.5 and 1.0 Hz (Figure 2a and 2b) with reduced ($p<0.05$) contractility observed at a stimulation rate of 3.0 Hz (Figure 2d). At the highest concentration

studied (100 μg/ml), BRB-I-28 significantly decreased maximal force development with paired pacing ($p<0.05$). BRB-I-28 (0.32-100 μg/ml) failed to alter the time to peak tension development or the duration of atrial muscle contraction observed at a stimulus rate of 1.0 Hz. The observed actions of BRB-I-28 were completely reversed within 15 min following removal of BRB-I-28 from the superfusion solution.

At concentrations of 0.32-32 μg/ml and at paced rates of 0.5-3.0 Hz, BRB-I-28 failed to significantly alter contractility in isometrically-contracting rabbit papillary muscles. Maximal force generation with paired pacing was not altered. At a concentration of 100 μg/ml, BRB-I-28 reduced ($p<0.05$) force development at 1.0-3.0 Hz. BRB-I-28 (0.32-100 μg/ml) failed to alter the time to peak tension development or the duration of ventricular muscle contraction observed at a stimulus rate of 1.0 Hz (data not shown). The observed actions of BRB-I-28 were completely reversed within 15 min following removal of BRB-I-28 from the superfusion solution.

Discussion

Results from this study demonstrate for the first time that BRB-I-28, SAZ-VII-22, and SAZ-VII-23, a novel group of antiarrhythmic agents, and tedisamil, a newer class III antiarrhythmic agent, produced a concentration-dependent inhibition of guinea-pig myocardial microsomal Na^+,K^+-ATPase activity. The inhibitory effects of these four compounds are much less than that of ouabain, a specific inhibitor of Na^+,K^+-ATPase, but are greater than that of lidocaine, a class I antiarrhythmic agent. The inhibition rank order was: ouabain > SAZ-VII-22 > BRB-I-28 > tedisamil > SAZ-VII-23 > lidocaine. In addition, it was found that these four agents inhibited

guinea pig myocardial Mg^{2+}-activated ATPase activity in a concentration-dependent manner. The inhibitory effects of these agents were greater than that of lidocaine. Ouabain failed to have any effect on Mg^{2+}-activated ATPase activity. The inhibition rank order was: BRB-I-28 > tedisamil > SAZ-VII-22 > SAZ-VII-23 > lidocaine.

Our results show that lidocaine had no significant inhibitory effects on Na^+,K^+-ATPase at a concentration below 13.5 m*M* which is not consistent with that of previous reports (Almotrefi et al., 1990). The difference in inhibitory concentrations for lidocaine between our study and the previous report could possibly be due to the slightly impure nature of our myocardial enzyme preparation and the difference in preincubation time used in our experiments.

The inhibitory effects of BRB-I-28, SAZ-VII-22, and SAZ-VII-23 on both myocardial Na^+,K^+-ATPase and Mg^{2+}-activated ATPase activities are significant and interesting in many respects. Previous studies have demonstrated that BRB-I-28, unlike lidocaine and most other class I antiarrhythmic drugs which depress blood pressure, produced a significant increase in mean arterial blood pressure when administered during sinus rhythm (Scherlag et al., 1988). BRB-I-28, produced a mild and fully reversible positive inotropic action on isolated rabbit muscle at paced rates of 0.5 to 1.0 Hz. However, it produced negative inotropic effects on both atrial and ventricular muscle preparations at 100 μg/ml, excess of the concentrations (1-10 μg/ml) needed to exert significant clinically relevant electrophysiologic and antiarrhythmic effects. The basis for this action is uncertain at present. Although BRB-I-28-induced release of catecholamines from cardiac tissues could mediate a positive inotropic of the drug, the time to peak tension and the duration of concentration were not altered in either atrial or ventricular tissues. A reduction in

the time to peak tension and contraction duration would be expected with the release of norepinephrine from adrenergic nerve endings. It is quite possible that the increase in systemic arterial blood pressure during sinus rhythm and contractile force of isolated atrial muscle could be mediated by the inhibitory effects of BRB-I-28 on myocardial Na^+,K^+-ATPase activity and increase Na/Ca exchange, which is similar to ouabain's effect on this enzyme (Hansen, 1984). The other possible mechanisms may be: (1) an increase in L-type calcium current; (2) a decrease in calcium extrusion via Na-Ca exchange; (3) a calcium ionophore action of BRB-I-28.

Our results also show that inhibitory effects on ATPase activity seem to be positively correlated with the retention time in reverse-phase HPLC and molar refractivity of the DHBCN derivatives. The retention time in reverse-phase HPLC is relatively proportional to the nonpolar nature of a compound, and log P is a measure of the nonpolar nature of compounds. Lipophilic properties of a drug play a crucial role in interaction between the drug molecule and a receptor (Eberlein, 1978). Our results show that the more nonpolar a drug, the more interaction occurs between the drug and receptor. Molar refractivity, a measure of steric bulk, has been used to express nonhydrophobic interaction between enzyme and substrate (Dunn, 1977). Based on our data, it appears that the lower the MR of DHBCN derivatives, the greater the inhibition of Na^+,K^+-ATPase activity, while no significant effect occurs on Mg^{2+}-ATPase activity. Therefore, inhibition on ATPases may be caused by easy integration of nonpolar compounds into microsome vesicles and more interaction with ATPase, while hydrophilic groups of the drugs may not be important for interaction between drugs and ATPases. We also found that all the DHBCN derivatives have a stronger inhibition on Mg^{2+}-activated ATPase than on Na^+,K^+-

ATPase, which may be mediated by the chelating properties of the DHBCN compounds with Mg^{2+} (Haller, 1965). Among the compounds studied, only GLG-IV-44 has specific inhibition on Mg^{2+}-activated ATPase. It is worthy of effort to continue the search for a specific Mg^{2+}-activated ATPase inhibitor, because no specific Mg^{2+}-activated ATPase inhibitor is currently available (Murphy, 1991).

It is interesting to note that tedisamil (KC8857), *N,N*-dicyclopropylmethyl-3,7-diazabicyclo[3.3.1]nonane·2HCl, which has been recently characterized as a class III antiarrhythmic agent (Beatch et al., 1991), produced a concentration-dependent inhibition on both Na^+,K^+-ATPase and Mg^{2+}-activated ATPase activity in a manner similar to that of BRB-I-28 and its analogues. The significance of the inhibitory effects of BRB-I-28, its analogues, and tedisamil on guinea pig myocardial Na^+,K^+-ATPase and Mg^{2+}-activated ATPase and their relationship with respect to electrophysiological actions on their antiarrhythmic properties are yet to be determined. Our findings that these four newer antiarrhythmic agents produced a significant inhibition on myocardial Mg^{2+}-activated ATPase raise the possibility of a link between Na/Mg exchange and intracellular Mg^{2+} ions of the myocardial cells. This should be further investigated as it is related to cardiac cellular Na^+, K^+, and Ca^{2+} channels supposedly involved in arrhythmogenesis (Murphy, 1991).

References

Akera, T. and Brody, T.M. (1971). Membrane adenosine triphosphatase: The effect of potassium on the formation and dissociation of the ouabain-enzyme complex. J. Pharmacol. Exp. Ther. 176, 545-557.

Akera, T., Larsen, FS. and Brody, T.M. (1969). The effect of ouabain on sodium-and potassium-activated adenosine triphosphatase from the hearts of several mammalian species. J. Pharmacol. Exp. Ther. 170, 17-26.

Almotrefi, A.A. and Dzimiri, N. (1991). Influence of lorcainide on microsomal Na^+,K^+-ATPase in guinea-pig isolated heart preparations. Br. J. Pharmacol. 102, 530-532.

Almotrefi, A.A. and Dzimiri, N. (1990). Effects of quinidine, lidocaine and encainide on guinea pig myocardial Na^+-K^+-ATPase activity. Res. Commun. Chem. Pathol. Pharmacol. 69, 253-256.

Bailey B.R., Berlin, K.D. et al. (1984). Synthesis, conformational analysis, and antiarrhythmic properties of 7-benzyl-3-thia-7-azabicyclo [3.3.1]nonan-9-one, 7-benzyl-3-azabicyclo [3.3.1]nonane hydroperchlorate, and 7-benzyl-9-thia-7-azabicyclo[3.3.1-]nonan-9-ol hydroperchlorates and derivatives: single-crystal x-ray diffraction analysis and evidence for chair-chair and chair-boat conformers in the solid state. J. Med. Chem. 27, 758-767.

Beach, G.N., Abraham S., McLeod, B.A., Yoshida, N.R. and Walker, M.J.A. (1991) Antiarrhythmic properties of tedisamil CKC8857) a putative transient K^+ current blocker. Br. J. Pharmacol. 102, 13-18.

Broekhuysen, J., Clinet, M. and Delisee, C. (1972). Action of amiodarone on guinea pig heart sodium and potassium activated adenosine triphosphate: comparison with ouabain. Biochem. Pharmacol. 21, 2951-2960.

Dunn, W.J. (1977). Molar refractivity as independent variable quantitative structure-activity studies. Eur. J. Med. Chem.-- Chimica Therapeutica. 12, 109-112.

Dzimiri, N., Fricke, U. and Klau, W. (1987). Influence of derivation on the lipophilicity and inhibitory actions of cardiac glycosides on myocardial Na^+-K^+-ATPase. Br. J. Pharmac. 91, 31-38.

Eberlein, W. (1978). Das molekulare konzept der pharmakonwirkung. In arzneimittelentwicklung, Grundlagen-Strategien-Perspektiven, ed. Kutter, E., Georg Thieme Verlag, Stuttgart. Pp.2-39.

Fisk, C.H. and SubbaRow, Y. (1925). The colorimetric determination of phosphorous. J. Biol. Chem. 66, 375-400.

Fricke, U., Klaus, W. and Rogatti, M. (1981). Lipophilicity and pharmacodynamics of cardiac glycosides. J. Mol. Cell. Cardiol. 13(suppl. 1), 29-34

Haller, R. (1965). Darstellung eines neuen bicyclischen ring system, des 3-thia-7-aza-9-oxobicyclo[3.3.1]nonanes. Arzneimittell-forschung. 13, 1117-8.

Hasen, O. (1984). Interaction of cardiac glycosides with $(Na^+ + K^{+})$-activated ATPase. A biochemical link to digitalis-induced inotropy. Pharmacological Reviews. 36 (suppl.), 143-163.

Jeyaraman, R. and Avila, S. (1981). Chemistry of 3-azabicyclo[3.3.1]nonanes. Chem. Rev. 81, 149-174.

Murphy, E., Freudenrich, C.C. and Lieberman, M. (1991). Cellular magnesium and Na/Mg exchange in heart cells. Annu. Rev. Physiol. 53, 273-287.

Oexle, B., Weinich, J. and Antoni, H. (1987). Electrophysiological profile of KC8857, a new bradycardic agent (Abstract). J. Mol. Coll. Cardiol. 19 (Supplement III), 194.

Patterson, E., Scherlag, B.J., Berlin, K.D. and Lazzara, R. (1991). Electrophysiological action of BRB-I-28 in canine myocardial tissues. J. Pharmacol. Exp. Ther. 259, 558-565.

Scherlag, B.J., Patterson E., Lazarra R., Bailey, B.R., Thompson, M.D. and Berlin, K.D. (1988). Comparative electrophysiological and hemodynamic actions of BRB-I-28 and lidocaine in the normal and infarcted dog heart. J. Electrophysiol. 2, 461-477.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K. et al. (1985). Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76-85.

Tallarida, R.J. and Murray, R.B. (1986). Manual of pharmacologic calculations with computer programes, Springer-Verlag, New York.

TABLE 1

INHIBITION OF BRB-I-28, SAZ-VII-22, SAZ-VII-23, TEDISAMIL, LIDOCAINE AND OUABAIN ON GUINEA PIG MYOCARDIAL Na^+,K^+-ATPase ACTIVITY. (MEAN ± SD; N = 6)

BRB-I-28		SAZ-VII-22		SAZ-VII-23		Tedisamil		Lidocaine		Ouabain	
Conc (mM)	I.R. (%)	Conc (mM)	I.R. (%)	Conc (mM)	I.R. (%)	Conc. (mM)	I.R. (%)	Conc (mM)	I.R. (%)	Conc (μM)	I.R. (%)
0.3	15.9±1.3*	0.3	14.5±3.2♦	0.3	8.6±4.6	0.30	20.0±1.4*	10.0	17.5±4.5*	0.1	15.9±7.2♦
0.6	17.6±6.3*	0.6	29.4±4.4§	0.6	15.8±1.5*	0.45	21.1±3.9*	12.0	20.7±2.2*	1.0	36.7±3.1§
0.9	47.6±1.8§	1.2	55.8±3.1§	1.2	34.5±4.7§	0.60	23.7±2.9§	13.5	62.1±1.1§	5.0	60.2±1.6§
1.2	65.9±5.8§	1.6	70.8±0.7§	1.6	56.2±3.0§	0.90	47.8±2.4§	15.0	86.8±2.5§	10	72.1±3.0§
1.6	91.1±4.0§	2.4	99.9±1.7§	2.0	74.6±1.1§	1.20	72.2±2.1§	20.0	105.6±2.8§	50	82.6±1.7§
2.4	102.2±2.5§			2.4	89.6±4.5§	1.60	87.4±4.6§			100	92.5±1.3§

The specific activity of Na^+,K^+-ATPase was 340.3±41.7 n*M* Pi/mg protein/min. I.R. = Inhibition Rate.
♦ $p<0.05$, * $p<0.01$, § <0.001

TABLE 2

EFFECTS OF BRB-I-28, SAZ-VII-22, SAZ-VII-23, TEDISAMIL, LIDOCAINE AND OUABAIN ON GUINEA PIG MYOCARDIAL Mg^{2+}-ACTIVATED ATPase ACTIVITY. (MEAN ± SD; N = 6)

BRB-I-28		SAZ-VII-22		SAZ-VII-23		Tedisamil		Lidocaine		Ouabain	
Conc (mM)	I.R. (%)	Conc (mM)	I.R. (%)	Conc (mM)	I.R. (%)	Conc (mM)	I.R. (%)	Conc (mM)	I.R. (%)	Conc (μM)	I.R. (%)
0.3	43.9±7.9§	0.3	34.5±7.7§	0.3	6.1±6.0$^{\blacklozenge}$	0.30	33.7±4.6§	10.0	1.8±1.2	0.1	0
0.6	83.9±5.8§	0.6	55.7±9.6§	0.6	20.5±3.7*	0.45	56.0±6.1§	12.0	2.6±1.2	1.0	0
0.9	90.8±1.3§	1.2	66.0±8.2§	1.2	30.4±4.7§	0.60	88.8±3.0§	13.5	2.4±2.0	5.0	0
1.2	97.4±1.1§	1.6	95.3±2.4§	1.6	43.0±9.7§	0.90	99.4±1.2§	15.0	32.3±2.4§	10	0
		2.4	98.7±2.8§	2.0	65.8±2.5§			20.0	69.8±5.2§	50	0
				2.4	94.2±3.0§					100	0

The specific activity of the magnesium-activated ATPase was 113.8±15.3 n*M* Pi/mg protein/min (It represents 25.1±2.3% total ATPase activity). I.R. = Inhibition Rate. $^{\blacklozenge}$ $p<0.05$, * $p<0.01$, § $p<0.001$

TABLE 3

MOLAR REFRACTIVITY (MR), LOG P, RETENTION TIME (RT) AND IC_{50} FOR DHBCN DERIVATIVES ON GUINEA PIG MYOCARDIAL Na^+,K^+-ATPase AND Mg^{2+}-ACTIVATED ATPase ACTIVITIES (MEAN ± SD; N = 6)

Compounds	MR	log P	RT (Min)	IC_{50} for Na^+,K^+-ATPase (m*M*)*	IC_{50} for Mg^{2+}-ATPase (m*M*)*
GLG-IV-17	8.24	2.48	24.30	0.547±0.041	0.433±0.027
GLG-IV-57	8.61	0.49	20.49	0.618±0.023	0.433±0.049
GLG-III-93	9.15	1.79	18.06	0.702±0.074	0.426±0.054
GLG-III-70	8.77	1.68	15.87	0.764±0.055	0.507±0.035
SAZ-VII-22	8.44	1.54	16.53	0.776±0.036	0.501±0.076
BRB-I-28	6.96	2.31	15.85	0.789±0.047	0.324±0.040
Tedisamil	9.50	3.95	15.91	0.792±0.060	0.378±0.010
GLG-II-5	9.82	3.50	10.6	0.857±0.017	0.356±0.011
SAZ-VII-23	7.95	0.75	11.35	1.219±0.125	1.217±0.103
GLG-IV-74	9.65	-0.08	6.03	1.315±0.103	1.010±0.060
GLG-III-96	8.28	0.88	11.78	1.341±0.130	1.137±0.103
GLG-III-86	10.52	1.78	9.91	1.562±0.105	0.810±0.063
GLG-I-97	9.60	3.79	12.97	1.706±0.051	0.175±0.007
Lidocaine	7.17	2.26	7.27	13.03±0.200	17.86 ±0.190
GLG-IV-44	8.32	0.08	7.5	No effect	1.771±0.049

Figure Legends

Figure 1. Chemical Structures of DBHCN, BRB-I-28, SAZ-VII-22 and SAZ-VII-23

Figure 2. Effects of BRB-I-28 on Contractile Force Development in Rabbit Atrial Muscle. *$p<0.05$

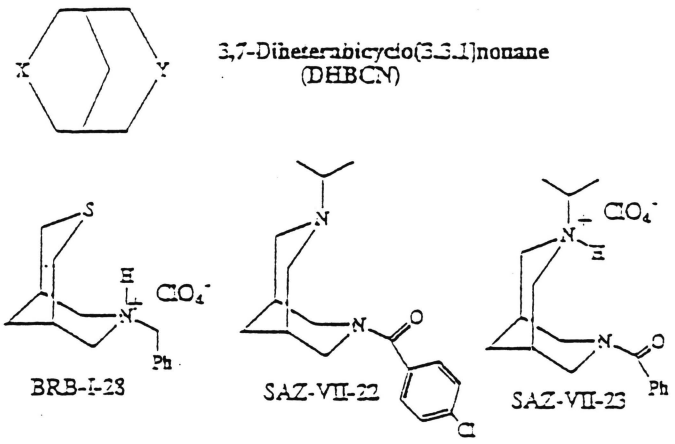

3,7-Diheteranbicyclo(3.3.1)nonane
(DHBCN)
BRB-I-23
SAZ-VII-22
SAZ-VII-23

Figure 1

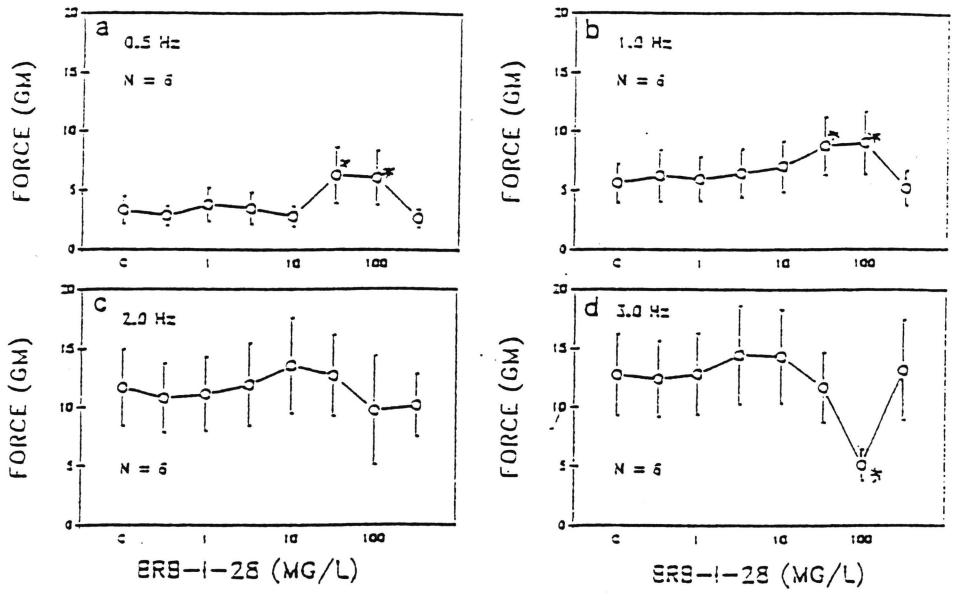

a
0.5 Hz
N = 6
b
1.0 Hz
N = 6
c
2.0 Hz
N = 6
d
3.0 Hz
N = 6
FORCE (GM)
FORCE (GM)
FORCE (GM)
FORCE (GM)
ERB-I-28 (MG/L)
ERB-I-28 (MG/L)

Figure 2

CHAPTER IX

EFFECTS OF NOVEL ANTIARRHYTHMIC AGENTS, BRB-I-28 AND ITS DERIVATIVES ON HEART MITOCHONDRIAL RESPIRATORY CHAIN AND SARCOPLASMIC RETICULUM Ca^{2+}-ATPase

Abstract

The effects of BRB-I-28 and its derivatives (GLG-V-13, SAZ-VII-22 and SAZ-VII-23), a novel group of antiarrhythmic agents, on rat heart mitochondrial respiratory chain were investigated. The results indicated that BRB-I-28 and its derivatives have concentration-dependent inhibitory effects on NADH oxidase and NADH-CoQ reductase (complex I), but they have no significant effects on succinate oxidase, succinate dehydrogenase (complex II), CoQ-cytochrome c reductase (complex III), cytochrome c oxidase (comple IV), and NADH-$K_3Fe(CN)_6$ reductase. The site of inhibition of BRB-I-28 and its derivatives on respiratory chain was localized between flavoprotein n (FPn) and CoQ, which is similar to the effect of rotenone and several other antiarrhythmic drugs such as amiodarone, propranolol etc. BRB-I-28 and its derivatives also have significant inhibition on mitochondrial ATPase activity as reported for other antiarrhythmic drugs such as amiodarone, propranolol, quinidine, and lidocaine, However, BRB-I-28 and its derivatives have no direct effects on sarcoplasmic reticulum Ca^{2+}-ATPase activity. The inhibitory effects of BRB-I-28 and its derivatives on mitochondrial oxidative phosphorylation may result

in the depletion of ATP. This effect, in combination with the effects on Na^+,K^+-ATPase could possibly produce an increase in Ca^{2+} concentration in cytosol. This may be another mechanism by which these DHBCN derivatives produce an increase in systemic arterial blood pressure and contractile force of isolated cardiac muscle. On the other hand, inhibition on mitochondrial respiration may account for some of the potential toxic effects of these diheterabicyclo[3.3.1]nonane derivatives.

Introduction

Compound BRB-I-28 {7-benzyl-7-aza-3-thiabicyclo[3.3.1]nonane hyperchlorate} (Figure 1) is a recently developed a novel class Ib antiarrhythmic agent (Bailey et al., 1984; Scherlag et al., 1988; Smith et al., 1990; Patterson et al., 1991; Fazakas et al., 1993a; Patterson et al., 1993). GLG-V-13, 3-[4-(1H-imidazol-1-yl)benzoyl]-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane dihydroperchlorate, (Figure 1) has been found to have combined class Ib and class III antiarrhythmic actions without hemodynamic depressant effect (Fazakas et al., 1992; 1993a; 1993b; 1994). Two other bicyclo[3.3.1]nonane derivatives, SAZ-VII-22 (3-94-chlorobenzoyl)-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane and SAZ-VII-23, [3-benzoyl-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane·$HClO_4$, also have been shown to have effective antiarrhythmic actions with modest class III antiarrhythmic properties (Zisman, 1989; Berlin et al., 1994). All of the four diheterabicyclo[3.3.1] nonane (DHBCN) derivatives have effective inhibition on induced ventricular tachycardia and less proarrhythmic activities than those of lidocaine. The special characteristics of BRB-I-28, SAZ-VII-22 and SAZ-VII-23 are their ability to produce a significant increase in mean blood pressure when administered during sinus rhythm (Scherlag et al., 1988; Berlin et al., 1994). However, GLG-V-13 causes slight reduction in mean blood pressure, but the effect was only of short duration (Fazekas et al., 1992; 1993a; 1993b; 1994; Garrison et al., 1994). It has been demonstrated that the increase in systemic arterial blood pressure and contractile force of isolated atrial muscle could be mediated by the inhibitory effects of BRB-I-28 on myocardial Na^+,K^+-ATPase activity and thus a possible increase Na/Ca exchange (Chen et al., 1992).

Mitochondrial and sarcoplasmic reticulum are two important organelle to control Ca^{2+} concentration in cytosol. In cardiac mitochondria, the key carriers for Ca are the uniporter pathway for influx via Ca^{2+}-ATPase and the Na/Ca antiporter pathway associated with efflux. The uniporter system primarily depends on mitochondrial respiration and/or on the hydrolysis of ATP (Lehninger et al., 1978). Most antiarrhythmic drugs have some effects (either inhibition or stimulation) on mitochondrial function *in vivo* and *in vitro* (Nagai et al., 1984; Kluppel et al., 1986; Nokin et al., 1987; Patterson et al., 1987; Fromenty et al., 1990; Almontrfi and Dzimiri, 1992; Baydoun et al., 1992;). The purpose of this study was to examine the effects of four diheterabicyclo[3.3.1]nonane derivatives on the submitochondrial enzymes activity and on sarcoplasmic reticulum Ca^{2+}-ATPase activity.

Materials and Methods

Chemicals

Compounds BRB-I-28, GLG-V-13, SAZ-VII-22 and SAZ-VII-23 were synthesized by established procedures (Zisman et al., 1990; Garrison et al., 1993). Oxidated and reduced $Q_{10}C_0Br$ were kindly provided by Dr. C-Y, Yu (Department of Biochemistry, Oklahoma State University). Cytochrome c, ß-NADH, succinate, and all other chemicals were purchased from the Sigma Chemical Company (St. Louis, MO).

Isolation of Rat Heart Submitochondrial Particles

Heart mitochondria were isolated from fasted (overnight) rats following

established procedures (Chen et al., 1988). The Sprague-Dawley rats were decapitated by guillotine. The hearts were removed immediately, and fat and connective tissue were removed, and then washed with ice-cooled 0.25 *M* sucrose-1m*M* Tris-1m*M* EDTA, pH 7.4. The heart was minced with scissors, and homogenized in 4 volumes of the above solution using a polytron homogenizer (Model PT 10/35, Brinkmann Instruments, Switzerland) for 30 seconds. The homogenate was centrifuged at 1,000 x g for 10 minutes. The supernatant was centrifuged at 15,000 x g for 15 minutes. Then the pellets were suspended in 0.25 *M* sucrose-5 m*M* Tris (pH 7.4) and centrifuged at 15,000 x g, for 15 minutes. The pellets were resuspended in the above solution and subjected to ultrasonification for 3 minutes (treated for 1 minute followed by a 2 minute pause for cooling down). The solution was centrifuged at 15,000 x g for 15 minutes to remove unbroken mitochondria. The supernatant was centrifuged at 105,000 x g for 1 hr to obtain submitochondrial particles. The submitochondrial particles are stored at -60 °C until analysis. All procedures were carried out at below 4 °C.

Preparation of Rat Cardiac Sarcoplasmic Reticulum (SR) Microsomes

The preparation of rat cardiac SR microsomes was carried out using the procedures described by Wang (Wang et al., 1986).

Determination of Enzyme Activities

NADH Oxidase. The activity of NADH oxidase was measured via a polarographic technique with a Clark electrode using the following reaction medium

(1.0 ml total volume): 50 m*M* KH_2PO_4-NaOH buffer (pH 7.4); 100 μg cytochrome c; and 0.1-0.3 mg Submitochondrial particles. The medium was incubated at room temperature (25±1 °C) for 5 minutes. Then the reaction was started by the addition of 1 m*M* NADH.

Succinate Oxidase. The activity of succinate oxidase was measured via a polarographic technique with a Clark electrode using the following reaction medium (1.0 ml total volume): 50 m*M* KH_2PO_4-NaOH buffer (pH 7.4); 200 μg cytochrome c; and 0.1-0.3 mg submitochondrial particles. The reaction was started by the addition of 30 m*M* sodium succinate.

NADH-CoQ Reductase. The activity was measured with a spectrophotometer at 340 nm with the following reaction medium (3.0 ml total volume) containing: 50 m*M* Tris-HCl buffer (pH 7.8); 1 m*M* $Q_{10}CoBr$; 2 m*M* NaN_3; and 0.1-0.3 mg submitochondrial particles. After incubation at room temperature for 5 minutes, the reaction was started by the addition of 1.0 m*M* of NADH.

NADH-Ferricyanide Reductase. The activity was measured with a spectrophotometer at 420 nm with the following reaction medium (3.0 ml total volume): 50 m*M* Tris-Hcl buffer (pH 7.8); 1.5 m*M*, $K_3Fe(CN)_6$; 2.0 m*M* NaN_3; and 0.1-0.3 mg submitochondrial particles. Afetr incubation at room temperature for 5 min, the reaction was started by the addition of 0.15 m*M* NADH.

Succinate Dehydrogenase. The activity was measured by a spectrophotometer at 600 nm with the following reaction medium (3.0 ml total volume): 50 m*M* KH_2PO_4-NaOH (pH 7.4); 2.0 m*M* NaN_3; 0.1-0.3 mg submitochondrial particles; and 30 m*M* sodium succinate. The medium was incubated at room temperature for 5

minutes. The reaction was started by the addition of 0.15 m*M* DCIP (2,6-dichlorophenol-indophenol).

CoQ-Cytochrome c Reductase and Cytochrome c Oxidase. The activities of both enzymes were measured with a spectrophotometer at 550nm as described previously (Chen et al, 1987).

ATPase Activity. The activity was measured with the following reaction medium (1 ml total volume): 10 m*M* Tris-HCl (pH 7.8); 2 m*M* $MgCl_2$; and 50-100 μg submitochondrial particles. After incubation at 37 °C for 5 minutes, the reaction was started by the addition of 3 m*M* ATP. The reaction was allowed to proceed for 10 minutes at 37 °C and then terminated with 1 ml of 15% trichloroacetic acid. After centrifugation, the inorganic phosphate liberated was measured by a previously established method (Chen et al., 1992).

Ca^{2+}-ATPase Activity. The activity was measured following the general procedures of Wang et al. (1986), except that the inorganic phosphate liberated was measured by a different method (Chen et al., 1992).

Protein Determination

Submitochondrial protein was assayed by using BCA (bicinchoninic acid) reagent with BSA (bovine serum albumin) as standard (Smith et al. 1985).

Analysis of Data

The concentration necessary to inhibit 50% of the maximal enzyme activity for individual drug was calculated using a selected computer software program (Tallarida,

1986).

Results

Effects of DHBCN Derivatives on Submitochondrial NADH Oxidase

Agent BRB-I-28, and its derivatives, GLG-V-13, SAZ-VII-22 and SAZ-VII-23, showed dose-dependent inhibition on oxidation of NADH by NADH oxidase, i.e. NADH respiratory chain (from NADH to O_2). The concentrations required for 50% inhibition rate were 2.10, 4.83, 0.124, and 1.61 m*M*, respectively (Figure 2).

Effects of DHBCN Derivatives on Submitochondrial Succinate Oxidase and Succinate Dehydrogenase

Compound BRB-I-28 and its derivatives did not produce significant effects on the oxidation of succinate by submitochondrial particles, i.e. succinate oxidase (from succinate to O_2) and succinate dehydrogenase (complex II).

Effects of DHBCN Derivatives on NADH-CoQ Reductase

Agent BRB-I-28 and its derivatives have potent inhibitory effects on NADH-CoQ reductase activity (complex I) of submitochondrial particles (Figure 3). The IC_{50} were 0.438 m*M* for BRB-I-28, 0.816 m*M* for GLG-V-13, 0.224 m*M* for SAZ-VII-22 and 1.40 m*M* for SAZ-VII-23.

Effects of DHBCN Derivatives on NADH-$K_3Fe(CN)_6$ Reductase

Compound BRB-I-28 and its derivatives failed to produce significant effects

on NADH-$K_3Fe(CN)_6$ reductase activity.

Effects of DHBCN Derivatives on Submitochondrial ATPase

Submitochondrial ATPase (Complex V) was markedly inhibited by the DHBCN derivatives (Figure 4). The concentrations for 50% inhibition rate were 0.963 m*M* for BRB-I-28, 0.521 m*M* for GLG-V-13, 0.812 m*M* for SAZ-VII-22 and 2.022 m*M* for SAZ-VII-23, respectively.

Effects of DHBCN Derivatives on Submitocondrial CoQ-Cytochrome c Reductase and Cytochrome c Oxidase

Agent BRB-I-28 and its derivatives failed to inhibit submitochondrial CoQ-cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV).

Effects of DHBCN Derivatives on Sarcoplasmic Reticulum Ca^{2+}-ATPase Activity

Compound BRB-I-28 and its derivatives have no significant effects on sarcoplasmic reticulum Ca^{2+}-ATPase activity.

Discussion

The DHBCN derivatives, a novel class of antiarrhythmic agents, produce concentration-dependent inhibition on NADH oxidase activity without any significant effect on succinate oxidase, succinate dehydrogenase (complex II), CoQ-cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV) of submitochondrial particles. The inhibition rank order was SAZ-VII-22 > SAZ-VII-23

> BRB-I-28 > GLG-V-13. From these results, one could conclude that complex I (NADH-CoQ reductase) must have been inhibited by the DHBCN derivatives. Unlike amiodarone, which has been shown to inhibit both complex I and II (Formenty et al., 1990), the DHBCN derivatives seem to inhibit specifically complex I, consistent with certain other antiarrhythmic drugs including perhexiline maleate, propranolol, lidoflazine, and iproveratril (Kluppel et al., 1978). Because NADH-$K_3Fe(CN)_6$ reductase activity was not affected by the DHBCN derivatives, the potential site of inhibition of DHBCN derivatives on the respiratory chain could be localized between FPn (flavoprotein n) and CoQ, but not between NADH and FPn. This phenomenion is similar to the inhibitory effects of rotenone (Burgos and Redfearn, 1965), amiodarone (Fromenty et al., 1990), perhexiline maleate, propranolol, lidoflazine, and iproveratril (Kluppel, et al., 1976; 1978) on complex I of the respiratory chain. However, the effect is different from those of rhein and emodin with localization of inhibition existing between NADH and FPn, since both agents display significant inhibition on NADH-$K_3Fe(CN)_6$ reductase (Chen et al. 1987; 1988).

Like the effects of several other antiarrhythmic drugs, such as lidocaine, procainamide, quinidine, propranolol and amiodarone (Komai and Berkoff, 1979; Chazotte et al., 1982; Guerriro et al., 1986; Dzimiri and Almotrefi, 1990), DHBCN derivatives also have strong inhibitory effects on submitochondrial ATPase activity. The concentration of the DHBCN derivatives required for 50% inhibition on respiration and ATPase are in the range very similar to those of other antiarrhythmic drugs. The effects of the DHBCN derivatives on mitochondrial respiratory chain and ATP synthesis have been summarized in Figure 5. The inhibition patterns of these

DHBCN derivatives on rat liver submitochondrial enzymes are the same as that on heart submitocondrial enzymes, suggesting that effects of DHBCN derivatives on submitochondrial enzymes may have no tissue specificity (Data are not shown).

The extent of inhibitory effects of the DHBCN derivatives on complex I (NADH-CoQ reductase) is not in agreement with that on NADH oxidase except with SAZ-VII-22. It was interesting to observe that the DHBCN derivatives had stronger inhibition on complex I than that on NADH oxidase. The reason for this is unknown at the present time and needs further investigation.

Previous results demonstrated that BRB-I-28 and its derivatives had moderate inhibition on cardiac microsomal Na^+,K^+-ATPase and Mg^{2+}-Activated ATPase activity, resulting in an increase in inotropic effect on myocardial muscle (Chen et al., 1992). It is well documented that Ca^{2+} is sequestered in the cell by the endoplasmic reticulum and mitochondria and is extruded from the cell by a plasma membrane Ca^{2+}-ATPase (Carafoli, 1987). BRB-I-28 and its derivatives had no direct inhibitions on sarcoplasma reticulum Ca^{2+}-ATPase activity. However, the Ca^{2+} pump in the inner mitochondrial membrane relies on respiratory chain, and/or ATP to take up calcium from the cytosol to mitochondria. The inhibition on submitochondrial NADH oxidase and ATPase activity by BRB-I-28 and its derivatives may result in depletion of ATP. Therefore, there could be indirect inhibitory effects on both Ca^{2+}-ATPase and Na^+,K^+-ATPase activities leading to a potential decrease in Ca^{2+} transportation from cytosol to mitochondria and an increase in cytosolic Ca^{2+} concentration (Figure 6). Another possible mechanism for an increase in cytosolic calcium concentration may be mediated by the calcium ionophore action of DHBCN derivatives because of their potential Ca^{2+} chelating properties (Haller, 1965).

However, the detailed mechanisms for a potential increase in cytosolic calcium concentration requires additional investigation. On the other hand, an increase in intracellular Ca^{2+} concentration may be responsible for cell injury suspected to be produced by amiodarone (Powis et al., 1990).

The plasma concentrations of BRB-I-28 and its derivatives in inhibiting induced ventricular arrhythmias in dogs were found to be 0.5-3 μg/ml (Chen et al., 1992; Fazekas et al., 1993a). However, the concentrations required to inhibit mitochondrial enzymes *in vitro* are significantly higher than pharmacologically effective plasma concentrations. Therefore, inhibitory effects on the mitochondrial respiration chain may not result in any significant therapeutic/toxic effects. However, several antiarrhythmic drugs have been found to be more effective in inhibiting Na^+,K^+-ATPase activity, mitochondrial respiration, and myocardial muscle in the pathological state than that in normal state (Lazzara et al., 1978; Hanaki et al., 1989; Almotrefi and Dzimiri, 1990). Consequently, it is quite possible that these antiarrhythmic agents might have profound effects on tissues subjected to various pathological conditions, in spite of their nonremarkable effects on normal healthy tissues,

In summary, BHDCN derivatives have significant inhibition on complex I, and thus on oxidation of NADH, and complex V, leading to a potential decrease in the generation of ATP. These effects may acount for some pharmacological/toxic effects of these novel antiarrhythmic agents. The significance of these findings related to potential therapeutics/toxic effects are yet to be addressed.

References

Almontrfi, A. A., and Dzimiri, N. Effects of ß-adrenoceptor blockers on mitochondrial ATPase activity in guinea pig heart preparations. Eur. J. Pharmacol. 215: 231-236, 1992.

Bailey, B. R., Berlin, K. D., Holt, E. M., Scherlag, B. J., Lazarra, R., Brachmann, J., Helm, D. V. D., Powell, D. R., Pantaleo, N. S., and Ruenitz, P. C. Synthesis, conformational analysis, and antiarrhythmic properties of 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane-9-one, 7-benzyl-3-thia-3-azabicyclo[3.3.1]nonane hydroperchlorate, and 7-benzyl-9-thia-7-azabicyclo[3.3.1]nonane-9-ol hydroperchlorates and derivatives: single-crystal x-ray diffraction analysis and evidence for chair-chair and chair-boat conformers in the solid state. J. Med. Chem. 27: 758-767,1984.

Baydoun, A. R., Markham, A., Morgan, R. M., and Sweetman, A. J. Bay K 8644, modifier of calcium transport and energy metabolism in rat heart mitochondria: a new intracellular site of action. Br. J. Pharmarcol92: 15-20, 1992.

Berlin, K. D., Garrison, G. L., Scherlag, B. J., Lazzara, R., Patterson, E., Sangiah, S., Chen, C. L. Unpublished data. 1994.

Carafoli, E. Intracellular calcium homeostasis. Annu. Rev. Biochem. 56: 395-433, 1987.

Chazotte, B., Vanderkooi, G., and Chignell, D. Futher studies on F_1-ATPase inhibition by local anesthetics. Biochemica and Biophysica Acta. 680: 310-316, 1982.

Chen, C. L., and Chen, Q. H. Biochemical study on Chinese Rhubarb XIX. Localization of inhibition of anthraquinone derivatives on the mitochondrial respiration chain. Acta Pharmaceutica Sinica 22(1): 12-18, 1987. Chem. Abstr. 106: 188504b.

Chen, C. L., He, B. F., and Chen, Q. H. Biochemical study on Chinese Rhubarb. Inhibition of anthraquinone derivatives on NADH oxidase and succinate oxidase of mitochondria. Chinese Biochem. J. 4(1): 36-38, 1988. 108: 200724.

Chen, C. L., Sangiah, S., Patterson, E., Berlin, K. D., Garrison, G. L., Dunn, W., Nan, Y., Scherlag, B. J., and Lazzara, R. Effects of BRB-I-28, a novel antiarrhythmic agent, and its derivatives on cardiac Na^+,K^+-ATPase, Mg^{2+}-ATPase activities and contractile force. res. Commun. chem. Pathol. Pharmacol. 78: 3-16, 1992.

Dzimiri, N., and Almotrefi, A. A. Actions of amiodarone in guinea pig myocardial ATPase preparations. Eur. J. Pharmacol. 183: 1772-1773, 1990.

Fazekas, T., Scherlag, B. J., Mabo, P., Berlin, K. D., Garrison, G. L., Chen, C. L., Sangiah, S., Patterson, E., and Lazzara, R. The comparative antiarrhythmic and proarrhythmic activity of a 3,7-diheterobicyclo[3.3.1]nonane, BRB-I-28, and lodocaine in the 1-4 day-old infarcted dog heart. Acta Physiol. Hung. 81: 297-307, 1993a.

Fazekas, T., Scherlag, B. J., Mabo, P., Berlin, K. D., Garrison, G. L., and Lazzara, R. Egy uj refrakter periodust megnyujto antiarimias vegyulet, a GLG-V-13 szivelektrofiziologiai jellemzese. Cardiologic Hungarica. 22: 9-15, 1993b.

Fazekas, T., Scherlag, B. J., Mabo, P., Berlin, K. D., Garrison, G. L., and Lazzara, R. Electrophysiological characterization of a new heart rate-slowing and refractoriness prolonging antiarrhythmic agent, GLG-V-13, in the 1-4 day-old infarcted dog heart. Acta Physiologica Hungarica. Submitted, 1994.

Fromenty, B., Fisch, C., Berson, A., Letteron, P., Larrey and Pessayre, D. Dual effect of amiodarone on mitochondrial respiration. Initial protonophoric uncoupling effect followed by inhibition of the respiratory chain at the levels of complex I and complex II. J. Pharmacol. Exp. Ther. 255: 1377-1384, 1990.

Garrison, G. L., Berlin, K. D., Scherlag, B. J., Lazzara, R., Patterson, E., Sangiah, S., Chen, C. L. Novel 3,7-diheterobicyclo[3.3.1]nonanes which possess multiple class antiarrhythmic activity in the 1-4 day infarcted dogs. J. Med. Chem. Submitted, 1994.

Guerreiro, M., Lopes, L. C. V., Campello, A. P., and Kluppel, M. L. W. Effect of amiodarone on mitochondrial energy-linked reactions and on mitochondrial morphology. Arq. Biol. Tecnol. 29: 621-631, 1986.

Haller, R. Darstellung eines neuen bicyclischen ring system, des 3-thia-7-aza-9-oxo-bicyclo[3.3.1]nonanes. Arzneimittell-forschung. 13: 1117-1118, 1965.

Hanaki, Y., Sugiyama, S., Hieda, N., Taki, K., Hayashi, H., and Ozawa, T. Cardioprotective effects of various class I antiarrhythmic drugs in canine hearts. J. Am. Coll. Cardiol. 14: 219-224, 1992.

Kluppel, M. L. W., Silveira, O., Vieira, L. C., and Campello, A. P. Biochemical aspects of the mechanism of action of antiarrhythmic drugs on heart mitochondria II-enzymatic activities. Arq. Biol. Tecnol. 21: 13-18, 1978.

Kluppel, M. L. W., Borba, H. R., Silveira, O., Lopes, L. C. V., and campello, A. D. P. Biochemical aspects of the mechanism of action of antiarrhythmic drugs on mitochondria. VII. Effect on energy-linked reactions and on membrane potential. Cell Biochem. Function. 4: 289-296, 1986.

Komai, H., and Berkoff, H. A. Effect of quinidine and propranolol on energy transduction in beef heart mitochondria. Biochem. Pharmacol. 28: 1501-1504, 1979.

Lazzara, R., Hope, R. R., EI-Sherif, N., Scherlag, B. J., Effects of lidocaine on hypoxic and ischemic cardiac cells. Am. J. Cardiol. 41: 872-879, 1978.

Lehninger, A. L., Vercesi, A., and Bababunni, E. A. Regulation of Ca^{2+} release from mitochondria by the oxidation-reduction state of pyridine nucleotides. Proc. Natl. Acad. Sci. U.S.A. 75: 1690-1694, 1978.

Nagai, S., Miyazaki, Y., Ogawa, K., Satake, T., Sugiyama, S., Ozawa, T. The effects of ß-blocking agents on mitochondrial function in ischemic myocardium. Jpn. Circ. J. 48: 32-36, 1984.

Nokin, P., Jungbluth, L., Mouton, J. Protective effects of amiodarone pretreatment on mitochondrial function and high energy phosphates in ischemic rat heart. J. Mol. Cell. Cardiol. 19: 603-614, 1987.

Patterson, E., Scherlag, B. J., Berlin, K. D., and Lazzara, R. Electrophysiological actions of BRB-I-28 in canine myocardial tissues. J. Pharmacol. Exp. Ther. 259: 558-565, 1991.

Patterson, E., Scherlag, B. J., Berlin, K. D., and Lazzara, R. Electrophysiological actions of BRB-I-28 in ischemically-injured canine myocardium. J. Cardiovasc. Pharmacol. 21: 637-646, 1993.

Patterson, E., Shlafer, M., Walden, K., Khazaeli, M. B., Montgometry, D. G., and Lucchesi, B. R. Changes in cardiac muscle function and biochemistry produced by long-term amiodarone and amodarone + triiodothyronine administration in the rabbit. Pharmacology. 35: 130-140, 1987.

Powis, G., Olsen, R., Standing, J. E., Kachel, D., and Martin II, W. J. Amiodarone-mediated increase in intracellular free Ca^{2+} associated with cellular injury to human pulmonary artery endothelial cells. Toxicol. Appl. Pharmacol. 103: 156-164, 1990.

Scherlag, B. J., Patterson, E., Lazarra, R., Bailey, B. R., Thompson, M. D., and Berlin, K. D. Comparative electrophysiological and hemodynamic actions of BRB-I-28 and lidocaine in the normal and infarcted dog heart. J. Electrophysiol. 2: 461-477, 1988.

Smith, G. S., Thompson, M. D., Berlin, K. D., Holt, E. M., Scherlag, B. J., Patterson, E., and Lazzara, R. A study of the synthesis and antiarrhythmic properties of selected 3,7-diheterabicyclo[3.3.1]nonanes with substituents of the 2,4-positions and at 9-position. Eur J Med Chem. 25: 1-8, 1990.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., Klenk, D. C. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150 : 76-85, 1985.

Tallarida, R. J., and Murray, R. b. Mannual of pharmacologic calculation with

computer programs. Springer-verlag, New York, 1986.

Wang, T., Nakamura, J., and Schwartz, A. Cardiac sarcoplasmic reticulum Ca^{2+}-ATPase. In "Methods in Pharmacology" (Schwartz, A., eds), pp 39-47, Plenum Press, New York, 1984.

Figure Legends

Figure 1. Chemical structures of BRB-I-28 (A), GLG-V-13 (B), SAZ-VII-22 (C) and SAZ-VII-23 (D)

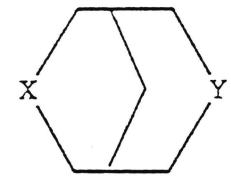
Figure 2. Effects of DHBCN derivatives on submitochondrial NADH oxidase. (a) BRB-I-28; (b) GLG-V-13; (c) SAZ-VII-22; and (d) SAZ-VII-23

Figure 3. Effects of DHBCN derivatives on submitochondrial NADH-CoQ reductase. (a) BRB-I-28; (b) GLG-V-13; (c) SAZ-VII-22; and (d) SAZ-VII-23

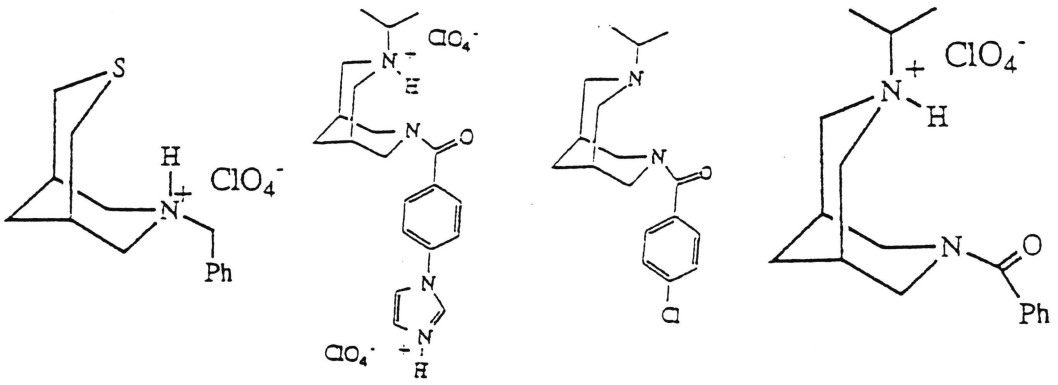
Figure 4. Effects of DHBCN derivatives on submitochondrial ATPase. (a) BRB-I-28; (b) GLG-V-13; (c) SAZ-VII-22; and (d) SAZ-VII-23

Figure 5. Localization of inhibition of DHBCN derivatives on reipiratory chain

Figure 6. The possible mechanisms for an increase in cytosolic Ca^{2+} concentration. (-) Inhibition; (+) Stimulation



3,7-Diheterabicyclo[3.3.1]nonane
(DHBCN)



A B C D

Figure 1

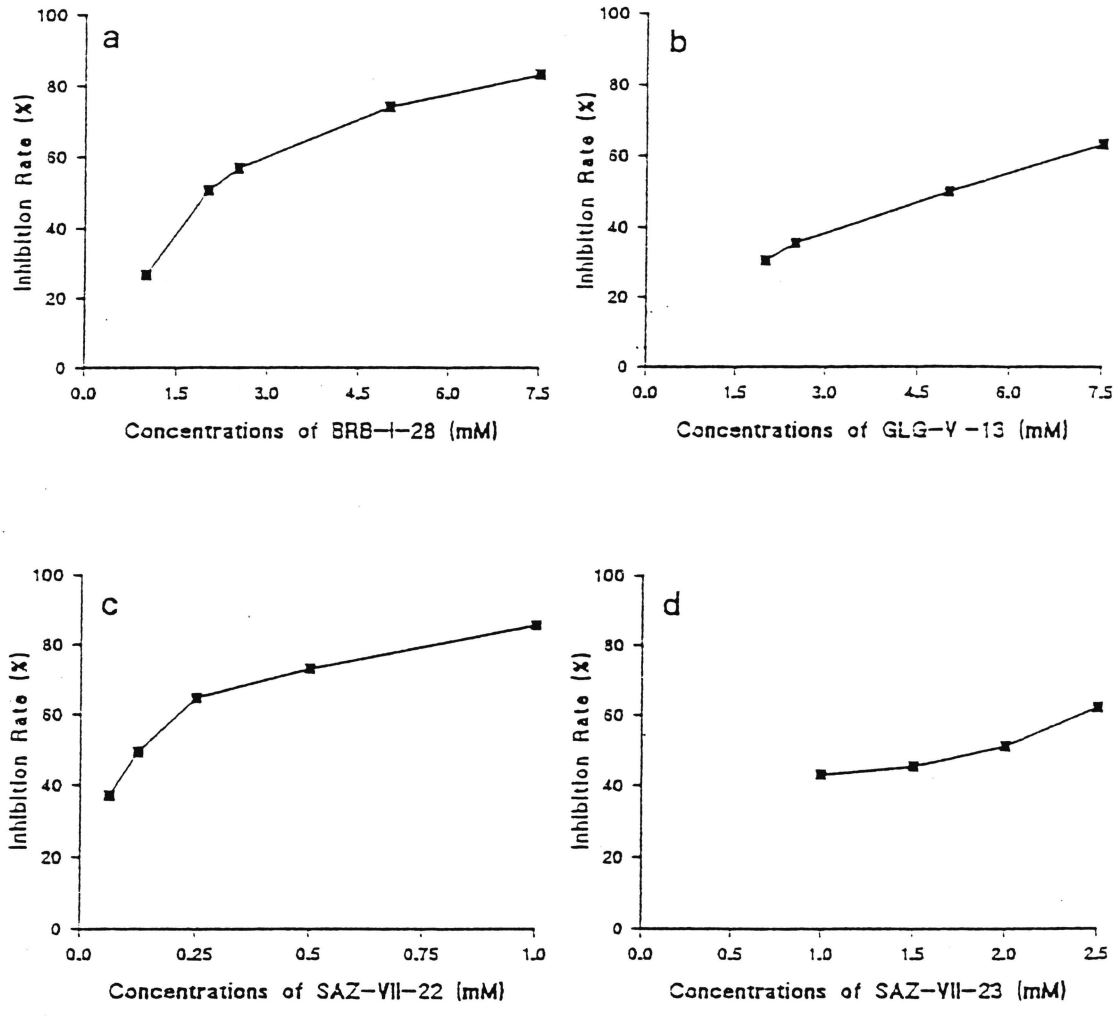

a
Inhibition Rate (%)
100
80
60
40
20
0
0.0
1.5
3.0
4.5
6.0
7.5
Concentrations of BRB-I-28 (mM)
b
Inhibition Rate (%)
Concentrations of GLG-V-13 (mM)
c
Inhibition Rate (%)
0.0
0.25
0.5
0.75
1.0
Concentrations of SAZ-VII-22 (mM)
d
Inhibition Rate (%)
0.0
0.5
1.0
1.5
2.0
2.5
Concentrations of SAZ-VII-23 (mM)

Figure 2

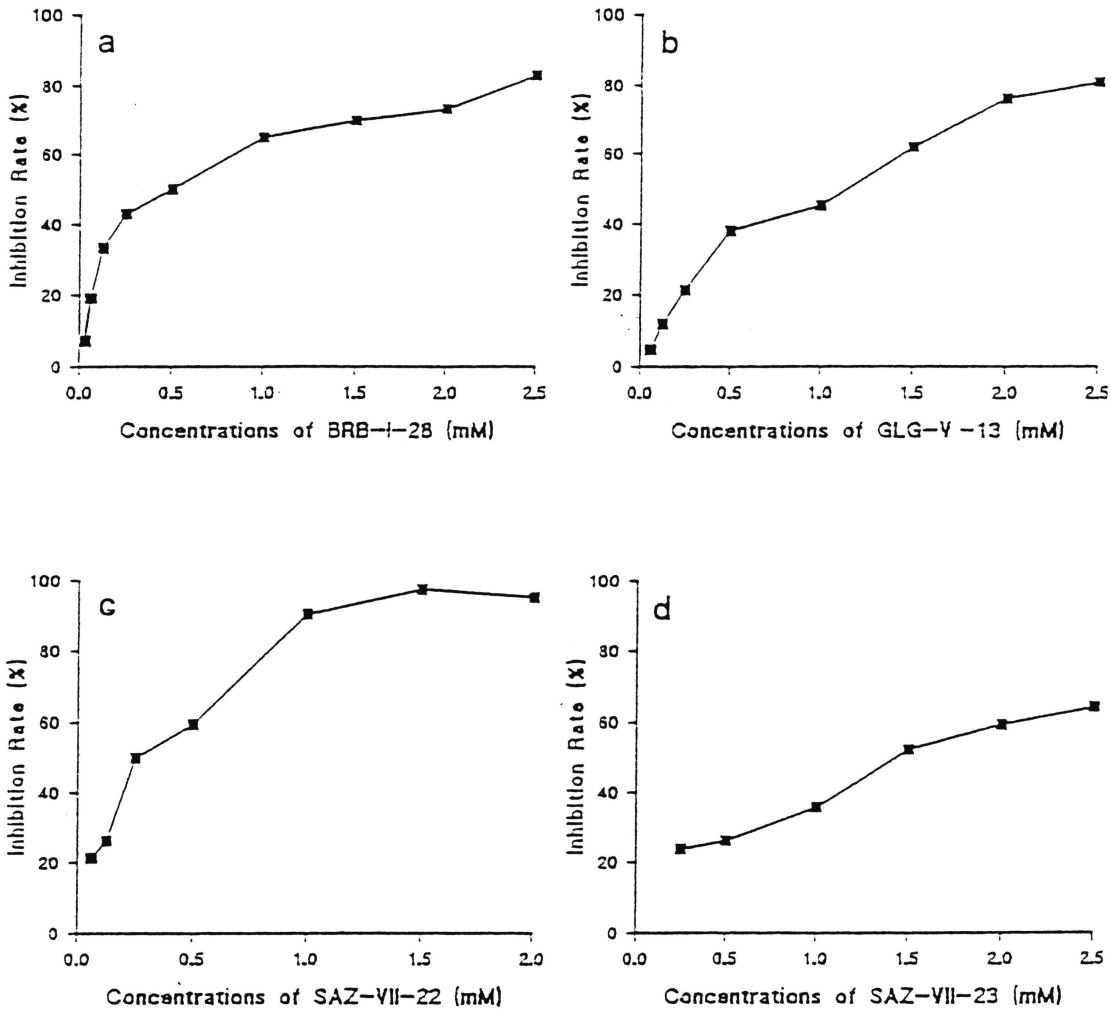

a
Inhibition Rate (%)
Concentrations of BRB-I-28 (mM)
b
Inhibition Rate (%)
Concentrations of GLG-V-13 (mM)
c
Inhibition Rate (%)
Concentrations of SAZ-VII-22 (mM)
d
Inhibition Rate (%)
Concentrations of SAZ-VII-23 (mM)

Figure 3

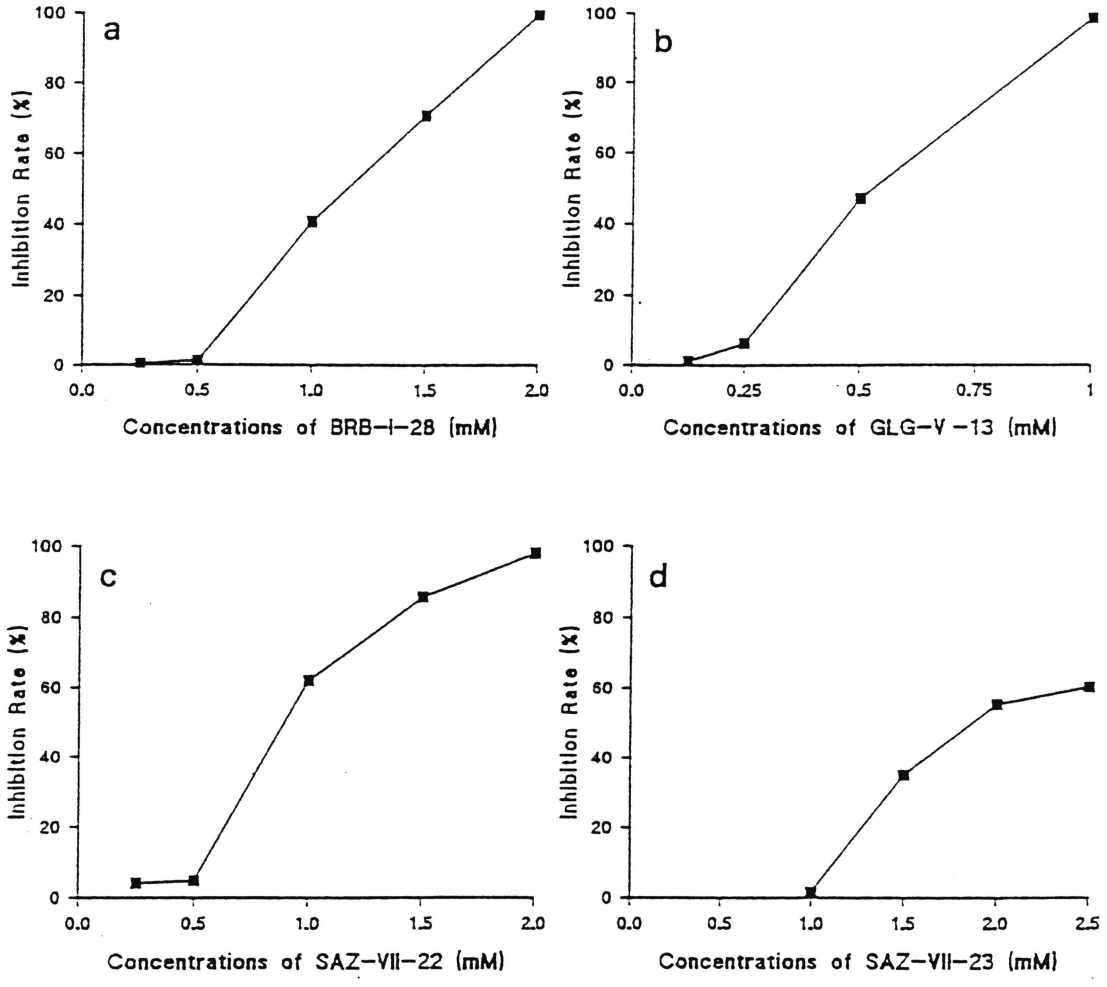

a
Inhibition Rate (%)
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
Concentrations of BRB-I-28 (mM)
b
Inhibition Rate (%)
0.0
0.25
0.5
0.75
1
Concentrations of GLG-V-13 (mM)
c
Inhibition Rate (%)
Concentrations of SAZ-VII-22 (mM)
d
Inhibition Rate (%)
0.0
0.5
1.0
1.5
2.0
2.5
Concentrations of SAZ-VII-23 (mM)

Figure 4

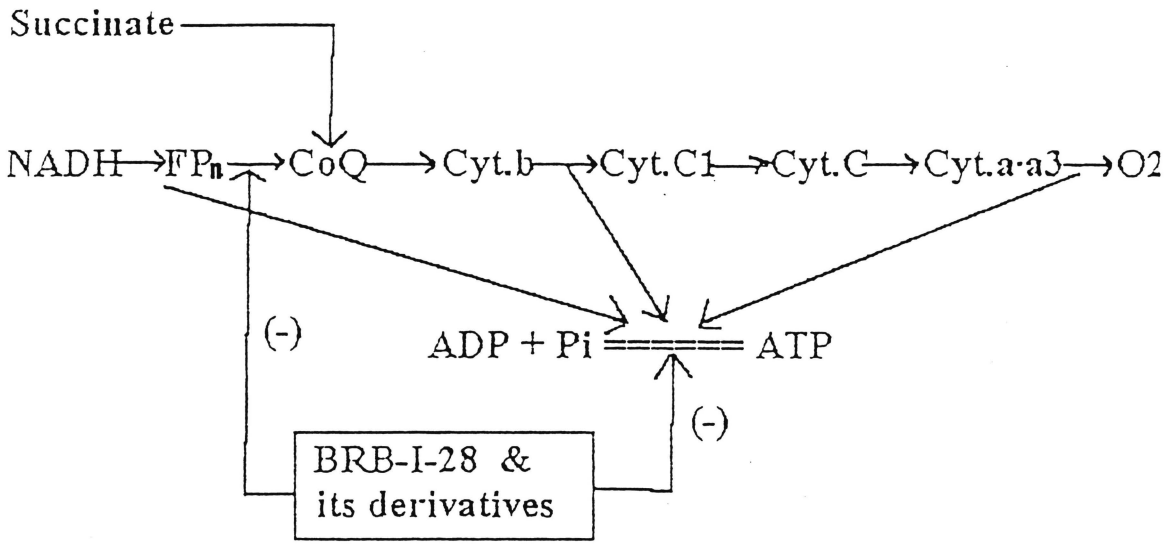

Succinate
NADH
FPn
CoQ
Cyt.b
Cyt.C1
Cyt.C
Cyt.a·a3
O2
(-)
ADP + Pi
ATP
(-)
BRB-I-28 &
its derivatives

Figure 5

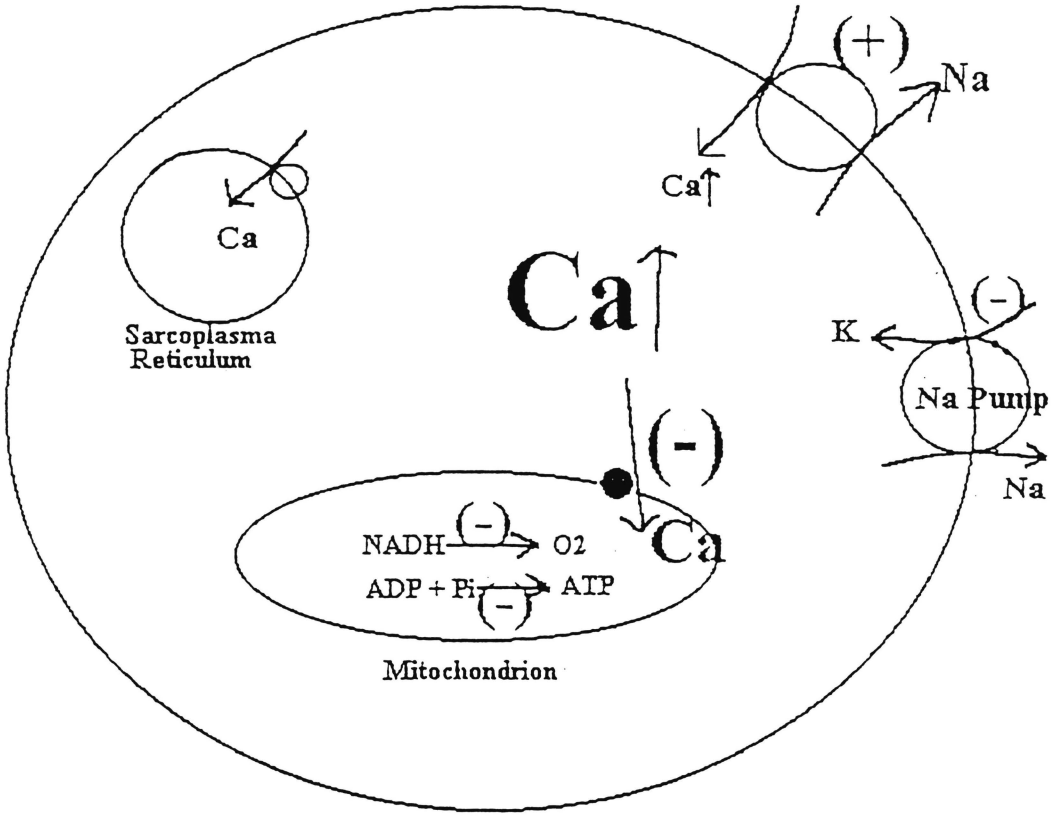

(+)
Na
Ca↑
Ca
Ca↑
Sarcoplasma
Reticulum
(-)
K
Na Pump
Na
(-)
Ca
NADH
(-)
O2
ADP + Pi
ATP
(-)
Mitochondrion

Figure 6

CHAPTER X

THE ACUTE AND SUBCHRONIC TOXICITY OF BRB-I-28, A NOVEL CLASS IB ANTIARRHYTHMIC AGENT, IN CD-1 MICE

Abstract

The acute and subchronic toxic effects of BRB-I-28 (7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane·HCl), a novel antiarrhythmic agent, were investigated in mice of both sexes. The estimated LD_{16}, LD_{50} and LD_{84} for BRB-I-28 given orally were 86, 128 and 189 mg/kg for male mice and 87, 131 and 196 mg/kg for female mice, respectively. The acute toxic signs appeared to be of CNS in origin. Four groups of mice (20/sex/group/dose) were fed daily with diet containing BRB-I-28 for 90 consecutive days. At interim of 45 days, 5 mice per group were used for the 45-day toxicity study. The approximate dose levels in male mice were 0, 11.7, 23.3, and 68.4 mg/kg/day for the 45-day toxicity study and 0, 16.1, 30.4, and 77.6 mg/kg/day for the 90-day toxicity study. The dose levels in female mice were 0, 14.0, 28.2 and 79.8 mg/kg/day for the 45-day toxicity study and 0, 17.5, 36.8, and 90.1 mg/kg/day for the 90-day toxicity study. All of the mice survived during 45 and 90 day studies. No significant toxic signs were observed during the studies. Food consumption per day was decreased, but water consumption per day was increased in a non-dose-dependent manner. However, both mean body weight and mean body weight gain were not significantly changed during the 45- and 90-day studies. An increase in feed

efficiency was observed with most of the drug treated groups. There were no significant changes in hematologic, biochemical values and the individual organ weights of drug-treated groups in the 45-day toxicity study. Significant gross and histopathological changes were not observed in the 45-day toxicity study. The amounts of MCHC and MCH were significantly increased, while the number of platelets was decreased in both male and female mice of the 90-day toxicity study. There were some significant changes in both absolute and relative weights of organs such as brain, heart, liver, kidney, spleen, and ovary plus uterus. Although there were some gross pathological changes confined to liver and lung in low and middle dose groups of the 90-day toxicity study, no significant histopathological and biochemical value changes were associated. However, there were significant gross and histopathological changes in high dose groups of the 90-day toxicity study, especially in the liver of male mice because of a significant increase in liver weight, liver/brain, and liver/body weight ratios. Fatty liver was observed in both sexes macroscopically. Hepatocellular necrosis was found in both male and female mice treated with high dose level of BRB-I-28, although there were no significant changes in the liver weight of female mice. Since there was no significant increase in serum liver enzyme activities, such as ALP, AST, ALT, and LDH, hepatocellular lesions could be mild in nature. Renal cortical vacuoles and myocardial necrosis with low numbers of lymphocytic infiltrations were present only in female mice treated with high dose level of BRB-I-28. The lesions in kidney and heart were mild as indicated by very little change in BUN, AST and LDH values. The data from the 45- and 90-day subchronic toxicity studies indicate that BRB-I-28 has limited toxicity potential. Based on the data, no-effect levels were tentatively determined at middle dose levels, i.e., 30.4

mg/kg/day for male mice and 36.8 mg/kg/day for female mice.

Introduction

BRB-I-28 (7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane·HCl) is a novel agent which has effective antiarrhythmic properties in dog and rat models (Bailey et al., 1984; Scherlag et al., 1988; Pugsley et al., 1992) and low proarrhythmic effects (Fazekas et al., 1992; 1993a; 1993b). It belongs to the family of 3,7-diheterabicyclo-[3.3.1]nonane (DHBCN) derivatives. Electrophysiological properties of BRB-I-28 were typical of class Ib antiarrhythmic drugs having use-dependent inhibition on sodium channels (Patterson et al., 1991; 1993). Unlike most other antiarrhythmic drugs which produce negative inotropic effects, BRB-I-28 was found to produce a significant elevation of systemic mean arterial blood pressure at the effective antiarrhythmic dose during sinus rhythm (Scherlag et al., 1988). BRB-I-28 has been demonstrated to have significant inhibitory effects on Na^+,K^+-ATPase and mitochondrial respiration, which may contribute to some of its electrophysiological properties and positive inotropic effects, leading to a moderate hypertensive response (Chen et al., 1992; 1994a).

The pharmacokinetic and metabolism profiles of BRB-I-28 in rats and dogs have been characterized (Alavi et al., 1991; Chen et al., 1993; 1994b). BRB-I-28 has reasonably good oral absorption, extensive wide distribution, and metabolism, and low protein binding properties with limited elimination rate as a parent compound. Two metabolites, sulfoxide and benzamide, were identified and were found to have less antiarrhythmic effects (Chen et al., 1994b). High accumulation of BRB-I-28 occurs

in the highly perfused organs such liver, heart and kidney but is low in the brain and fat. The soluble form of BRB-I-28 (7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane·HCl) was recently synthezied (Garrison, 1993), which may be more useful in clinical therapy. During the pharmacokinetic study, no significant systemic toxicity with intravenous dose of 10 mg/kg and oral dose of 20 mg/kg in dogs was observed. However, detailed toxicity profiles of this compound have not been completed. The objectives of this study were to characterize both acute and subchronic toxicity profiles of this novel antiarrhythmic agent in CD-1 mice of both sexes.

Materials & Methods

Chemicals

A soluble form of BRB-I-28 was synthesized by a method previously reported (Garrison, 1993). The purity of the compound was >99.0% as determinated by thin-layer chromatography (TLC). Mice pelleted food (Purina Lab Chow 5001) was purchased from Purina Mills Inc. (Richmond, IN).

Animals

Four week old, CD-1 strain mice (Charles River Breeding Labs) of both sexes weighing 18 to 25 g were housed in a controlled environment (12-hour light/12-hour dark photoperiod changing at 0800 and 2000 hr, 22±1°C, 50±10% relative humidity). The mice used for the acute toxicity study were allowed free access to pelleted food (Purina Mills Inc., Richmond, IN). The mice were fed powdered food for the 45- and 90-day toxicity studies. The mice had free access to city tap water *ad libitum*. All

mice were preconditioned for 2 weeks prior to initiation of dosing. A preliminary health check indicated that no gross abnormalities were present in these mice.

Acute Toxicity

Twelve hours before initiation of the experiment, food was withdrawn. Mice were randomly allotted in 5 groups (10 mice/sex/group/dose). BRB-I-28 was dissolved in deionized water processed through Milli-Q Water System (Millipore Corp., Marlborough, MA). Individual mice within each group was gavaged (ca 0.2 ml/10 g) different doses (76.8, 96, 120, 150, 187, 233.8 mg/kg) of aqueous solution of BRB-I-28 using a No. 18 stainless-steel ball-tipped feeding needle. Systemic toxic signs were monitored for a period of three days, and the body weight of survivors were determined daily.

Determination of LD_{16}, LD_{50} and LD_{84}

The number of animals which died acutely within groups for each dose was counted. The LD_{16}, LD_{50} and LD_{84} with 95% confidence limits were calculated by a computer program PHARM/PCS based on Litchfield and Wilcoxon method (Tallarida and Murray, 1986). The data were expressed as LD_{50} (mg/kg) with 95% confidence limits.

Diets Preparation

Powdered diets containing 0, 75, 150 and 440 mg/kg of BRB-I-28 were prepared by mixing together preweighed quantities of BRB-I-28 and powdered diets in a HandyBlender II (Balck & Decker Inc., Shelton, CT) for 15 min. The diet was

prepared once in every two days. Analytical determinations with HPLC indicated that this procedure resulted in a homogenous mixture. Due to lack of significant toxicity in the 45-day toxicity studies, the amount of BRB-I-28 in the powdered diets was increased to 0, 150, 300, and 700 mg/kg for feeding the mice in the remaining 45 days.

Forty-five- and Ninety-Day Toxicity Studies

After adaptation for 2 weeks, the mice were randomly divided into four groups (20 mice/sex/group/dose). Test animals were approximately 6 weeks old with mean body weight of 26.0±2.1 g at the beginning of dosing. Five mice were housed together in suspended 11" x 6.7" x 4.7" solid-bottom polycarbonate cages fitted with hardwood chips as cage bedding. The mice were allowed free access to food and city tap water. Animals were fed with powdered food containing three different dose levels of BRB-I-28 between 1300 and 1500 hr, and the control group was fed the diet without BRB-I-28. The physical condition and overt signs of toxicity of the individual mice within a group were assessed daily. Body weights were measured prior to the first dosing and thereafter once a week during the entire test period. Food and water consumption for a group of 5 mice in each cage were assessed once every two days. Feed efficiency (g total body weight gain/g total feed consumption x 100) was calculated from body weight gain and food consumption.

Blood Sampling. Of the 20 mice per group, 5 mice were used for the 45-day toxicity study and remaining 15 mice for the 90-day toxicity study. Blood samples were collected from ocular venous plexus of the individual mice under light CO_2 anesthesia on the days 46 and 91. The portions (ca. 0.3 ml) of the whole blood were

collected in microtainers with EDTA·K_2 (Becton Dickinson and Company, Rutherford, NJ), and the remaining portions were placed in separate tubes containing no anticoagulant (Vacutainer, Becton Dickinson and Company, Rutherford, NJ) for separation of serum. Then, the mice were terminated by cervical dislocation of spinal cord with cerebrum. Both whole blood and serum samples from individual mice were used for determination of hematological and clinical chemistry profiles, respectively.

Determination of Hematological Values. The number of white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets counts, and total protein on whole blood were measured with SYSTEM 9000, Baker Instrument (Serono-Baker Diagonistics Inc., Allentown, PA). Differential counts of WBC, morphologies of both WBC and RBC were determinated from blood smear by light microscopy.

Determination of Clinical Pathological Values. Serum biochemical values including blood urea nitrogen (BUN), alkaline phosphatase (ALP), asparate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) activity, and sodium, potassium, albumin, and cholesterol concentrations were determined by using a Roche Cobas Mira (Roche Analytical Instruments, Nutley, NJ) at 46th and 91th days.

Gross Pathological Findings. The weights of brain, heart, liver, kidney, spleen, testis, epididymis, ovary plus uterus, adrenal, and lungs were measured. The organ/brain and organ/body weight ratios were calculated. Any significant gross pathological changes in individual mouse were observed and noted.

Histopathological Examination. At necropsy, the following tissues were collected in 10% neutral buffered formalin: adrenal glands, brain, caecum, colon, duodenum, heart (with major vessels), ileum, kidney, liver, lung, lymph node, pancreas, pituitary, skeletal muscle, spleen, stomach, and urinary bladder. The eye, epididymis, and testis were fixed in Boune's solution and then transferred to 70% ethanol. Following dehydration and embedding, all tissues were routinely processed, sectioned at 5-6 microns, and stained with hematoxylin and eosin (H&E) and then examined microscopically.

Statistical Analysis of the Data

Mean and standard deviations were calculated for body weight, food, water consumption, clinical pathologic, and organ weight data. Statistical analysis was carried out using an appropriate Student t test (equal or unequal variance) and a computer program PHARM/PCS (Tallarida and Murray, 1986). Differences between the treatment groups and controls were considered significant at $p<0.05$, and highly significant at $p<0.01$.

Results

Oral LD_{50} Study

Although a given dose of BRB-I-28 produced a different number of deaths in male and female mice, the median acute lethal dose was similar in both male and female mice with LD_{50} of 128 and 131 mg/kg, respectively. LD_{16} and LD_{84} were 86, 189 mg/kg for male mice, and 87, 196 mg/kg for female mice, respectively (Table 1).

Body Weight, Food, Water, Drug Consumption, and Feed Efficiency

The initial mean body weight, body weight and body weight gain at 46th day, food, water, and drug consumption in the 45-day toxicity study are presented in Table 2. In the 45-day toxicity study, the approximate BRB-I-28 consumption was calculated to be 11.7, 23.3, and 68.4 mg/kg/day for male mice, and 14.0, 28.2, and 79.8 mg/kg/day for female mice. Figures 1 and 2 show mean body weight and cumulative body weight gains in male and female mice fed BRB-I-28 for 45 days, respectively. Initial body weights were almost the same for all groups. After treating with BRB-I-28 for 45 days, there were no significant differences in mean body weight between control and all the drug-fed groups. However, there was a significant increase in body weight gain in mice fed with low level of both male and female mice. A decrease in food consumption in high and low dose level groups of male mice and in all three dose level groups of female mice was observed, but no change in middle dose level of male mice. There was no marked difference in water consumption observed in three dose level groups of both male and female mice. Feed efficiency was significantly increased in all dose levels of male mice, however, feed efficiency in female mice was increased only in the low dose level group (Figure 3). In male mice with low dose level of BRB-I-28, feed efficiency significantly increased beginning 21 day to 45 day; with middle dose level beginning 28 day to 45 day; and with high dose beginning 35 day to 45 day. However, in female mice, feed efficiency was increased with low dose level only at final week. A decrease in food consumption and an increase in feed efficiency were not dose-related. The initial mean body weight, body weight gain and body weight at 91th day, food, water and drug consumption in 90 day toxicity study are presented in Table 3. Mean body weight

and cumulative body weight gains in male and female mice fed BRB-I-28 for 90 days are presented in Figures 4 and 5, respectively.

In 90 day toxicity study, the BRB-I-28 consumption was calculated to be 16.1, 30.4 and 77.6 mg/kg/day for male mice and 17.5, 36.8 and 90.1 mg/kg/day for female mice. Initial body weights were approximately the same for all groups. Body weight and body weight gain during 90 days were not significantly different in male and female mice at all three dose levels compared with the control group, except the body weight of male mice fed with middle dose of BRB-I-28, which was significantly higher than that of the control group. Food consumption of mice fed BRB-I-28 markedly reduced in all three dose levels groups of both male and female mice. In male mice fed BRB-I-28, water consumption increased markedly in all three dose levels, but no change was observed in the female mice. An increase in feed efficiency was observed only in male mice treated with middle dose level of BRB-I-28 (Figure 6). Like the situation in the 45-day toxicity study, food consumption was significantly decreased in all three dose level groups of both male and female mice. However, this change was not dose-dependent. In male mice with a low dose level, feed efficiency was increased beginning the 3rd week to the 6th week and then remaining the same as that of controls. With the middle dose level, feed efficiency was increased from the 2nd week to the 13th week. With the high dose level, feed efficiency was increased beginning 10th week to 13th week. In female mice fed with low and middle dose levels of BRB-I-28, feed efficiency was increased beginning the 6th week to 11th week, while with the high dose level of BRB-I-28, feed efficiency was increased beginning the 6th week to the 13th week. However, water consumption was not markedly changed with all three dose levels of female mice.

Hematology

Mean hematological values in male and female mice fed BRB-I-28 for 45 days have been presented in Tables 4 and 5, respectively. There were no significant changes in hematological values of male and female mice fed BRB-I-28 for 45 days, except a significant elevation in the percentage of eosinophils and absolute numbers of lymphocytes observed in female mice fed a middle dose level of BRB-I-28. However, the absolute number of eosinophils and the percentage of lymphocytes were *not* significantly changed.

Tables 6 and 7 show the mean hematological values in male and female mice fed BRB-I-28 for the 90-day toxicity study, respectively. Most of the hematological values were not significantly different between control and all the three dose groups in the 90-day toxicity study. The amount of MCHC was markedly increased in male and female mice fed middle and high dose levels of BRB-I-28. The number of platelets was reduced significantly in male mice fed with middle and high dose levels of BRB-I-28, and the reduction also occurred in female mice in all three dose level groups. A significant elevation of both the percentage and absolute number of monocytes were observed only in the middle dose level group of both male and female mice. In male mice, a significant elevation in white blood cells was found in the middle dose level group and a decrease in the percentage of segmented neutrophils along with an increase in absolute number of lymphocytes were observed in the high dose level group. In female mice, a reduction in both mean corpuscular hemoglobin of the high dose level group and total protein content of the low dose level group were observed.

Clinical Biochemical Values

The clinical biochemical values in mice fed at various levels of BRB-I-28 for 45 and 90 days have been presented in Tables 8 and 9, respectively. No significant changes in clinical biochemical values were observed during the 45-day toxicity study.

In 90 days toxicity study, a significant decrease in serum Na^+ was observed in male mice treated with high dose level of BRB-I-28 but no change in female mice (Table 9). The amount of serum K^+ was found to decrease only in female mice treated with a high dose level of BRB-I-28 with no change in male mice. There was a significant reduction in serum alkaline phosphatase in female mice treated with all three dose levels of BRB-I-28 (Table 9). There were no significant changes in BUN, LDH, AST, ALT, albumin, and cholesterol in both sexes fed at all dose levels of BRB-I-28.

Organ Weight Changes

Absolute, relative organ weights to brain and to body weight of both male and female mice fed BRB-I-28 for 45 days are presented in Tables 10 and 11, respectively. In male mice, no significant change in absolute and relative weights of organs including brain, liver, kidney, lung, epididymis, spleen, testis, and adrenal were observed. In female mice, there were some changes but only in brain, adrenal and liver. Absolute brain weight was decreased significantly but only in middle dose level. Although absolute adrenal weight was not markedly altered, adrenal to brain weight and adrenal to body weight ratios were significantly increased only in female mice treated with a low dose level of BRB-I-28. A significant change in relative liver to body weight ratio occurred in the middle dose level of female mice.

Tables 12 and 13 show the absolute and relative organ weights in male and female mice treated with BRB-I-28 for 90 consecutive days, respectively. In male mice treated with low dose level of BRB-I-28, organ weight changes were limited to heart and testis, absolute heart weight, heart/brain weight and heart/body weight ratios were elevated markedly, and an increase in absolute testis weight was observed. With the middle dose level, absolute brain, kidney, spleen, and testis weights were found to increase, but the elevation was only observed in liver/body weight and spleen/body weight ratios. With a high dose level, absolute liver and lungs weights, and their relative to brain weight and to body weight ratios were increased significantly. In female mice, there were some changes in ovary plus uterus without significant changes in all other organs. The marked reduction in absolute ovary and uterus weight was observed in all three dose levels, while a reduction in ovary plus uterus/brain weight ratio and ovary plus uterus/body weight ratio were observed in both low and high dose groups, but no change occurred in the middle dose group.

Gross Pathological Changes

There were some significant gross pathological changes during the 45-day toxicity study. In male mice, mild pulmonary edema/congestion was observed in 1/5, 2/5, and 2/5 mice of low, middle and high dose groups, respectively. One mouse in the high dose group showed significant enlargement of the kidney. A similar phenomena of mild pulmonary congestion/hemolysis was observed in 1/5 female mice of all the three dose group, respectively.

In the 90-day toxicity study, gross pathological changes were observed mainly in liver and lung. The pulmonary congestion/edema was observed in 1/15 in all 3

dose groups of male and female, respectively. Pale, fatty liver was observed in middle and high dose groups with 3/15 and 4/15 male mice and 1/15 and 1/15 female mice, respectively.

Histopathological Changes

Abnormal histopathological changes of any kind were not observed in all tissues of mice fed all levels of BRB-I-28 during the 45-day toxicity study. Significant histopathological changes were limited in high dose groups of the 90-day toxicity study. Histologic examination of tissue sections from male mice administrated BRB-I-28 at the highest dose group (77.6 mg/kg/day for 90 days) revealed multifocal, randomly scattered hepatocellular necrosis. The necrotic focus contained few (between 1-4) shrunken necrotic hepatocytes with pyknotic nuclei and condensed eosinophilic cytoplasm (Figure 7). The focus contained mild infiltrates of mononuclear cells predominantly lymphocytes. This lesion was observed in 8/10 mice administrated BRB-I-28. The extent (number or size) of the lesions did not differ between the mice. Male mice supplemented with BRB-I-28 at the other 2 lower doses (16.1 and 30.4 mg/kg/day) did not have significant histopathologic lesions. Significant histopathological lesions were *not* present in any other tissues examined.

Focal hepatocellular necrosis with infiltration of mononuclear cells similar to that of male mice was observed in 4/10 mice of high dose group (90.1 mg/kg/day for 90 days). One female mouse in the high dose group had several renal cortical (proximal convoluted tubule) vacuoles. Within this dose group, 3/10 mice had focal myocardial necrosis with low numbers of lymphocytic infiltration. The lesion was

mild, with a maximum of 2 foci observed in a section. The lesion was confined to the left ventricles. A fourth mice had scattered vacuolated myofibers without any inflammatory cells. Significant histopathological lesions were *not* present in any other tissues of male and female mice examined.

Discussion

Acute Toxicity

During the acute toxicity study, most cases of death in mice were observed within 10 minutes after oral administration of BRB-I-28. This finding is significantly different from that observed in the mice treated with acute dose of GLG-V-13 in which most cases of death were over 30 minutes period (Chen et al., 1994), but similar to that of lidocaine in which most cases of death were within 15 minutes (Smith and Duce, 1971). The acute toxic signs including sedation, tremors, convulsion, twitching, catalepsy, and ataxia, followed by dyspnea and apnea leading to death, were of CNS in origin. The systemic signs were very similar to those treated with GLG-V-13 (Chen et al., 1994) and lidocaine (Smith and Duce, 1971), two other antiarrhythmic agents. There was no significant changes in body weight within 3 days. Since the mice were not subjected to necropsy, gross and histopathological status of the individual target organ were unknown.

Forty-five-Day Toxicity

In the 45-day toxicity study, all the mice fed with different levels of the drug survived and showed no overt signs of toxicity. Some change in differential counts

of WBC of female mice was observed, although there was no significant change in the total number of white blood cells. However, a change in the percentage was accompanied by no change in absolute number, or a change in the absolute number was accompanied by no change in the percentage. Therefore, these changes may have no significant toxicological consequences. In necropsy, there were some organ weight and gross pathological changes in the liver. However, neither histological lesions nor changes in serum enzymes such as ALP, LDH, AST and ALT indicative of liver dysfunction were observed. Therefore, no significant organ toxicity was found in the 45-day toxicity study. Gross pathological alterations unaccompanied by histopathological changes in the lungs seem to be a common finding in both male and female mice of all dose groups. It is quite possible that changes in the lungs may be an artifact. It could have been caused by inhalation of CO_2 or by the procedure of taking blood samples.

Ninety-Day Toxicity

In the 90-day toxicity study, there was no drug-related mortality during the course of the study, and toxic signs were unremarkable. There was no significant change in body weight and body weight gain except a marked increase in body weight of male mice fed with a middle dose of the drug. Interestingly, water consumption was significantly increased in all three dose levels of male mice even though food consumption was significantly *decreased*. Since there were not significant changes in hematocrit and serum constituents (serum albumin, urea nitrogen), it is obvious that the normal water balance is maintained throughout the study. However, the sodium ion is the most important component of the osmotic factors involved in the

distribution of water between extra- and intracellular tissue spaces. Significant hyponatremia observed in male mice fed with the high dose level could have resulted in increased water consumption. The observed relationship between sodium ion loss and increased water consumption is uncertain, and the toxicological importance for this is unknown. Water consumption and serum sodium ion content remained the same in control and drug-treated groups of female mice.

A consistent increase in mean corpuscular hemoglobin concentration with the middle and high dose levels of male and female mice was observed. However, mean corpuscular hemoglobin was increased only in female mice treated with high dose levels of BRB-I-28. The erythrocytic indices including both MCHC and MCH, which are calculated from the red blood cell count, hemoglobin concentration, and volume of packed red blood cells were relatively inaccurate (Woodliff and Herrmann, 1973). The lack of a dose-dependent change, in combination with the lack of any change in the number of red blood cells, hemoglobin, and HCT, suggest that BRB-I-28 may have no significant toxicologic effects on the red blood cells and hemoglobin. A significant decrease in platelet counts of both male mice treated with middle and high dose levels and female mice treated with all three dose levels of BRB-I-28 indicate that thrombocytopenia caused by BRB-I-28 may have some effects on platelet plug formation, coagulation, and clot retraction. However, this effect is not dose-related. A decrease in platelet counts may be caused by either deceased platelet production or increased platelet destruction, exact mechanisms for which remain unknown. Further investigation on the potential toxicological impact of BRB-I-28 is warranted.

No gross pathological lesions of vital target organs, except pulmonary edema/congestion and fatty liver in low- and middle-dose groups of the 90-day toxicity

study were observed. Moreover, histopathological lesions were *not* found in any organs even in liver and lung. Therefore, although weights of several organs, such as brain, kidney, liver, and lung of male mice in the low- and middle-dose groups, were changed significantly, these changes were of questionable toxicologic significance. In female mice, only the ovary plus the uterus showed significant reduction in their absolute and relative weights. But there were no gross and histopathological alterations associated therewith. Therefore, these may have no significant toxicologic importance.

Attention should be paid to some histopathological changes of the liver, kidney, and heart in the *high-dose group*. In male mice, the hepatocellular necrosis was associated with significant increase in liver weight and liver/brain weight ratio and liver/body weight ratio. The focal necrosis of hepatocytes was observed *only* at the high dose level of 77.6 mg/kg/day for male mice and 90.1 mg/kg/day for female mice. The necrosis was of a coagulative type characterized by shrunken hypereosinophilic hepatocytes with degenerating nuclei. Liver cell death may be caused by variety of drugs and chemicals. Although this patten of necrosis has been described following the exposure of humans to a variety of drugs and toxicants, it is infrequently observed in toxicologic studies in animals. Focal necrosis has been described in detail following the acute treatment of rats with phalloidin, carbon tetrachloride, and D-galactosamine (Plaa, 1986; Popp and Cattley, 1991). The necrotic hepatocytes are replaced by regeneration of adjacent hepatocytes *without* fibrosis. Since repair occurs by regeneration, without inciting fibrosis, the lesion may be undetectable histologically with the lower doses of the drugs. Furthermore, since several liver enzymes such as ALP, LDH, AST and ALT, did not change significantly from basal values, the

hepatocellular lesion is of mild nature. This hypothesis was further confirmed by no changes in albumin and cholesterol content in control and drug treated groups.

Female mice administrated BRB-I-28 at the *high dose* of 90.1 mg/kg/day had individual necrotic myocytes with minimal inflammatory infiltrates of mononuclear cells. The lesion seems to have healed by fibrosis with deposition of connective tissue. Myocardial lesions without marked changes in serum enzymes such as AST, ALT, and LDH elevations implied no serious toxicological significance.

Although there were vacuoles observed in the renal cortex of female mice fed the high dose of BRB-I-28, the fact that there was no change in BUN makes the drug relatively free of renal toxicity. Pharmacokinetic disposition data of BRB-I-28 in rats have indicated that this drug is extensively accumulated in highly perfused organs including liver, kidneys, and heart (Alavi, et al., 1991). The histopathological changes confined to these organs could possibly be due to the increased levels of this compound or its metabolites in liver, kidney and heart. Unlike GLG-V-13, a combined class Ib and class III antiarrhythmic agent which produced marked vacuolation of the X zone in the adrenal gland with mild to moderate deposition of ceroid pigment (brown degeneration), the adrenal gland was free of any lesion in BRB-I-28 treated mice (Chen et al., 1994c).

It was interesting to find that ALP in female mice was markedly decreased, *but not in dose-dependent manner.* However, decreased activity of serum ALP has little clinical significance (Cohn and Kaplan, 1971). Whether it was a favorable effect/undesirable toxic effect was unclear. In the high dose level, sodium ion content in male mice and potassium ion content in female mice were significantly decreased. Sodium and potassium reabsorption and excretion were mainly controlled through the

kidney. Mild kidney lesions and excessive intake of water may make some contribution to these electrolyte changes. A decrease in serum sodium and potassium ions may be due to the inhibitory effects of BRB-I-28 on renal Na^+,K^+-ATPase. Exact mechanisms for this phenomenon must be further investigated.

In summary, the results of both acute and subchronic toxicity studies indicate that this class Ib antiarrhythmic agent, BRB-I-28, has a low potential for systemic toxicity. No evidence of systemic toxicity, in combination with its low proarrhythmic actions and desirable cardiovascular effects, make this compound worthy of further development as a potential antiarrhythmic drug.

References

Alavi, F. K., Clarke, C. R., Sangiah, S., Berlin, K. D., Zisman, S. A., Chen, C. L., Garrison, G. L., Scherlag, B. J., and Lazzara, R. Disposition of BRB-I-28 (7-benzyl-7-aza-3-thiabicyclo[3.3.1]nonane hydroperchlorate), a novel antiarrhythmic agent. Drug Invest. 3: 317-323, 1991.

Bailey, B. R., Berlin, K. D., Holt, E. M., Scherlag, B. J., Lazzara, R., Brachmann, J., Helm, D. V. M., Powell, D. R., Pantaleo, N. S., and Ruenitz, P. C. Synthesis, conformational analysis, and antiarrhythmic properties of 7-benzyl-3-thia-7-azabicyclo [3.3.1]nonan-9-one, 7-benzyl-3-azabicyclo [3.3.1]nonane hydroperchlorate, and 7-benzyl-9-thia-7-azabicyclo[3.3.1]nonan-9-ol·hydroperchlorates and derivatives: single-crystal x-ray diffraction analysis and evidence for chair-chair and chair-boat conformers in the solid state. J. Med. Chem. 27: 758-767, 1984.

Chen, C. L., Sangiah, S., Patterson, E., Berlin, K. D., Garrison, G. L., Dunn, W., Nan, Y., Scherlag, B. J., and Lazzara, R. Effects of BRB-I-28, a novel antiarrhythmic agent, and its derivatives on cardiac Na^+,K^+-ATPase, Mg^{2+}-ATPase activities and contractile force. Res. Commun. Chem. Pathol. Pharmcol. 78: 3-16, 1992.

Chen, C. L., Sangiah, S., Roder, J. D., Chen, H., Berlin, K. D., Garrison, G. L., Scherlag, B. J., lazzara, R., and Patterson, E. Pharmacokinetics and plasma protein binding of BRB-I-28, a novel antiarrhythmic agent, in dog. Drug Invest. 6: 237-243, 1993.

Chen, C. L., Sangiah, S., Yu, C-A., Chen, H., Roder, J. D., Berlin, K. D., Garrison, G. L., Scherlag, B. J. and Lazzara, R. Effects of novel antiarrhythmic agents, GLG-V-78 and its derivatives, on mitochondrial respiration. Abstract. XII International Congress of Pharmacology. Montreal, Canada, 1994a.

Chen, C. L., Sangiah, S., Bourne, D. W. A., Roder, J. D., Chen, H., Alavi, F. K., Clarke, C. R., Garrison, G. L., Berlin, K. D., Couch, K. M., Zisman, S. A., Scherlag, B. J., Lazzara, R., Patterson, E., Hossain, M. B., and Helm, D. V. D. *In vivo* and *in vitro* metabolism studies on 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane, an antiarrhythmic agent. Drug Metab. Dispos. submitted, 1994b.

Chen, C. L., Sangiah, S., Chandra, A. M. S., Qualls, C. W., Chen, H., Roder, J. D., Garrison, G. L., Berlin, K. D., Scherlag, B. J., and Lazzara, R. The acute and subchronic toxicity of GLG-V-13, a combined class Ib and class III antiarrhythmic agent, in CD-1 mice. In preparation, 1994c.

Cohn, C., and Kaplan, A. Blood chemistry. In "Textbook of Clinical Pathology", (Miller, S. E., and Weller, J. W., ed), pp 235-278, The Williams & Wilkins Company, Baltimore, 1971.

Fazekas, T., Mabo, P., Berlin, K. D., Scherlag, B. J., and Lazzara, R. Comparative

electrophysiological effects of a new chemical class of antiarrhythmic agents, 3,7-diheterabicyclononanes, in the 1-4-day-old infarcted dog heart. The Physiologists. 35: 221, 1992.

Fazekas, T., Scherlag, B. J., Mabo, P., Berlin, K. D., Garrison, G. L., Chen, C. L., Sangiah, S., Patterson, E., and Lazzara, R. The comparative antiarrhythmic and proarrhythmic activity of a 3,7-diheterabicyclo[3.3.1]nonane, BRB-I-28, and lidocaine in the 1-4-day-old infarcted dog heart. Acta Physiologica Hungary. 81: 297-307, 1993a.

Fazekas, T., Mabo, P., Berlin, K. D., Garrison, G. L., Patterson, E., Scherlag, B. J., and Lazzara, R. A lidokain es a BRB-I-28 osszehasonlito szivelektrofiziologiai vizsgalata experimentalis szivizom infarktusban. Cardiologia Hungarica. 22: 17-21, 1993b.

Patterson, E., Scherlag, B. J., Berlin, K.D. and Lazzara, R. Electrophysiological action of BRB-I-28 in canine myocardial tissues. J. Pharmacol. Exp. Ther. 259: 558-565, 1991.

Patterson, E., Scherlag, B. J., Berlin, K. D., and Lazzara, R. Electrophysiological actions of BRB-I-28 in ischemically-injured canine myocardium. J. Cardiovas. Pharmacol. 21: 637-646, 1993.

Plaa, G. L. Toxic responses of the liver. In "Toxicology", (Klaassen, C. D., et al., ed), 3rd edition, pp 286-309, Macmillan Publishing Company, New York, 1986.

Popp, J. A., and Cattley, R. C. Hepatobiliary system. In "Handbook of Toxicologic Pathology" (Haschek, W. M., and Rousseaux, C. G., eds), pp. 279-314, Academic Press, New York, 1991.

Pugsley, M. K., Walker, M. J. A., Garrison, G. L., Howard, P. G., Lazzara, R., Patterson, E., Scherlag, B. J., Berlin, K. D., The cardiovascular and antiarrhythmic properties of a serious of novel sparteine analogs. Proc. West. Pharmacol. Soc. 35: 87-91, 1992.

Scherlag, B.J., Patterson E. , Lazzara R., Bailey B.R., Thompson M.D. and Berlin K.D. (1988). Comparative electrophysiological and hemodynamic actions of BRB-I-28 and lidocaine in the normal and infarcted dog heart. J. Electrophysiol. 2, 461-477.

Smith, E. R., and Duce, B. R. The acute antiarrhythmic and toxic effects in mice and dogs of 2-ethylamino-2′,6′-acetoxylidine (L-86), a metabolite of lidocaine. J. Pharm. Exp. Ther. 179: 580-585, 1971.

Tallarida, R.J. and Murray, R.B. (1986). Manual of pharmacologic calculations with computer programs. Springer-Verlag, New York.

Woodliff, H. J., and Herrmann, R. P. (ed.). Concise Hematology. The Williams & Wilkins, Baltimore, 1973.

TABLE 1

ORAL LETHAL DOSES OF BRB-I-28 IN CD-1 MICE

Dose (mg/kg)	Male (Died/Used)	Female (Died/Used)
76.8	1/10	----
96	3/10	3/10
120	4/10	3/10
150	5/10	7/10
187	9/10	7/10
233.8	----	10/10
LD_{16} (mg/kg)	86 (83-90)	87 (84-91)
LD_{50} (mg/kg)	128 (107-151)	131 (109-156)
LD_{84} (mg/kg)	189 (181-196)	196 (188-205)

The data are presented as acute lethal dose (95% confidence limit).

TABLE 2

BODY WEIGHT, BRB-I-28, FOOD AND WATER CONSUMPTION OF CD-1 MICE FED FOR 45 DAYS (MEAN±SD, N = 5)

	Control group	Treatment groups		
		Low Dose	Middle dose	High dose
Male				
Approximate BRB-I-28 consumption (mg/kg/day)	0	11.7±1.6	23.3±3.4	68.4±9.3
Initial body wt (g)	28.1±1.4	29.6±2.4	27.6±1.5	27.0±1.1
B.wt at 45 days (g)	34.4±1.4	37.4±2.9	36.2±2.8	34.9±1.9
B.wt gain (g)	6.3±1.2	7.8±0.8*	8.6±2.1	7.9±1.9
Food Consumption (g/day/mouse)	5.97±0.50	5.13±0.48**	5.30±0.47	4.97±0.50**
Water Consumption (g/day/mouse)	6.93±0.59	7.43±0.90	7.81±0.74	7.43±0.70
Female				
Approximate BRB-I-28 consumption (mg/kg/day)	0	14.0±2.0	28.2±3.7	79.8±9.7
Initial body wt (g)	26.0±1.1	24.5±1.3	24.1±1.7	25.4±1.2
B.wt at 45 days (g)	32.2±1.1	32.1±1.6	31.7±4.9	31.7±2.4
B.wt gain (g)	6.2±1.0	7.6±0.6*	7.6±3.5	6.3±1.7
Feed Consumption (g/day/mouse)	6.39±0.38	5.59±0.55**	5.54±0.43**	5.36±0.34**
Water Consumption (g/day/mouse)	7.96±0.60	7.90±0.43	8.07±0.46	8.00±0.53

* $p<0.05$; ** $p<0.01$

TABLE 3

BODY WEIGHT, BRB-I-28, FOOD AND WATER CONSUMPTION OF CD-1 MICE FED FOR 90 DAYS (MEAN±SD, N = 5)

	Control group	Treatment groups		
		Low Dose	Middle dose	High dose
Male				
Approximate BRB-I-28 consumption (mg/kg/day)	0	16.1±5.1	30.4±8.8	77.6±14.2
Initial body wt (g)	26.9±1.3	27.3±1.8	27.6±1.5	27.4±2.1
B.wt at 90 days (g)	36.1±1.9	36.9±3.1	39.5±4.3**	36.8±3.9
B.wt gain (g)	9.2±2.1	9.6±3.0	11.9±4.4**	9.4±2.7
Food Consumption (g/day/mouse)	5.82±0.49	5.11±0.39**	5.15±0.48**	4.78±0.54**
Water Consumption (g/day/mouse)	6.99±0.51	7.72±0.78**	7.76±0.64**	7.44±0.58**
Female				
Approximate BRB-I-28 consumption (mg/kg/day)	0	17.5±4.8	36.8±10.6	90.1±15.4
Initial body wt (g)	25.7±1.4	24.8±1.3	24.8±1.4	24.8±1.4
B.wt at 90 days (g)	35.2±2.4	34.3±2.6	34.8±2.7	34.1±2.2
B.wt gain (g)	9.6±2.3	9.5±2.3	9.8±2.4	9.4±2.1
Feed Consumption (g/day/mouse)	6.25±0.60	5.22±0.77**	5.39±0.50**	5.11±0.50**
Water Consumption (g/day/mouse)	7.83±0.61	7.94±0.37	8.07±0.42	7.98±0.52

* $p<0.05$; **$p<0.01$

TABLE 4

HEMATOLOGICAL VALUES (MEAN±SD) IN MALE CD-1 MICE FED BRB-I-28 FOR 45 DAYS (N = 5)

Dose (mg/kg/day)	0	11.7	23.3	68.4
WBC (10^3/mm^3)	10.6±3.76	8.80±3.66	8.74±3.45	11.2±3.51
RBC (10^6/mm^3)	8.70±0.47	9.45±1.55	9.69±0.98	8.31±1.66
HGB (g/100ml)	14.5±0.50	15.6±2.08	15.6±1.83	14.5±2.47
HCT (%)	42.5±2.30	46.5±6.85	47.0±5.58	41.5±9.48
MCV (fl)	48.9±1.76	49.3±1.19	48.4±1.17	48.7±2.07
MCH (pg)	16.6±0.79	16.6±0.62	16.0±0.37	17.2±1.34
MCHC (g/100 ml)	34.0±1.34	33.7±0.55	33.1±0.56	35.4±3.31
PLT (10^5/mm^3)	11.3±1.80	12.7±2.28	12.3±1.63	10.4±1.35
TP (g/100 ml)	6.12±0.36	6.24±0.67	6.40±0.45	5.88±0.30
Differential				
SEG (%)	22.8±20.6	25.2±12.1	26.8±13.9	35.0±10.5
(Absolute #)	2433±2238	2268±1678	2570±1738	4018±1818
Lymph (%)	73.6±18.3	70.4±12.3	70.4±14.4	63.0±10.0
(Absolute #)	7753±3521	6064±2574	5909±2203	7033±2629
Mono (%)	2.2±3.4	3.4±2.7	2.4±2.1	1.0±1.4
(Absolute #)	221±279	242±200	239±223	80±124
Eosin (%)	1.4±1.7	0.8±0.8	0.4±0.5	0.6±0.5
(Absolute #)	153±235	86±87	22±33	57±57
Band (%)	0	0.2±0.4	0	0.2±0.4
(Absolute)	0	20±44	0	12±27
Baso (%)	0	0	0	0.2±0.4
(Absolute #)	0	0	0	19±42

Abbreviation: WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean cell hemoglobin; MCHC, mean cell corpuscular hemoglobin concentration; PLT, platelet; TP, total protein; SEG, Segmented neutrophils; Lymph, lymphocytes; Mono, monocytes; Eosin, eosinophils; Band, neutrophilic band; Baso, basophils.

TABLE 5

HEMATOLOGICAL VALUES (MEAN±SD) IN FEMALE CD-1 MICE FED BRB-I-28 FOR 45 DAYS (N = 5)

Dose (mg/kg/day)	0	14.0	28.2	79.8
WBC (10^3/mm^3)	9.64±1.38	9.84±4.56	7.16±2.22	7.50±3.07
RBC (10^6/mm^3)	8.53±0.66	8.64±0.53	8.73±0.89	8.17±0.91
HGB (g/100ml)	14.7±0.96	15.4±0.41	14.2±3.31	14.8±0.91
HCT (%)	41.8±3.47	42.7±1.90	42.1±5.01	39.9±4.76
MCV (fl)	49.0±1.51	49.5±2.01	48.1±1.21	48.9±3.05
MCH (pg)	17.3±1.07	17.9±0.98	15.7±2.87	18.2±1.23
MCHC (g/100 ml)	35.2±1.51	36.0±1.72	32.8±5.51	37.4±2.37
PLT (10^5/mm^3)	11.6±1.36	12.1±0.68	11.8±0.87	12.5±1.12
TP (g/100 ml)	6.26±0.27	6.28±0.16	6.30±0.43	6.08±0.47
Differential				
SEG (%)	13.4±5.5	12.8±5.45	13.8±4.97	14.6±5.32
(Absolute #)	1281±600	1229±824	916±361	1152±576
Lymph (%)	82.8±8.1	78.6±7.09	79.4±8.38	77.8±6.34
(Absolute #)	7989±1470	7600±3059	5445±1836*	5790±2397
Mono (%)	2.6±2.7	3.8±4.1	3.6±3.9	2.8±1.8
(Absolute #)	260±303	342±383	227±199	208±173
Eosin (%)	1.0±0.7	4.8±5.9	3.0±1.6*	4.6±3.1
(Absolute #)	91±60	670±1048	217±176	326±220
Band (%)	0.2±0.4	0	0	0.2±0.4
(Absolute)	19±43	0	0	23±51
Baso (%)	0	0	0.2±0.4	0
(Absolute #)	0	0	15.4±34.4	0

*$p<0.05$

Abbreviation: WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean cell hemoglobin; MCHC, mean cell corpuscular hemoglobin concentration; PLT, platelet; TP, total protein; SEG, segmented neutrophils; Lymph, lymphocytes; Mono, monocytes; Eosin, eosinophils; Band, neutrophilic band; Baso, basophils.

TABLE 6

HEMATOLOGICAL VALUES (MEAN±SD) IN MALE CD-1 MICE FED BRB-I-28 FOR 90 DAYS (N = 15)

Dose (mg/kg/day)	0	16.1	30.4	77.6
WBC (10^3/mm^3)	8.15±1.92	8.55±3.42	10.3±3.10*	10.0±3.68
RBC (10^6/mm^3)	9.66±0.84	9.12±2.70	9.44±1.31	9.92±0.85
HGB (g/100ml)	15.4±1.22	16.2±1.64	15.2±1.91	16.4±1.40
HCT (%)	46.7±3.56	48.5±4.47	44.4±5.64	47.9±3.77
MCV (fl)	48.4±1.58	48.3±1.86	47.1±1.85	48.3±1.42
MCH (pg)	16.0±0.70	16.1±0.98	16.2±0.64	16.5±0.57
MCHC (g/100 ml)	33.1±0.67	33.4±1.07	34.3±0.95**	34.1±0.61**
PLT (10^5/mm^3)	13.5±2.04	13.2±2.04	11.4±2.84*	10.9±2.04**
TP (g/100 ml)	6.61±0.36	6.57±0.45	6.43±0.31	6.61±0.35
Differential				
SEG (%)	21.1±6.67	21.0±7.46	19.7±11.9	16.9±4.00*
(Absolute #)	1729±778	1685±1647	2100±1665	1704±871
Lymph (%)	75.3±6.93	75.3±6.80	74.3±11.7	78.5±4.90
(Absolute #)	6123±1528	6539±2999	7599±2593	7825±2738*
Mono (%)	2.8±2.0	2.7±1.5	4.3±2.0*	3.6±2.4
(Absolute #)	231±187	249±182	445±230**	364±302
Eosin (%)	0.8±0.92	1.0±1.5	1.7±1.6	0.9±1.2
(Absolute #)	71±77	72±81	156±152	91±128
Band (%)	0	0	0	0.1±0.4
(Absolute)	0	0	0	16±40
Baso (%)	0	0	0	0
(Absolute #)	0	0	0	0

*$p<0.05$; **$p<0.01$

Abbreviation: WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean cell hemoglobin; MCHC, mean cell corpuscular hemoglobin concentration; PLT, platelet; TP, total protein; SEG, segmented neutrophils; Lymph, lymphocytes; Mono, monocytes; Eosin, eosinophils; Band, neutrophilic band; Baso, basophils.

TABLE 7

HEMATOLOGICAL VALUES (MEAN±SD) IN FEMALE CD-1 MICE FED BRB-I-28 FOR 90 DAYS (N = 15)

Dose (mg/kg/day)	0	17.5	36.8	90.1
WBC (10^3/mm^3)	6.83±2.20	8.14±4.25	6.60±2.48	7.93±3.02
RBC (10^6/mm^3)	8.80±0.85	9.07±0.85	8.52±0.78	8.49±0.94
HGB (g/100ml)	15.5±1.46	16.0±0.95	15.4±1.18	15.7±1.29
HCT (%)	44.3±4.00	44.4±3.29	41.7±3.92	41.8±5.27
MCV (fl)	50.3±1.70	49.1±2.18	48.9±0.92*	48.8±1.63*
MCH (pg)	17.7±1.19	17.7±0.99	18.1±0.98	18.6±1.02*
MCHC (g/100 ml)	35.1±1.53	36.1±1.22	37.0±1.99**	37.8±2.17**
PLT (10^5/mm^3)	13.1±2.05	11.5±2.17*	11.3±1.61*	11.1±1.34**
TP (g/100 ml)	6.47±0.28	6.20±0.36*	6.43±0.31	6.40±0.29
Differential				
SEG (%)	19.9±11.3	15.5±8.46	13.7±5.29	19.2±10.4
(Absolute #)	1371±1031	1140±756	931±385	1526±1098
Lymph (%)	77.5±10.8	82.0±9.48	81.5±6.51	77.8±10.0
(Absolute #)	5273±1763	6795±3892	5798±1703	6185±2653
Mono (%)	1.2±1.4	1.2±1.7	3.7±3.2*	1.8±1.9
(Absolute #)	84±104	94±128	255±222*	133±146
Eosin (%)	1.4±1.4	1.2±1.3	1.1±1.1	1.1±1.4
(Absolute #)	105±148	102±110	82±89	68±92
Band (%)	0	0.1±0.3	0	0.1±0.3
(Absolute)	0	3.0±13	0	9.0±27
Baso (%)	0	0	0	0
(Absolute #)	0	0	0	0

*$p<0.05$; **$p<0.01$

Abbreviation: WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean cell hemoglobin; MCHC, mean cell corpuscular hemoglobin concentration; PLT, platelet; TP, total protein; SEG, segmented neutrophils; Lymph, lymphocytes; Mono, monocytes; Eosin, eosinophils; Band, neutrophilic band; Baso, basophils.

TABLE 8

BIOCHEMICAL VALUES (MEAN±SD, N = 5) FOR CD-1 MICE FED BRB-I-28 FOR 45 DAYS

Dose (mg/kg/d)	BUN (mg/dl)	ALP (IU/L)	LDH (IU/L)	GOT/AST (IU/L)	PT/ALT (IU/L)	Na (meq/L)	K (meq/L)
Male							
0	28.2±5.8	48.6±15.5	1013.8±581.2	231.6±51.3	81.4±53.1	152.2±1.3	8.0±0.5
11.7	29.0±0.4	43.8±19.3	1348.0±283.6	234.4±76.7	96.0±47.2	152.6±0.9	8.0±1.6
23.3	29.6±5.8	42.4±17.5	1217.8±335.8	228.6±120.7	81.6±39.0	152.8±2.8	8.5±2.9
68.4	23.2±3.4	52.0±44.6	1290.2±265.9	310.6±123.4	102.2±45.8	153.2±3.4	8.1±2.1
Female							
0	28.4±6.1	51.8±12.2	1489.2±354.5	222.4±71.1	42.2±10.1	153.0±2.7	7.8±1.1
14.0	25.8±5.5	38.8±18.5	1120.0±352.0	218.8±65.0	44.8±18.9	151.2±2.2	6.9±1.0
28.2	35.0±4.6	63.6±8.47	1257.2±241.3	190.0±123.8	52.8±31.3	149.6±2.3	7.5±0.7
79.8	27.8±1.9	55.0±12.9	1090.0±212.9	216.8±66.5	42.0±7.40	152.6±1.52	7.3±1.0

Abbreviation: BUN, blood urea nitrogen; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; AST, asparate aminotransferase; ALT, alanine aminotransferase.

TABLE 9

BIOCHEMICAL VALUES (MEAN±SD, N = 15) FOR CD-1 MICE FED BRB-I-28 FOR 90 DAYS

Dose (mg/kg/d)	BUN (mg/dl)	ALP (IU/L)	LDH (IU/L)	GOT/AST (IU/L)	GPT/ALT IU/L)	Na (meq/L)	K (meq/L)	Alb (g/dl)	Chole (mg/dl)
Male									
0	28.0±3.6	37.2±8.43	1400.1±434.4	237.1±94.3	88.8±101.8	155.9±3.51	7.5±0.7	3.4±0.2	140±17
16.1	27.5±4.5	38.7±6.60	1303.4±396.0	210.0±68.1	83.1±100.6	153.8±1.94	7.3±0.6	3.2±0.2	131±26
30.4	27.9±3.0	45.9±31.8	1466.6±392.9	236.3±170.4	79.2±44.6	153.9±2.81	7.5±0.8	3.3±0.2	136±25
77.6	27.7±4.3	41.7±24.6	1572.0±254.8	230.5±45.8	128.9±115	152.7±2.8**	7.5±0.8	3.3±0.2	144±33
Female									
0	25.3±3.6	54.2±17.6	1398.4±207.2	216.5±69.0	44.5±9.90	153.3±3.6	6.8±0.6	3.4±0.2	96±17
17.5	25.9±3.3	42.5±10.3*	1485.8±380.9	218.4±54.7	58.6±32.5	152.1±4.2	6.7±0.7	3.3±0.3	89±18
36.8	26.7±3.4	40.3±13.3*	1287.5±326.2	226.6±77.8	55.9±30.7	151.8±5.5	6.4±0.7	3.4±0.2	97±21
90.1	26.2±4.0	40.6±14.2*	1291.0±297.0	222.3±90.9	52.1±20.2	152.7±3.2	6.3±0.6*	3.3±0.2	98±28

*$p<0.05$; **$p<0.01$

Abbreviation: BUN, blood urea nitrogen; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; AST, asparate aminotransferase; ALT, alanine aminotransferase; Alb, albumin; Chole, cholesterol.

TABLE 10

ABSOLUTE AND RELATIVE ORGAN WEIGHTS (MEAN±SD) OF MALE CD-1 MICE FED BRB-I-28 FOR 45 DAYS (N = 5)

Dose (mg/kg/day)	0	11.7	23.3	68.4
Absolute organ weight (mg)				
Brain	463.4±38.2	492.1±17.8	464.1±29.2	499.7±18.5
Heart	179.6±22.9	214.3±41.3	193.9±23.6	195.3±45.7
Liver	2032.4±136.0	2042.3±231.6	2159.7±226.8	1925.1±328.8
Kidney	523.1±11.4	637.0±103.4	571.1±58.6	677.9±227.1
Spleen	93.5±12.2	108.6±18.8	114.4±29.2	90.8±20.3
Testis	229.7±30.0	263.6±85.6	254.7±11.9	240.3±25.0
Epididymis	114.1±35.8	106.9±21.1	116.0±11.7	111.7±5.59
Adrenal	9.0±1.6	10.5±1.90	16.1±9.73	8.67±2.81
Lung	230.6±49.4	302.9±88.1	320.0±126.9	256.5±72.2
Relative organ weight (mg/g brain)				
Heart	390.9±68.7	494.9±76.9	418.3±48.7	392.0±95.4
Liver	4289.8±332.1	4157.6±527.0	4669.6±347.1	3849.7±613.7
kidney	1135.5±106.6	1294.6±213.9	1230.0±93.5	1353.2±436.1
Spleen	201.7±21.3	221.5±42.7	245.8±60.6	182.0±41.4
Testis	499.5±85.5	582.4±84.5	549.8±29.5	480.7±42.1
Epididymis	244.2±73.8	218.4±49.2	249.7±16.0	223.9±15.8
Adrenal	19.5±3.1	21.2±3.69	34.8±20.6	17.4±5.80
Lung	502.8±133.1	615.1±175.6	688.2±265.0	516.3±156.3
Relative organ weight (mg/g body weight)				
Brain	13.5±1.5	13.2±1.0	12.8±0.4	14.3±0.9
Heart	5.2±0.6	5.7±0.9	5.4±0.6	5.6±1.1
Liver	59.3±5.8	54.8±5.4	60.0±5.1	54.9±6.3
Kidney	15.2±0.6	17.0±1.7	15.8±1.3	19.2±5.4
Spleen	2.7±0.4	2.9±0.4	3.2±0.8	2.6±0.5
Testis	6.7±0.9	7.7±1.1	7.1±0.5	6.9±1.0
Epididymis	3.3±1.2	2.9±0.6	3.2±0.2	3.2±0.2
Adrenal	0.26±0.05	0.28±0.06	0.44±0.27	0.25±0.08
Lung	6.7±1.4	8.0±1.8	8.8±3.2	7.3±1.9

TABLE 11

ABSOLUTE AND RELATIVE ORGAN WEIGHTS (MEAN±SD) OF FEMALE CD-1 MICE FED BRB-I-28 FOR 45 DAYS (N = 5)

Dose (mg/kg/day)	0	14.0	28.2	79.8
Absolute organ weight (mg)				
Brain	514.7±11.3	493.4±26.9	481.7±23.4*	526.4±26.8
Heart	167.8±14.7	170.9±41.9	165.0±27.4	177.5±44.4
Liver	1901.3±179.6	1709.1±173.7	1631.5±248.4	1715.8±217.5
Kidney	405.5±19.7	400.0±49.8	390.7±27.9	419.7±28.4
Spleen	123.1±2.3	123.5±33.3	127.3±23.6	108.8±12.2
Ovary + Uterus	246.4±58.3	279.2±51.0	247.2±103.7	211.7±16.7
Adrenal	12.7±3.63	17.0±2.37	11.6±3.75	13.8±4.11
Lung	234.9±40.0	212.9±55.0	278.2±77.6	216.1±52.7
Relative organ weight (mg/g brain)				
Heart	326.3±32.6	344.9±70.8	345.3±65.1	336.1±72.9
Liver	3697.3±375.7	3474.4±422.4	3408.5±663.0	3278.1±552.2
kidney	788.4±47.3	811.3±96.3	813.60±83.4	799.2±71.0
Spleen	239.3±8.4	250.8±66.8	266.4±62.2	207.4±28.6
Ovary + Uterus	478.7±110.8	563.9±86.2	521.1±240.4	401.9±14.6
Adrenal	24.5±6.80	34.5±5.75*	24.3±8.40	26.4±8.66
Lung	456.8±80.8	436.4±137.1	581.5±181.4	415.4±126.9
Relative organ weight (mg/g body weight)				
Brain	16.0±0.5	15.4±1.3	15.5±2.7	16.7±1.8
Heart	5.2±0.5	5.3±1.3	5.3±0.9	5.6±1.2
Liver	59.1±5.1	53.2±4.7	51.5±1.7**	54.0±3.9
Kidney	12.6±0.8	12.5±1.5	12.5±1.6	13.3±0.7
Spleen	3.8±0.2	3.8±1.0	4.0±0.7	3.5±0.5
Ovary + Uterus	7.7±1.9	8.8±1.9	7.6±2.3	6.7±0.9
Adrenal	0.39±0.10	0.53±0.05*	0.36±0.10	0.43±0.10
Lung	7.3±1.4	6.6±1.4	8.7±1.1	6.8±1.3

* $p<0.05$; ** $p<0.01$

TABLE 12

ABSOLUTE AND RELATIVE ORGAN WEIGHTS (MEAN±SD) OF MALE CD-1 MICE FED BRB-I-28 FOR 90 DAYS (N = 15)

Dose (mg/kg/day)	0	16.1	30.4	77.6
Absolute organ weight (mg)				
Brain	481.8±45.3	487.9±71.6	531.1±33.1**	480.7±50.3
Heart	206.3±33.8	256.1±67.4*	227.1±34.0	212.0±39.5
Liver	2027.2±229.1	2168.9±273.0	2072.9±225.3	2273.3±333.8*
Kidney	590.9±77.2	659.7±148.7	672.5±95.0*	609.1±102.4
Spleen	101.8±18.6	114.8±16.6	130.5±32.6**	114.2±27.2
Testis	251.8±49.8	282.5±28.3*	291.8±55.4*	268.8±41.0
Epididymis	136.8±35.5	142.8±37.3	154.8±31.8	149.6±42.2
Adrenal	12.7±5.3	15.7±12.5	16.5±9.9	15.3±8.6
Lung	292.8±68.7	340.5±66.1	337.7±70.4	371.8±86**
Relative organ weight (mg/g brain)				
Heart	430.3±67.7	533.2±143.2*	430.1±77.5	446.8±96.1
Liver	4148.1±755.3	4521.6±758.3	3912.2±510.2	4754.2±716.8*
kidney	1230.4±166.7	1368.9±296.9	1273.5±216.1	1246.1±185.9
Spleen	212.0±33.6	240.8±51.7	247.4±66.2	238.0±48.5
Testis	511.9±113.0	597.2±154.6	550.1±99.7	553.8±73.3
Epididymis	299.2±89.2	297.1±80.6	292.6±62.8	308.5±78.5
Adrenal	27.7±9.9	31.7±22.6	31.6±20.1	31.5±17.7
Lung	606.8±122.5	726.6±258.2	632.6±104.4	760.3±171**
Relative organ weight (mg/g body weight)				
Brain	13.4±1.3	13.2±1.7	13.6±1.8	13.2±1.6
Heart	5.7±0.8	6.9±1.8*	5.8±0.9	5.8±0.9
Liver	56.2±5.5	58.8±3.7	52.6±3.5*	61.7±5.1**
Kidney	16.3±1.5	17.8±2.7	17.0±1.6	16.5±2.0
Spleen	2.8±0.5	3.1±0.4	3.3±0.6*	3.1±0.5
Testis	7.0±1.3	7.7±0.7	7.4±1.5	7.3±1.0
Epididymis	3.8±0.9	3.9±0.9	3.9±0.8	4.1±1.1
Adrenal	0.35±0.14	0.43±0.33	0.41±0.23	0.42±0.26
Lung	8.1±1.8	9.3±1.8	8.7±2.2	10.1±1.85**

* $p<0.05$; ** $p<0.01$

TABLE 13

ABSOLUTE AND RELATIVE ORGAN WEIGHTS (MEAN±SD) OF FEMALE CD-1 MICE FED BRB-I-28 FOR 90 DAYS (N = 15)

Dose (mg/kg/day)	0	17.5	36.8	90.1
Absolute organ weight (mg)				
Brain	499.3±54.4	500.0±30.7	510.3±45.1	507.0±28.7
Heart	197.0±27.3	212.3±30.1	198.1±27.0	187.6±26.2
Liver	1929.1±230.1	1924.7±305.9	2004.8±218.9	1915.8±249.9
Kidney	486.1±46.1	462.9±39.8	472.7±93.4	460.7±27.8
Spleen	143.9±27.4	137.8±24.7	152.2±30.4	145.9±32.6
Ovary + Uterus	398.1±91.9	307.5±95.4*	330.6±71.9*	308.4±76.3**
Adrenal	19.1±3.8	18.9±6.9	20.5±5.1	17.8±4.3
Lung	322.9±79.3	317.3±55.3	340.3±89.0	319.0±79.5
Relative organ weight (mg/g brain)				
Heart	399.2±70.1	428.1±77.3	392.7±73.7	370.2±48.7
Liver	3925.4±769.0	3879.8±736.7	3952.4±533.9	3787.9±539.9
kidney	986.9±162.9	931.4±113.4	929.0±183.8	910.3±58.6
Spleen	290.0±55.5	276.6±50.9	300.4±67.8	287.4±58.0
Ovary + Uterus	805.4±198.4	614.2±184.6*	678.8±153.9	610±159**
Adrenal	38.5±8.83	38.1±14.7	40.1±8.77	35.5±9.13
Lung	642.4±136.3	639.5±130.0	670.0±183.0	632.8±167.1
Relative organ weight (mg/g body weight)				
Brain	14.2±1.6	14.6±1.5	14.7±1.7	15.0±1.3
Heart	5.6±0.7	6.2±1.1	5.7±0.7	5.5±0.8
Liver	55.0±6.3	56.1±8.5	57.6±4.5	56.2±5.7
Kidney	13.9±1.3	13.5±1.3	13.6±2.7	13.6±1.2
Spleen	4.1±0.7	4.0±0.7	4.4±0.7	4.3±0.9
Ovary + Uterus	11.4±2.8	9.0±2.7*	9.98±2.5	9.1±2.1**
Adrenal	0.54±0.13	0.55±0.19	0.59±0.13	0.52±0.13
Lung	9.2±2.2	9.3±1.7	9.7±2.4	9.4±2.2

* $p<0.05$; ** $p<0.01$

Figure Legends

Figure 1. Mean body weight change (a) and cumulative body weight gains (b) in male CD-1 mice fed BRB-I-28 at doses of 0, 11.7, 23.2, and 68.4 mg/kg/day for 45 days. Points are the mean for groups of 5 mice.

Figure 2. Mean body weight change (a) and cumulative body weight gains (b) in female CD-1 mice fed BRB-I-28 at doses of 0, 14.0, 28.2, and 79.8 mg/kg/day for 45 days. Points are the mean for groups of 5 mice.

Figure 3. Feed efficiency of male (a) and female (b) mice fed diets containing BRB-I-28 for 45 days. Points are the mean for groups of 5 mice.

Figure 4. Mean body weight change (a) and cumulative body weight gains (b) in male CD-1 mice fed BRB-I-28 at doses of 0, 16.1, 30.4, and 77.6 mg/kg/day for 90 days. Points are the mean for groups of 15 mice.

Figure 5. Mean body weight change (a) and cumulative body weight gains (b) in female CD-1 mice fed BRB-I-28 at doses of 0, 17.5, 36.8, and 90.1 mg/kg/day for 90 days. Points are the mean for groups of 15 mice.

Figure 6. Feed efficiency of male (a) and female (b) mice fed diets containing BRB-I-28 for 90 day. Points are the mean for groups of 15 mice.

Figure 7. Light micrograph of the liver of a male mouse given BRB-I-28 at the dose of 77.6 mg/kg/day for 90 days. Note multifocal, randomly scattered necrosis. Similar hepatocellular necrosis was observed in a female mouse given BRB-I-28 at the dose of 90.1 mg/kg/day for 90 days.

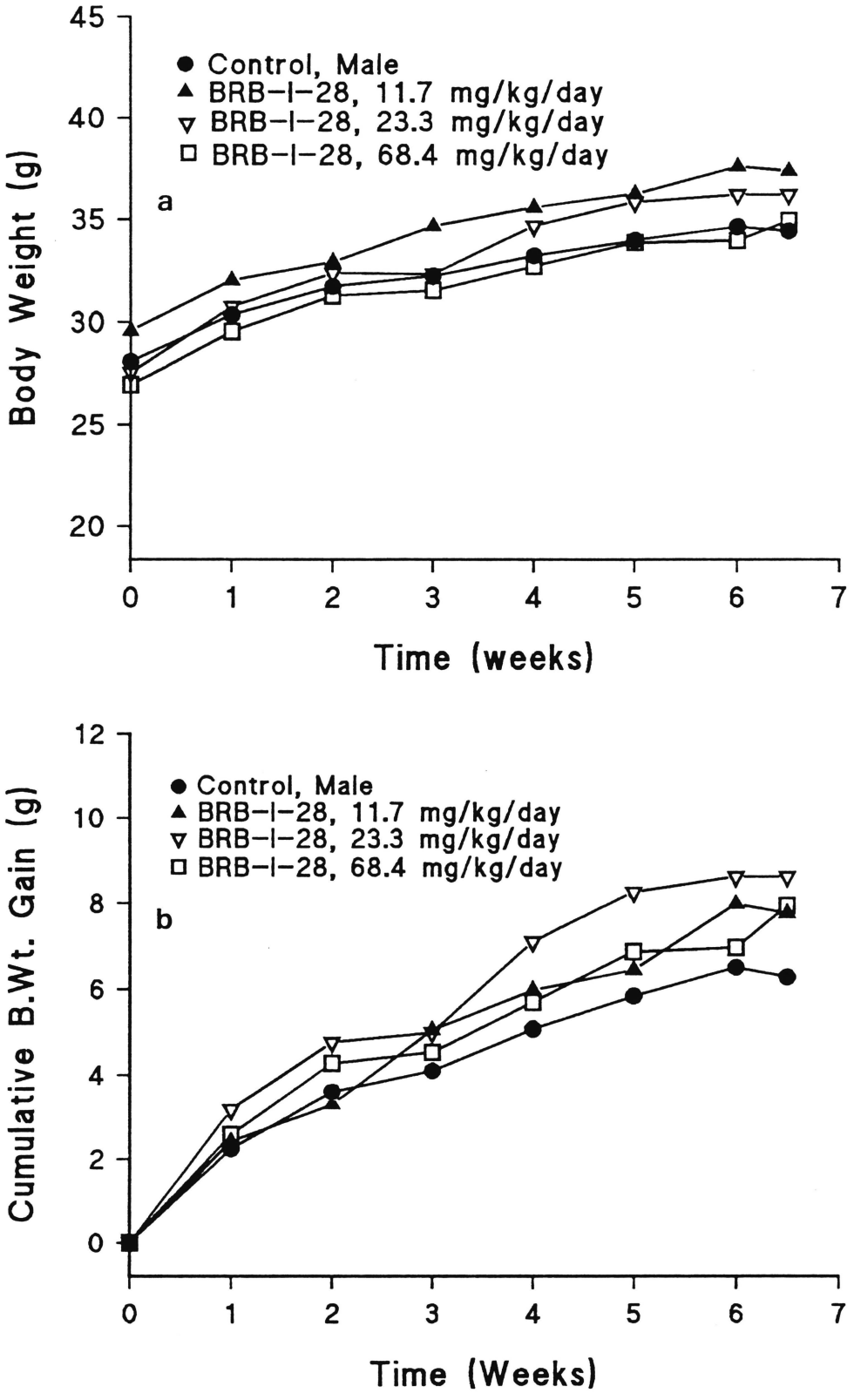
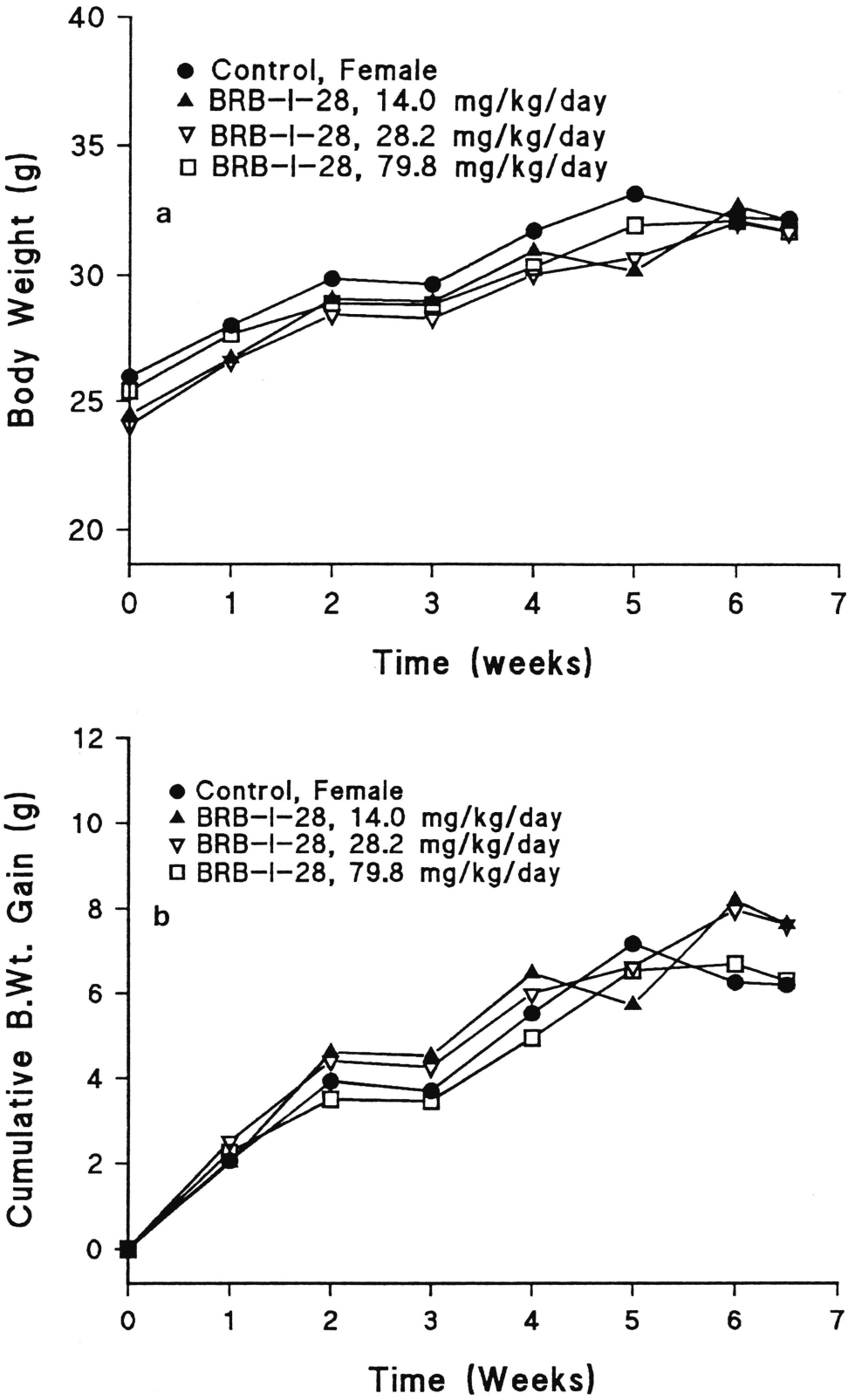

45
40
35
30
25
20
Body Weight (g)
● Control, Male
▲ BRB-I-28, 11.7 mg/kg/day
▽ BRB-I-28, 23.3 mg/kg/day
□ BRB-I-28, 68.4 mg/kg/day
a
0
1
2
3
4
5
6
7
Time (weeks)
12
10
8
6
4
2
0
Cumulative B.Wt. Gain (g)
● Control, Male
▲ BRB-I-28, 11.7 mg/kg/day
▽ BRB-I-28, 23.3 mg/kg/day
□ BRB-I-28, 68.4 mg/kg/day
b
0
1
2
3
4
5
6
7
Time (Weeks)

Figure 1


Body Weight (g)
40
35
30
25
20
● Control, Female
▲ BRB-I-28, 14.0 mg/kg/day
▽ BRB-I-28, 28.2 mg/kg/day
□ BRB-I-28, 79.8 mg/kg/day
a
0
1
2
3
4
5
6
7
Time (weeks)
Cumulative B.Wt. Gain (g)
12
10
8
6
4
2
0
● Control, Female
▲ BRB-I-28, 14.0 mg/kg/day
▽ BRB-I-28, 28.2 mg/kg/day
□ BRB-I-28, 79.8 mg/kg/day
b
0
1
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3
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6
7
Time (Weeks)

Figure 2

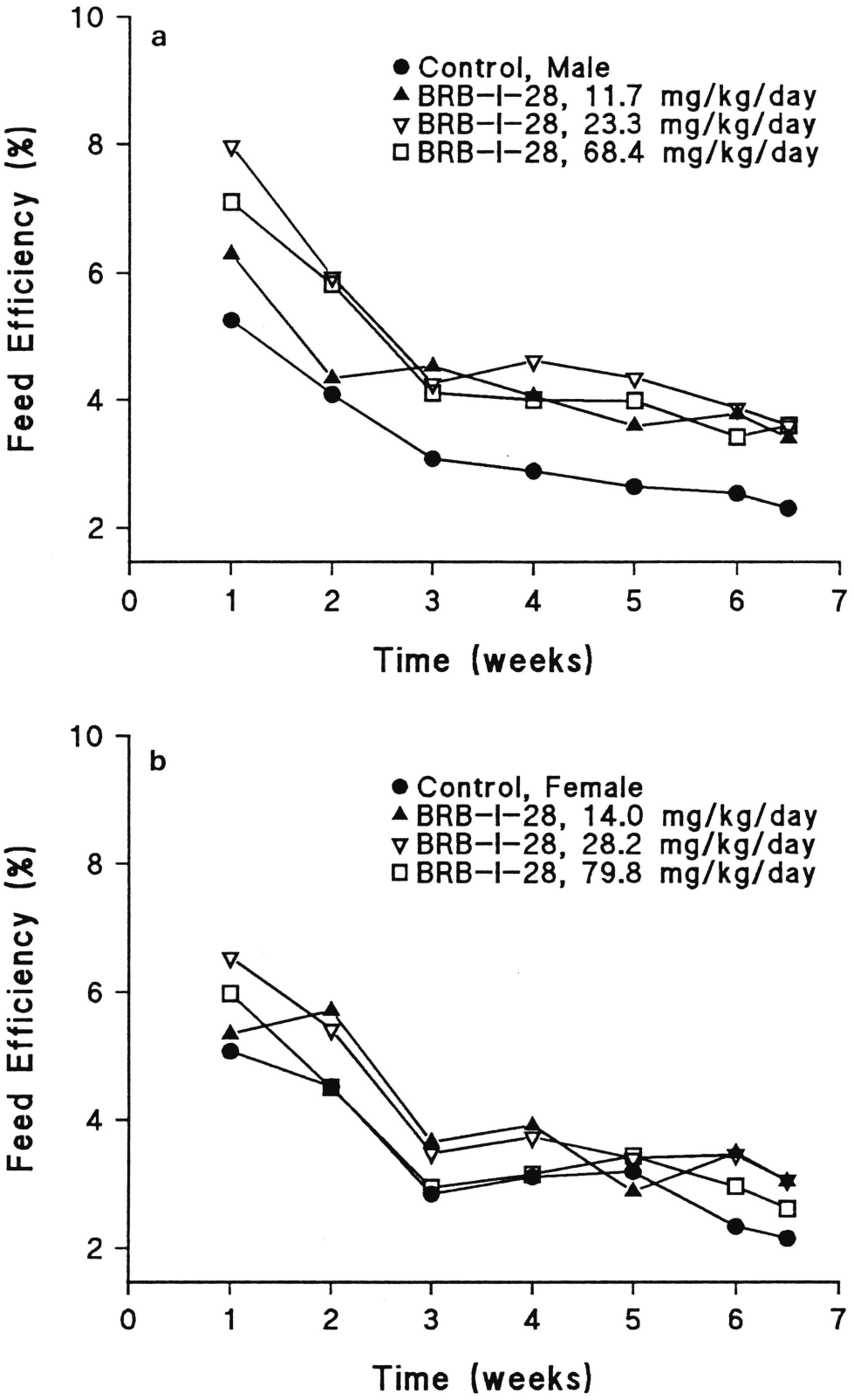

a
Control, Male
BRB-I-28, 11.7 mg/kg/day
BRB-I-28, 23.3 mg/kg/day
BRB-I-28, 68.4 mg/kg/day
Feed Efficiency (%)
Time (weeks)
b
Control, Female
BRB-I-28, 14.0 mg/kg/day
BRB-I-28, 28.2 mg/kg/day
BRB-I-28, 79.8 mg/kg/day
Feed Efficiency (%)
Time (weeks)

Figure 3

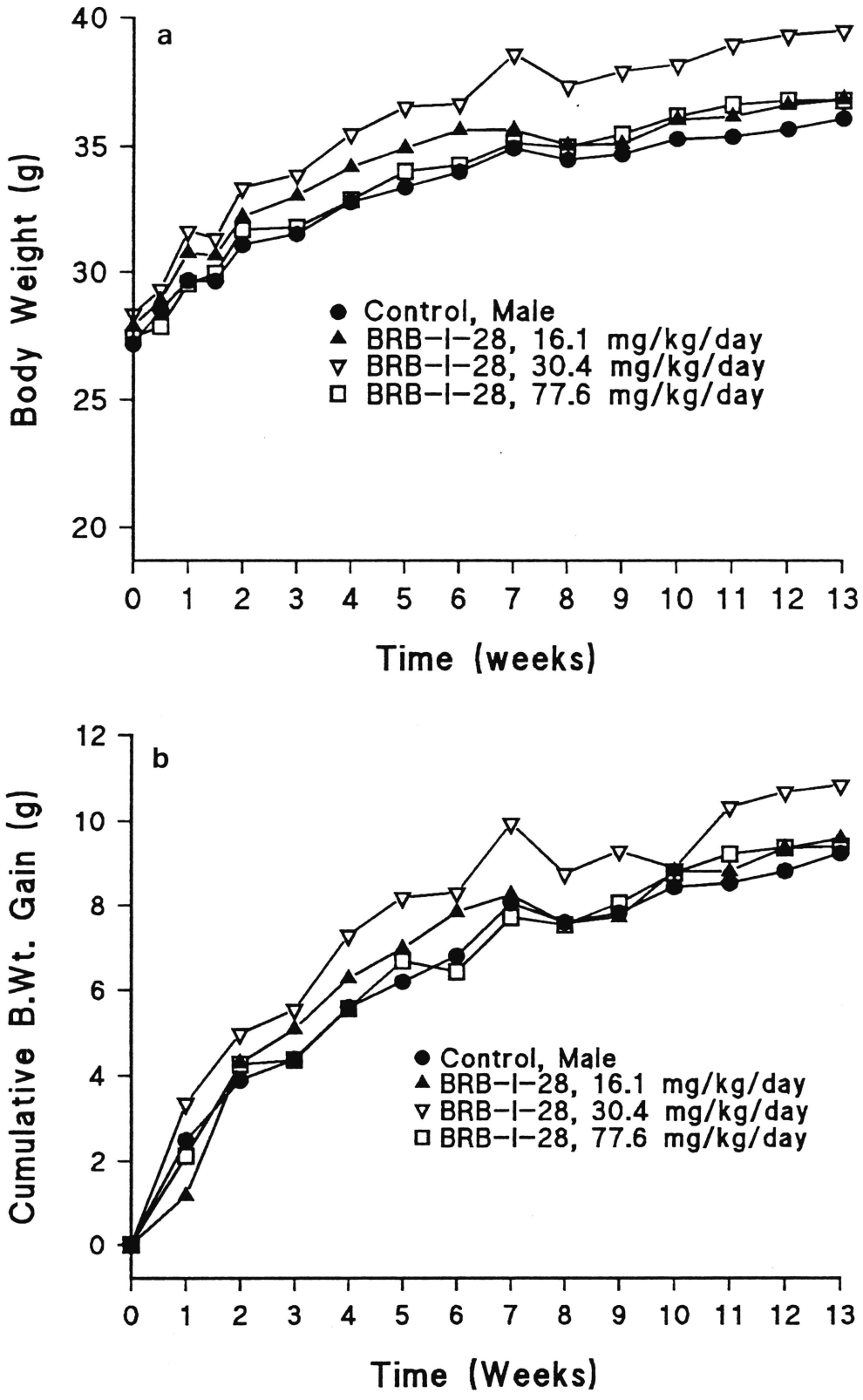

a
Body Weight (g)
40
35
30
25
20
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Time (weeks)
Control, Male
BRB-I-28, 16.1 mg/kg/day
BRB-I-28, 30.4 mg/kg/day
BRB-I-28, 77.6 mg/kg/day
b
Cumulative B.Wt. Gain (g)
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Time (Weeks)
Control, Male
BRB-I-28, 16.1 mg/kg/day
BRB-I-28, 30.4 mg/kg/day
BRB-I-28, 77.6 mg/kg/day

Figure 4

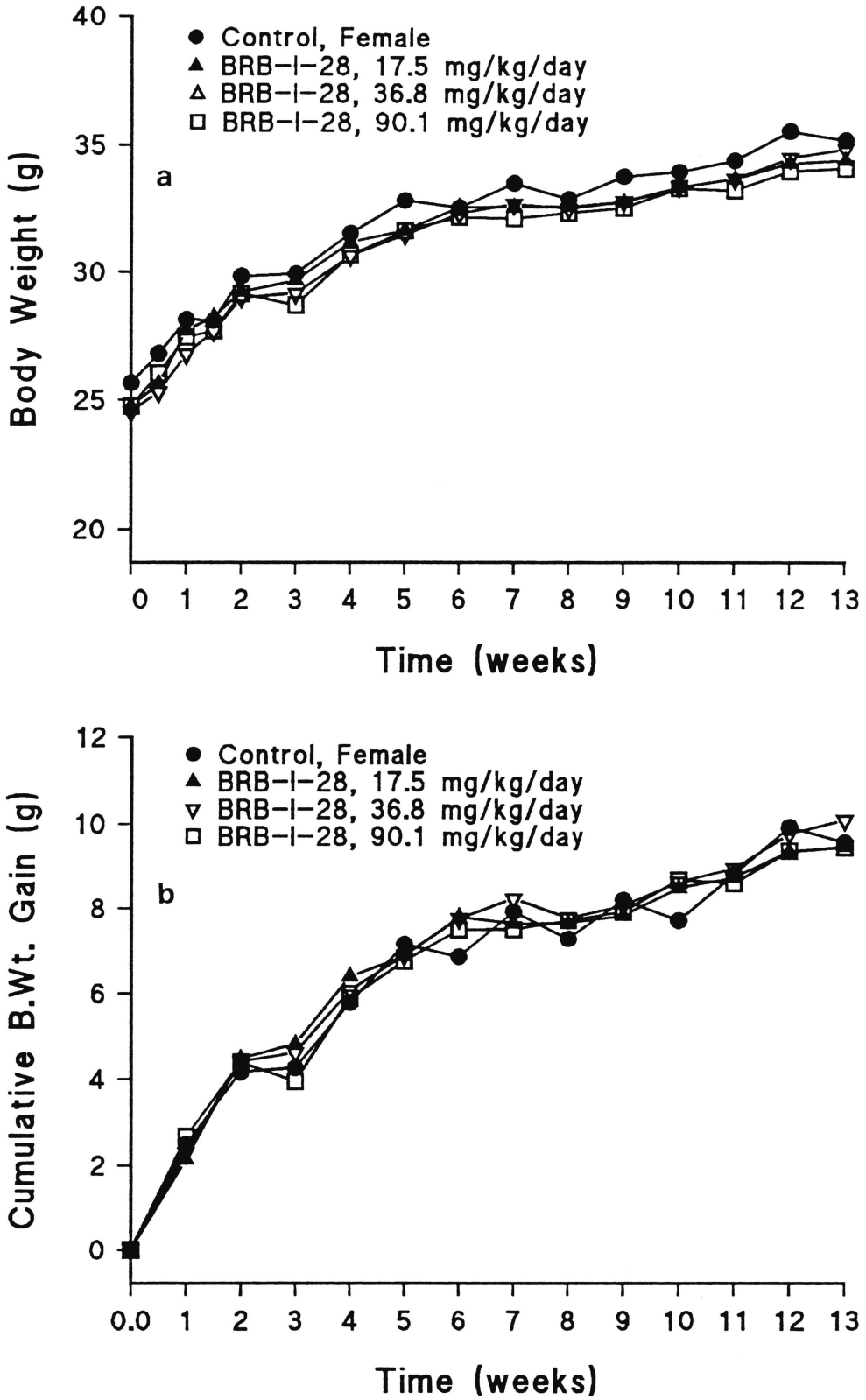

Control, Female
BRB-I-28, 17.5 mg/kg/day
BRB-I-28, 36.8 mg/kg/day
BRB-I-28, 90.1 mg/kg/day
a
Body Weight (g)
40
35
30
25
20
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Time (weeks)
Control, Female
BRB-I-28, 17.5 mg/kg/day
BRB-I-28, 36.8 mg/kg/day
BRB-I-28, 90.1 mg/kg/day
b
Cumulative B.Wt. Gain (g)
12
10
8
6
4
2
0
0.0 1 2 3 4 5 6 7 8 9 10 11 12 13
Time (weeks)

Figure 5

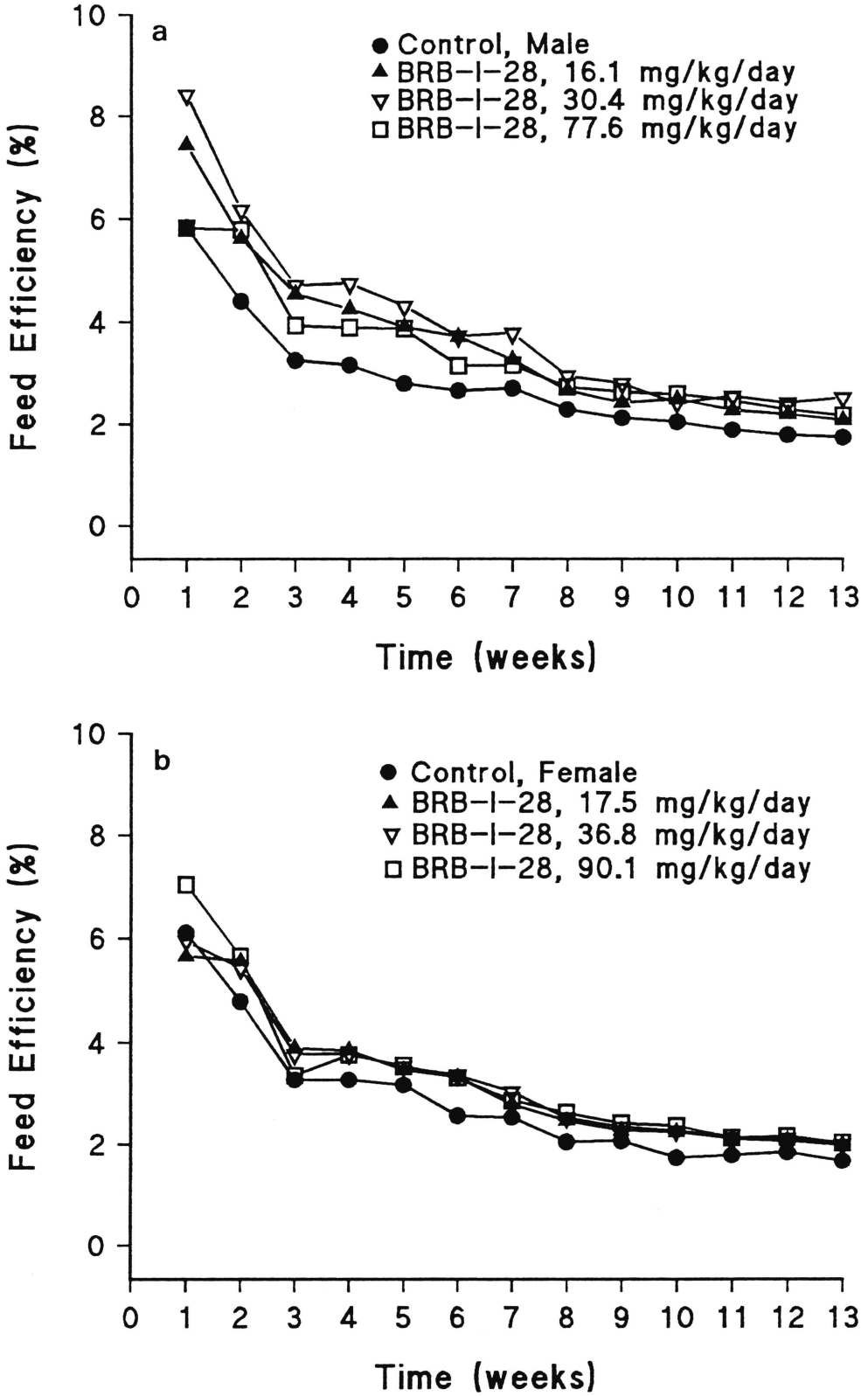

a
Control, Male
BRB-I-28, 16.1 mg/kg/day
BRB-I-28, 30.4 mg/kg/day
BRB-I-28, 77.6 mg/kg/day
Feed Efficiency (%)
Time (weeks)
b
Control, Female
BRB-I-28, 17.5 mg/kg/day
BRB-I-28, 36.8 mg/kg/day
BRB-I-28, 90.1 mg/kg/day
Feed Efficiency (%)
Time (weeks)

Figure 6

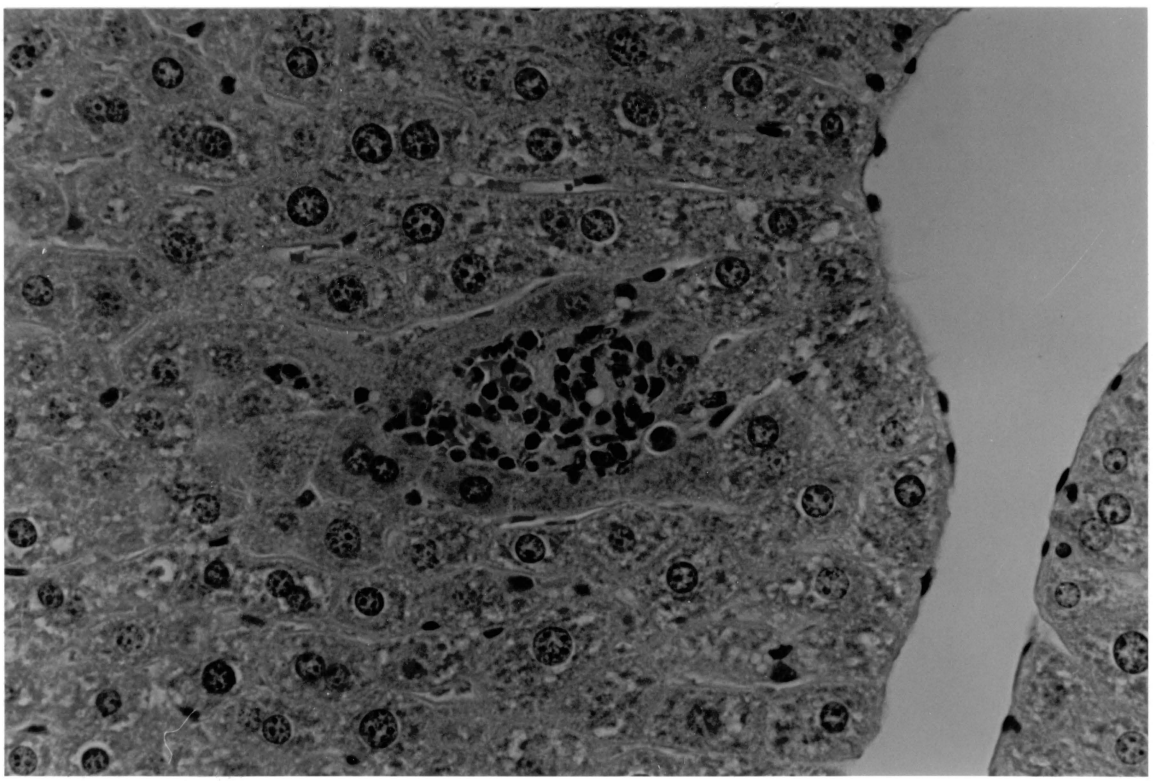


Figure 7

CHAPTER XI

THE ACUTE AND SUBCHRONIC TOXICITY OF GLG-V-13, A COMBINED CLASS IB AND CLASS III ANTIARRHYTHMIC AGENT, IN CD-1 MICE

Abstract

The acute and subchronic toxic effects of GLG-V-13 {3-[4-(1*H*-imidazol-1-yl)benzoyl]-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane dihydroperchlorate}, a novel combined class Ib and class III antiarrhythmic agent, were investigated in mice. The estimated LD_{16}, LD_{50}, and LD_{84} for GLG-V-13 given orally were 319, 419 and 551 mg/kg for male mice and 278, 383 and 528 mg/kg for female mice, respectively. The acute toxic signs appeared to be of CNS in origin. Four groups of mice (20/sex/group/dose) were fed daily with diets containing GLG-V-13 for 90 consecutive days. At the interim of 45 days, 5 mice per group were used for the 45-day toxicity study. The approximate dose levels in male mice were 0, 15.6, 39.0 and 106.8 mg/kg/day for the 45-days toxicity study and 0, 21.6, 50.5 and 121.3 mg/kg/day for the 90-day toxicity study. The dose levels for female mice were 0, 20.3, 49.4 and 123.4 mg/kg/day for the 45-day toxicity study and 0, 26.7, 59.9 and 136.3 mg/kg/day for the 90-day toxicity study. All of the mice survived during the 45- and 90-day study. No significant toxic signs were observed during the period of the studies. Food consumption was decreased in all high dose groups and some low and middle dose groups of the 45- and 90-day toxicity studies. However, mean body weight and body

weight gain were *not* significantly changed. An increase in feed efficiency was found only in female mice treated with the low dose level of the 90-day study with no changes in all other groups. No significant hematological changes were observed in both sexes of the 45-day toxicity study. There were some changes in either absolute or relative weight of some organs. Gross pathological changes, especially in the lungs and liver, were found at middle and high dose groups. However no significant histopathological alterations were detected in the 45-day toxicity study. Consistent increased MCHC and decreased MCH were observed in all dose groups of the 90-day toxicity study. *High dose levels* of the compound produced gross pathological lesions in the liver, kidney and adrenal glands during the 90-day toxicity study. Hepatocellular necrosis was found in both male and female mice but only when treated with the high dose level of GLG-V-13. Since there was no significant increase in the serum ALP, AST, and ADH, the hepatocellular necrosis is likely mild. However ALT was increased significantly in female mice, and they may have severe hepatocellular lesions. Histopathological lesions in the kidneys were found only in male mice without a significant elevation in BUN, suggesting that the compound is free of nephrotoxicity. Marked vacuolation of the X zone in the adrenal gland with mild to moderate deposition of ceroid pigments (Brown degeneration) was observed in female mice at the high dose level. Lesions in the kidneys and adrenal glands may be a possible reason for changes in serum sodium and potassium ions concentrations leading to increase water intake. A significant reduction in cholesterol in the high dose group may be a favorable pharmacological effect of GLG-V-13. Based on these data, GLG-V-13 appears to have has limited systemic toxicity potential with no-effect levels being tentatively calculated at 50.5 mg/kg/day for male

and 59.9 mg/kg/day for female mice respectively.

Introduction

Most currently available antiarrhythmic drugs are less than ideal for therapeutic management of clinical cases of various arrhythmias (Nygaard et al., 1986; Woosley, 1991). Since the Cardiac Arrhythmias Suppression Trial (CAST) (Rogers et al., 1989; Echt, 1991; Rogers et al., 1991), Class III antiarrhythmic agents were found to have better therapeutic results than those of other class agents (Mason et al., 1989; Burkurt et al., 1990; Cairns, 1991; Campbell, 1993; Greene et al., 1993). However, current class III agents, such as sotalol and amiodarone, possess several other actions and serious side effects typical of class III agents (Bigger and Hoffman, 1990). Therefore, efforts are directed towards synthesizing newer agents possessing class III activity, especially a combination of class III/I antiarrhythmic properties with low toxicity (Hondeghem and Snyders, 1990).

Several 3,7-diheterobicyclo[3.3.1]nonane (DHBCN) derivatives have been demonstrated to have significant antiarrhythmic properties (Jeyaraman and Avila, 1981; Bailey et al., 1984; Hartenstein and Wager, 1986; Thompson et al, 1987; Zisman, 1989; Smith et al., 1990; Beatch et al., 1991; Berlin et al., 1992; Garrison, 1993). Tedisamil, a new class III antiarrhythmic agent, was studied extensively (Oexle et al., 1987). Recently, GLG-V-13, 3-[4-(1*H*-imidazol-1-yl)benzoyl]-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane·$HClO_4$, was shown to prevent sustained monomorphic ventricular tachycardia in 5/6 dogs with proarrhythmic action seen only in one dog (Fazekas et al., 1993a). The drug significantly increases the refractory period of the

right ventricle and prolongs AH, HV, and QT intervals (Fazekas, 1992: 1993a; 1993b; 1994). GLG-V-13 appears to have some class Ib and predominant class III antiarrhythmic activities without hemodynamic depressant effects (Fazekas et al., 1992; 1993a; 1993b; 1994). Additional characterization of electrophysiological effects of GLG-V-13 in normal and ischemically-injured myocardial cells is in progress (Patterson et al., 1994).

The pharmacokinetic data demonstrated that GLG-V-13 has reasonably good oral bioavailability, wide distribution, and relatively slow elimination in dogs and rabbits (Chen, et al., 1994a). In addition, this compound has a long duration of pharmacological effects, low proarrhythmic activity, and cardiodepressant actions. All of these effects make this compound a most promising candidate as an antiarrhythmic agent. The objective of this study was to characterize both acute and subchronic toxicity profiles of this novel antiarrhythmic agent.

Materials & Methods

Chemicals

GLG-V-13 was synthesized by a method previously described (Garrison et al., 1993). The purity of the compound was >99.9% as determined by a thin-layer chromatography (TLC). Mice pelleted food (Purina Lab Chow 5001) was purchased from Purina Mills Inc. (richmond, IN).

Animals

Four week old, CD-1 strain mice (Charles River Breeding Labs) of both sexes

weighing 18 to 25 g were housed in a controlled environment (12-hour light/12-hour dark photoperiod changing at 0800 and 2000 hr, 22±1°C, 50±10% relative humidity). The mice for the acute toxicity study were allowed free access to pelleted food (Purina Mills Inc., Richmond, IN) and tap water. For the 45- and 90-day toxicity studies, the mice were fed powdered food. All mice had free access to city tap water *ad libitum*. All the mice were preconditioned for 2 weeks prior to initiation of dosing. A preliminary physical examination indicated that no gross abnormalities were present in these mice.

Acute Toxicity

Twelve hours before initiation of the experiment, food was withdrawn. Mice were randomly allotted in 5 groups (10 mice/sex/group). GLG-V-13 was dissolved in deionized water processed through a Milli-Q Water System (Millipore Corp., Marlborough, MA). Individual mice within each group were gavaged (ca 0.2 ml/10 g) different doses (307.1, 361.3, 425, 500, 588 mg/kg) of the drug using a No. 18 stainless-steel ball-tipped feeding needle. Systemic toxic signs were monitored for a period of three days, and the body weights of survivors were determined daily.

Determination of LD_{16}, LD_{50} and LD_{84}

The number of animals died acutely within groups for each dose was counted. The LD_{16}, LD_{50} and LD_{84} values were calculated by a computer program PHARM/PCS based on Litchfield and Wilcoxon method (Tallarida and Murray, 1986). The data were expressed as LD50 (mg/kg) with 95% confidence limits.

Diets Preparation

Powdered diets containing 0, 100, 250, and 650 mg/kg of GLG-V-13 were prepared by mixing together preweighed quantities of GLG-V-13 and powdered diets in a HandyBlender II (Black & Decker Inc., Shelton, CT) for 15 min. The diet was prepared every two days. Analytical determinations of feed samples with HPLC indicated that this procedure resulted in a homogenous mix. Due to lack of significant toxicity in the 45-day toxicity studies, the amount of GLG-V-13 in the powdered diets was increased to 0, 200, 450, and 900 mg/kg for feeding the mice in the remaining 45 days.

Forty-five and Ninety-Day Toxicity Studies

After adaptation for 2 weeks, the mice were randomly divided into four groups of mice (20 mice/sex/group/dose). Test animals were approximately 6 weeks old with a mean body weight of 26.0±2.1 g at the beginning of dosing. Five mice were housed together in suspended 11" x 6.7" x 4.7" solid-bottom polycarbonate cages fitted with hardwood chips as cage bedding. The mice were allowed free access to food and water. Animals were fed with food containing three different dose levels of GLG-V-13 between 1300 and 1500 hr, and the control group was fed with diet without GLG-V-13. During the experiments, the physical condition and overt signs of toxicity of the individual mice within a group were assessed daily. Body weights were measured prior to the first dosing and thereafter once a week during the entire test period. Food and water consumption for a group of 5 mice in each cage was measured once every two days. Feed efficiency (g total body weight gain/g total feed consumption x 100) was calculated from body weight gain and food consumption.

Blood Sampling. Of the 20 mice per group, 5 mice were used for the 45-day toxicity study and remaining 15 mice for the 90-day toxicity study. Blood samples were collected from ocular venous plexus of individual mice under light CO_2 anesthesia on the days 46 and 91. The portions (ca 0.3 ml) of whole blood were collected in Microtainers with EDTA·K2 (Becton Dickinson and Company, Rutherlord, NJ), and the remaining portions were placed in separate tubes containing no anticoagulant (Vacutainer, Becton Dickinson and Company, Rutherlord, NJ) for serum. Then, the mice were terminated by cervical dislocation of the spinal cord from the cerebrum. Both whole blood and serum from individual mice were used for hematological and clinical chemistry profiles, respectively.

Determination of Hematological Values. The number of white blood cells (WBC), red blood cells (RBC), hemoglobin, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets counts, and total protein were measured by using a SYSTEM 9000, Baker Instrument (Serono-Baker Diagonistics Inc., Allentown, PA). Differential counts of WBC, morphologies of both WBC and RBC were determined from blood smear by light microscopy.

Determination of Clinical Pathological Values. The determination of serum biochemical values including blood urea nitrogen (BUN), alkaline phosphatase, asparate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) activity, and sodium, potassium, albumin and cholesterol concentrations were determined by using a Roche Cobas Mira (Roche Analytical Instruments, Nutley, NJ).

Gross Pathological Findings. The weights of brain, heart, liver, kidney, spleen,

testis, epididymis, ovary plus uterus, adrenal, and lungs were determined. The organ/brain weight and organ/body weight ratios were calculated. Any significant gross pathological changes in individual mouse were observed and noted.

Histopathological Examination. At necropsy, the following tissues were collected in 10% neutral buffered formalin: adrenal glands, brain, caecum, colon, duodenum, heart (with major vessels), ileum, kidneys, liver, lung, lymph node, pancreas, skeletal muscle, spleen, stomach, and urinary bladder. The eye, epididymis and testis were collected in Boune's solution and then transferred to 70% ethanol. Following dehydration and embedding, all tissues were routinely processed, sectioned at 5-6 microns, and stained with hematoxylin and eosin (H&E) and then examined microscopically.

Statistical Analysis of the Data

Means and standard deviations were calculated for body weight, food, water consumption, clinical pathologic, and organ weight data. Statistical analysis was carried out using an appropriate Student t test (equal or unequal variance) and a computer program PHARM/PCS (Tallarida and Murray, 1986). Differences between the treatment and control groups were considered significant at $p<0.05$, and highly significant at $p<0.01$.

Results

Acute Toxicity Study - Determination of LD_{50}

The same doses were used for male and female mice. There was a little

difference in median acute lethal dose in male and female mice with LD_{50} of 419 and 383 mg/kg, respectively. LD_{16} and LD_{84} were 319 and 551 mg/kg for male mice and 278 and 529 mg/kg for female mice, respectively (Table 1).

Body Weight, Food, Water, Drug Consumption, and Feed Efficiency

The initial mean body weight, body weight gain and body weight at the 46th day, food, water, and drug consumptions during the 45-day toxicity study have been presented in Table 2. In the 45-day toxicity study, the approximate GLG-V-13 consumption was calculated to be 15.6, 39.0 and 106.8 mg/kg/day for male mice and 20.3, 49.4 and 123.4 mg/kg/day for female mice. Figures 1 and 2 show mean body weight and cumulative body weight gains in male and female mice fed GLG-V-13 for 45 days, respectively. Mean body weights and body weight gain in male and female mice fed GLG-V-13 were the same as those of the controls, except there was a significant reduction in body weight of the female mice fed the *middle dose* of GLG-V-13. Decrease in food consumption in drug-fed groups was observed in middle and high dose groups of male mice and in high dose group of female mice. Feed efficiency in the 45-day toxicity study was increased only in male mice fed low and high dose of GLG-V-13 (Figure 3). Water consumption was not markedly different between drug-fed and control groups. In male mice, food consumption was decreased significantly in all three dose groups, but feed efficiency was increased only in low and high dose groups. However, feed efficiency was increased between the 5th and 6th weeks in low dose groups and between 3rd and 5th week in high dose groups. Nevertheless, there was no significant change in water consumption.

The initial mean body weight, body weight gain and body weight at the 90th

day, food, water and drug consumptions during the 90-day toxicity study have been presented in Table 3. In the 90-day toxicity study, the GLG-V-13 consumption was calculated to be 21.6, 50.5 and 121.3 mg/kg/day for male mice and 26.7, 59.9 and 136.3 mg/kg/day for female mice. Figures 4 and 5 show mean body weight and cumulative body weight gains in mice fed GLG-V-13 for 90 days. Body weight and body weight gain during the 90 days were not significantly different between male and female mice compared with the control groups. However, significant reduction in body weight and body weight gain was found between the 4th week and 10th week in the male mice fed a middle dose level of GLG-V-13. Similar results were observed in female mice of the high dose group between the 4th to 9th week. Food consumption was markedly reduced in both male and female mice fed different dose levels of GLG-V-13 except there was no significant change in female mice fed a low dose level of drug. An increased feed efficiency was observed only in low dose groups of both male and female mice (Figure 6). In male mice, water consumption was increased markedly only in those fed a high dose level of GLG-V-13. However, there was a significant increase in water consumption in female mice fed low, middle and high dose levels of GLG-V-13.

Hematology

Mean hematological values in male and female mice fed GLG-V-78 for 45 day are presented in Tables 4 and 5, respectively. There were no significant changes in hematological values of male and female mice fed low and high dose levels of GLG-V-13 for 45 days. However, some significant changes in hematology were observed but only in the middle dose level groups. In male mice fed the middle dose level, an

elevation of hemoglobin was observed. In female mice fed with the middle dose level of GLG-V-13, the percentages of segmented neutrophils (SEG) and eosinophils were increased with an increase in absolute number of eosinophils but not in SEG.

Tables 6 and 7 show the mean hematological values in male and female mice fed GLG-V-13 for 90 days. In the 90-day toxicity study, the amount of mean corpuscular hemoglobin concentration (MCHC) was markedly increased in male and female mice fed low, middle, and high dose level of GLG-V-13. A moderate increase in MCH was observed in male and female mice fed low and high dose levels of GLG-V-13. In male mice, the absolute number of segmented neutrophils was decreased in the high dose level group without changing its percentage. Both percentage and absolute number values of eosinophils were increased in the low dose group. In female mice, hemoglobin, HCT, platelets, total protein, and percentage of SEG were decreased significantly but only in the high dose level group. The percentage and absolute number values of monocytes were increased in the low and middle dose level groups.

Clinical Biochemical values

The clinical biochemical values of both male and female mice fed GLG-V-13 for 45 and 90 days are presented in Tables 8 and 9, respectively. In general, there were no significant changes in clinical biochemical values of both male and female mice observed during the 45-day toxicity study except that there was a reduction in LDH in female mice fed a middle dose level of drug.

In the 90-day toxicity study, there were no significant changes in all biochemical values determinated in male mice except for a significant reduction in

serum Na^+ which was observed in male mice treated with middle and high dose levels of GLG-V-13, but similar changes were not observed in female mice. Several other clinical biochemical values were altered in female mice, in which BUN was significantly increased in all dose levels. ALT activities were elevated only in low and middle dose groups. The activity of alkaline phosphatase was decreased in female mice fed a low dose level of drug. There was a significant reduction in serum cholesterol content in female mice fed with a high dose level of GLG-V-13. The amount of serum K^+ was found to increase only in female mice treated with the high dose level of GLG-V-13. However, this change did not occur in male mice.

Organ Weight Changes

Absolute and relative organ weights of male mice fed GLG-V-13 for 45 days have been presented in Table 10. No significant changes were observed in organ weights of brain, heart, spleen, testis, epididymis, and adrenal gland of male mice in the 45-day toxicity study. Absolute kidney weight and kidney/brain ratio were decreased in the middle dose group. In contrast, absolute kidney weight and kidney/body weight ratio were increased in the high dose group. The absolute lung weight and lung/body weight ratio were increased markedly only in middle dose group. The liver/brain ratio was decreased in the low dose group.

Absolute and relative organ weights of female mice fed GLG-V-13 for 45 days have been presented in Table 11. In female mice fed with a low dose of GLG-V-13, absolute kidney weight, kidney/brain weight, and kidney/body weight ratios were reduced significantly. However spleen/brain and spleen/body weight ratios were increased markedly with no change in absolute spleen weight. In mice fed with

middle dose level of GLG-V-13, the absolute liver and liver/brain ratio were decreased significantly. Absolute spleen weight was reduced with no significant change in relative spleen weights.

Tables 12 presents the data on absolute and relative organ weights in male mice treated with GLG-V-13 for 90 consecutive days. Significant increases in absolute organ weights and organ/body weight ratios in brain, heart, liver, kidney epididymis, adrenal and lung were found in high dose groups. However, only the adrenal/brain weight ratio was increased in this group of mice. Heart/body weight and lung/body weight ratios were elevated in the mice treated with the middle dose level of GLG-V-13. Absolute lung weight and lung/body weight ratio were also significantly increased in the low dose group.

In female mice, significant reduction in absolute ovary plus uterus weight, and their relative weights to brain and body weight, was observed in all three dose level groups. Absolute adrenal weight was increased only in the middle dose group without changes in its relative weight to brain and body weight. However, no significant changes in all other organ weights were observed in female mice of the 90-day toxicity study.

Gross Pathological Changes

Pulmonary congestion/edema was found in both male and female mice of all dose groups (usually 1-2 mice per group) of the 45- and 90-day toxicity studies. In the 45-day toxicity study, no significant gross pathological changes were present in male mice. However, discoloration of the liver was a consistent finding in 20% female mice fed all dose levels. In 90 day toxicity study, a pale liver was found in

both male and female mice (1-2/15) of middle and high dose groups.

Histopathological Changes

Histologic examination of tissue sections from male mice administered GLG-V-13 at the high dose of 121.3 mg/kg/day revealed multifocal, randomly scattered hepatocellular necrosis (Figure 7). The necrotic focus contained a few (between 1-4) shrunken necrotic hepatocytes with pyknotic nuclei and condensed eosinophilic cytoplasm. The focus contained mild infiltrates of mononuclear cells predominately of lymphocytes. This lesion was observed in 5/10 mice. Additionally 5/10 mice had vacuolated renal cortical tubules, of which one mouse had protein droplets in the tubules. The lesion was mild and was confined to the proximal convoluted tubules. Male mice supplemented with GLG-V-13 at the other 2 lower doses did not have any significant histopathologic lesions. *No other tissues examined had any histopathological lesions.*

In female mice, focal hepatocellular necrosis was observed in 4/10 mice. The adrenal glands of 7/10 mice administered GLG-V-13 at the high dose of 136.3 mg/kg/day for 90 days had marked vacuolation of the X zone (involution) (Figure 8). This involution of the X zone was observed in 4/10 mice in the intermediate dose of 59.9 mg/kg/day and 2/10 mice in the lowest dose of 26.7 mg/kg/day of GLG-V-13. In comparison, all the 10 control female mice still had the X zone composed of basophilic cells. However, 3/10 control mice had a few scattered vacuoles in the X zone along with the presence of the basophilic cells, indicating the beginning of involution of the X zone. *There were no significant histopathological lesions in any other tissues examined.*

Discussion

Acute Toxicity

During estimation of median acute lethal values, most cases of death in the mice were observed between 30 min and a 4 hour period, but only few deaths occurred in less than 30 min. This finding is significantly different from that observed in the mice treated with an acute dose of BRB-I-28, in which most instances of death were within a 10 min period after administration of the compound (Chen et al., 1994c). The acute toxic signs, including sedation, tremors, convulsion, twitching, catalepsy, ataxia followed by dyspnea, and apnea leading to death, were of CNS in origin. The systemic signs were very similar to those treated with BRB-I-28, one of the novel antiarrhythmic agents. No significant changes in body weight were observed within the 3-day period. Since the mice were not subjected to necropsy, gross and histopathological status of the individual target organs were unknown.

Forty-five-Day Toxicity

In the 45-day toxicity study, both male and female mice survived, and no overt toxic signs were observed overall. There were no significant changes in body weight, body weight gains, hematological and clinical biochemical values in male mice treated with three different dose levels of GLG-V-13 except for some alterations in absolute and relative organ weights of kidneys and lungs. However, no histopathological lesions were observed in kidney and lung.

Significant changes in body weight, body weight gains, food consumption, feed efficiency, water consumption, hematological, and clinical biochemical values were not

observed in female mice treated with low and high dose levels of GLG-V-13 except for a reduction in absolute and relative kidney weight and an elevation in relative spleen weights in the low dose group. No gross and histopathological lesions of toxicological importance were found in these groups.

Although there were some gross pathological changes noticed in the liver of female mice, neither histopathological changes nor significant alteration of liver enzymes such as ALP, AST, ALT, and LDH were observed, suggesting low potential for hepatotoxicity. Pulmonary edema/congestion associated with very small histopathological changes indicate GLG-V-13 is relatively free of pneumotoxicity It is quite possible that the observed pulmonary edema/congestion may be induced by inhalation of CO_2 or by the procedure for taking the blood samples.

Ninety-Day Toxicity

In the 90-day toxicity study, there were no drug-related deaths and no significant toxic signs during the course of study. It appears that initial body weight, body weight at the 90th day, and body weight gain were not significantly changed, although food consumption was decreased in male mice fed the three dose levels of GLG-V-13 and female mice fed with middle and high dose levels of GLG-V-13. There was no significant increase in feed efficiency in these mice. In contrast, the food consumption was not significantly changed in female mice fed the low dose level of GLG-V-13, but the feed efficiency was increased beginning from 6th week to 13rd week.

Water consumption was significantly increased in male mice of the high dose group and female mice of all three dose groups. An increase in water consumption

in male mice may be directly related to sodium loss, because the sodium ion is the most important component of osmotic factors involved in the distribution of water between extra- and intracellular tissue spaces. The vacuolation in the renal cortical tubules may be another reason for an increase in water uptake. In female mice, water consumption was elevated in all three dose groups. Since serum sodium and renal cortical tubules were normal, one possible mechanism for an elevation in water consumption may be related to dysfunction of adrenal glands. However, the mechanisms and toxicological importance of an increase in water consumption in the drug-treated mice are unknown.

Like BRB-I-28, GLG-V-13 caused a consistent increase in mean corpuscular hemoglobin (MCH) in male and female mice fed low and high dose levels of GLG-V-13 and a decrease in mean corpuscular hemoglobin concentration (MCHC) in both sexes of all three dose groups. MCH and MCHC, the two erythrocytic indices, which are calculated from the total red blood cell number, hemoglobin concentration and volume of packed red blood cells, are relatively inaccurate (Woodliff and Herrmann, 1973). The lack of any change in the total number of red blood cells, hemoglobin, and MCV suggests that GLG-V-13 may have little significant toxicological effects on erythropoiesis in the male and female mice. It is important to note that there is a marked reduction in concentration of hemoglobin, HCT, platelet number, and total protein in male and female mice fed the high dose of GLG-V-13. However, the importance of these changes in toxicity induced by GLG-V-13 is uncertain. The total number of white blood cells was the same between control and drug-treated mice, but an elevation in percentage and absolute number of monocytes and eosinophils was observed in female mice of the low and middle dose group and in the low dose group

of female mice, respectively. Since these effects were not dose-related, they may not have significant toxicological consequence.

Since there were no noticeable histopathological changes in the low- and middle-dose groups of the 90-day toxicity study, changes in brain, lung, and spleen weights of the low and middle dose groups and gross pathological alterations in liver may have no significant toxicological consequence.

The focal necrosis of hepatocytes observed in mice administered GLG-V-13 predominantly at the high dose of 121.3 mg/kg/day for male mice and 136.3 mg/kg/day for female mice for 90 days was of a coagulative type (Figure 7). It was characterized by shrunken hypereosinophilic hepatocytes with degenerating nuclei. A variety of toxicants and therapeutic agents produce necrosis of liver (Plaa, 1986). Although this pattern of necrosis has been described following the exposure of humans to a variety of compounds, it is infrequently observed in animals exposed to a variety of toxicants. Focal necrosis has been described in detail following acute treatment of rats with phalloidin, carbon tetrachloride, and D-galactosamine (Popp and Cattley, 1991; Plaa, 1986). The necrotic hepatocytes are replaced by regeneration of adjacent hepatocytes without fibrosis. Since repair is by regeneration, without inciting fibrosis, the lesion may be undetectable histologically in mice exposed to lower dose levels. An increase in serum ALT activity may be caused by hepatocellular lesion. However, other key enzymes such as ALP, LDH, and AST were indicative of liver function and did not increase significantly, suggesting that hepatocellular lesions are of a mild nature.

Male mice administrated GLG-V-13 at the high dose of 121.3 mg/kg/day had vacuolization of the proximal convoluted tubule in the renal cortex. This

vacuolization represents hydropic swelling of the phagolysosome and has been designated "osmotic nephrosis". The functional impact of this change is minimal and the vacuolization actually may be a transient adaptive response (Alden and Frith, 1991). These vacuoles are probably pinocytic vacuoles containing substrate and water absorbed from the tubular lumen and represent a physiological effect rather than a true degeneration. Since there was no significant elevation of BUN in male mice treated with GLG-V-13, the renal lesions may be mild in nature. It was interesting to note that an elevation of BUN was observed in female mice fed with three dose levels, but it was not accompanied by renal lesions. Thus, GLG-V-13 could be potentially toxic to the kidneys. In man, vacuolation in the renal cortex has been associated with long standing potassium depletion (Glaister, 1986). A significant increase in serum potassium was observed only in female mice fed with the high dose level. Decreased serum Na^+ may possibly be related to renal lesion. This might have resulted in an increase in water intake, since renal lesions may affect absorption and secretion of electrolytes through kidney. The exact toxicological/pathological mechanisms relating to renal lesions with alterations in serum Na^+ and water consumption induced by high dose of GLG-V-13 is unknown.

Female mice treated with GLG-V-13 had marked vacuolation of the X zone in the adrenal gland with mild to moderate deposition of ceroid pigment (brown degeneration), in contrast to the controls (and the BRB-I-28 group) where minimal vacuolation was present. The vacuoles in the X zone of the adrenal gland indicate involution of the zone. The involution of the X zone occurred between 30-83 days in virgin females (Dunn, 1970). This exaggerated normal fatty degeneration has also been observed with thyroxin and methanol (Gopinath et al., 1987; Ribelin, 1984).

The brown degeneration has been reported as a true degeneration and not a spontaneous production of ceroid pigment (Moore and Callas, 1975). This lesion in the adrenal gland was not observed in the male mice. The X zone appears 10 days after birth in both male and female mice, reaching a maximum at 3 weeks in males. However, in the males degeneration occurs at 5 weeks when the animal reaches sexual maturity. A significant elevation in serum K^+, with a trend for reduction in sodium may possibly be related to the lesions in the adrenal gland via changes in secretion of mineralcorticoids. The role of brown degeneration of adrenal glands and the mineralcorticoids in the pathogenesis of hyperkalemia induced by this novel antiarrhythmic agent needs further investigation.

A significant reduction in LDH was found in mice of the low dose group. The beneficial or harmful effects of decreased LDH activity in mice is unknown. It should be pointed out that a marked reduction of cholesterol in male mice fed the middle dose of GLG-V-13 and female mice fed a high dose of GLG-V-13 will have potential beneficial effects in patients with arrhythmias, since hypercholesterolemia was found to be major factor in pathogenesis of atherosclerosis and coronary artery diseases (Stryer, 1988).

In summary, data collected from the 45- and the 90-day subchronic toxicity studies indicates that GLG-V-13, a combined class Ib and class III antiarrhythmic agent, has a limited potential for systemic toxicity. Target organs susceptible to toxic insult include liver, kidney, and adrenal gland, but gross pathological lesions in these organs are mild. The reduction in cholesterol may be a favorable effect of GLG-V-13. Low toxicity, in combination with effective antiarrhythmic effects, and low proarrhythmic actions make this compound worthy of further development as a potential antiarrhythmic agent.

References

Alden, C. L., and Frith, C. H. Urinary system. In "Handbook of Toxicologic Pathology", (Haschek, W. M., and Rousseaux, C. G., eds), Academic Press, New York, 1991.

Bailey, B. R., Berlin, K. D., Holt, E. M., Scherlag, B. J., Lazzara, R., Brachmann, J., Helm, D. V. D., Powell, D. R., Pantaleo, N. S., and Ruenitz, P. C., Synthesis, conformational analysis, and antiarrhythmic properties of 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonan-9-one, 7-benzyl-3-azabicyclo[3.3.1]nonane hydroperchlorate, and 7-benzyl-9-thia-7-azabicyclo[3.3.1]nonan-9-ol hydroperchlorates and derivatives: single-crystal x-ray diffraction analysis and evidence for chair-chair and chair-boat conformers in the solid state. Journal of Medicinal Chemistry 27: 758-767, 1984.

Beatch, G. N., Abreham, S., Mac Lead, B. A., Yoshida, N. R., Walker, M. J. A. Antiarrhythmic properties of tedisamil (KC8857). A putative transient outward K^+ current blocker. Eur. J. Pharmacol. 102: 13-18, 1991.

Berlin, K. D., Scherlag, B. J., Clarke, C. R., Otiv, S. R., Zisman, S. A., Sangiah, S., Mulekar, S. V., Salts of 3-azabicyclo[3.3.1]nonanes as potential antiarrhythmic agents, and precursors thereof. U.S. Patent 5,084,572, 1992; Chem. Abstr. 115: 114550c, 1991.

Bigger, Jr. J. T., and Hoffman, B. F. Antiarrhythmic drugs. In "The Pharmacological Basis of Therapeutics", (Gilman, A. G., et al., eds), 8th edition, pp. 840-873, Pergamon Press, New York, 1990.

Burkart, F., Pfisterer, M., Kiowski, W. Effects of antiarrhythmic therapy on mortality in survivors of myocardial infarction with asymptomatic complex ventricular arrhythmias: Basel antiarrhythmic study of infarct survival (BASIS). J. Am. Coll. Cardiol. 16: 1711-1718, 1990.

Cairns, J. A. Post-myocardial infarction mortality in patients with ventricular premature depolarizations: Canadian amiodarone myocardial infarction arrhythmia trial pilot study. Circulation. 84: 550-557, 1991.

Campbell, T. J. Class III antiarrhythmic action: the way forward? the Medical Journal of Australia. 158: 732-733, 1993.

Chen, C. L., Sangiah, S., Roder, J. D., Chen, H., Berlin, K. D., Garrison, G. L., Scherlag, B. J., and Lazzara, R. Oral, intravenous pharmacokinetics and plasma protein binding of GLG-V-13, a combined class Ib and class III antiarrhythmic agent, in dog. (unpublished) 1994a.

Chen, C. L., Sangiah, S., Roder, J. D., Chen, H., Berlin, K. D., Garrison, G. L., Scherlag, B. J., and Lazzara, R. Determination of GLG-V-13, a novel antiarrhythmic

agent, in plasma and urine by high-performance liquid chromatography. J. Liquid Chromatogr. 17(4): 913-927, 1994b.

Chen, C. L., Sangiah, S., Chandra, A. M. S., Qualls, C. W., Chen, H., Roder, J. D., Garrison, G. L., Berlin, K. D., Scherlag, B. J., and Lazzara, R. Acte and subchronic toxicity of BRB-I-28, a novel class Ib antiarrhythmic agent. (unpublished), 1994c.

Dunn, T. B. Normal and pathologic anatomy of the adrenal gland of the mouse, including neoplasms. J. Natl. Cancer Inst. 44: 1323-1389, 1970.

Echt, D, S., Liebson, P. R., Mitchell, L. B., Peters, R. W., Obias-Manno, D., Barker, A. H., Arensburg, D., Baker, A., Friedman, L., Greene, H. L., Huther, M. L., Richardson, D. W., and the CAST Investigators. Mortality and morbidity in patients receiving encainide, flecainide, or placebo. The cardiac arrhythmia suppression trial. N. Engl. J. Med. 324: 781-788, 1991.

Fazekas, T., Scherlag, B. J., Mabo, P., Patterson, E., Berlin, K. D., Garrison, G. L. and Lazzara, R. Egy uj refrakter periodust megnyujto antiarimias vegyulet, a GLG-V-13 szivelektrofiziologiai jellemzese. Cardiologic Hungarica. 22: 9-15, 1993a.

Fazekas, T., Scherlag, B. J., Mabo, P., Berlin, K. D., Garrison, G. L., Chen, C. L., Sangiah, S., Patterson, E., and Lazzara, R. The comparative antiarrhythmic and proarrhythmic activity of a 3,7-diheterobicyclo[3.3.1]nonane, BRB-I-28, and lidocaine in the 1-4-day-old infarcted dog heart. Acta Physiologica Hungary. 81: 297-307, 1993b.

Fazekas, T., Scherlag, B. J., Mabo, P., Patterson, E., Berlin, K. D., Garrison, G. L., and Lazzara, R. Electrophysiological characterization of a new heart rate-lowering and refractoriness prolonging antiarrhythmic agent, GLG-V-13, in the 1-4 day-old infarcted dog heart. Acta Physiologica Hungarica. Submitted, 1994.

Garrison, G. L. Selected derivatives of 3,7-diheterobicyclo[3.3.1]nonanes which possess multi-class antiarrhythmic activity. Ph.D. Dissertation. Oklahoma State University, 1993.

Glaister, J. R. Target-organ pathology. In "Principles of Toxicological Pathology" (Glaister, J. R., eds), pp. 51-130, Taylor and Francis, London and Philadelphia, 1986.

Gopinath, C., Prentice, D. E., and Lewis, D. J. (ed). "Atlas of Experimental Toxicological Pathology", MTP Press, Lancaster, 1987.

Greene, H. L., and The CASCADE Investigators. Randomized antiarrhythmic drug therapy in survivors of cardiac arrest (The CASCADE Study). Am. J. Cardiol. 72: 280-287, 1993.

Hartenstein, J., and Wager, B. Antiarrhythmic agents. In "Cardiovascular Drugs", (Bristol, J. A., ed.), pp. 305-415, John Wiley & Sons, New York, 1986.

Hondeghem, L. M., and Snyders, D. J. Class III antiarrhythmic agents have a lot of potential but a long way to go. Circulation. 81: 686-690, 1990.

Jeyaraman, R., and Avila, S. Chemistry of 3-azabicyclo[3.3.1]nonanes. Chemical Reviews. 81: 149-174 (1981).

Mason, J. W., and The ESVEM Investigators. The ESVEM Trial. Circulation. 79: 1354-1360, 1989.

Moore, N. A., and Callas, G. Observations on the fine structure of propylthiouracil induced "brown degeneration" in the zona reticularis of mouse adrenal cortex. Anat. Rec. 183: 293-302, 1975.

Nygaard, T. W., Sellers, T. D., Cook, T. S., and DiMarco, J. P. Adverse reactions to antiarrhythmic drugs during therapy for ventricular arrhythmias. J. Am. Med. Assoc. 256: 82-84, 1986.

Oexle, B., Weinich, J., and Antoni, H. Electrophysiological profile of KC8857, a new bradycardiac agent (Abstract). J. Mol. Coll. Cardiol. 19(Supplement III), 194.

Patterson, E., Scherlag, B. J., Berlin, K. D., Garrison, G. L., Sangiah, S., Chen, C. L., and Lazzara, R. Electrophysiological action of GLG-V-13 in canine myocardial tissues and in ischemically-injured canine myocardium. Unpublished data, 1994.

Plaa, G. L. Toxic responses of the liver. In "Toxicology", (Klaassen, C. D., et al., eds), 3rd edition, pp 286-309, Macmillan Publishing Company, New York, 1986.

Popp, J. A., and Cattley, R. C. Hepatobiliary system. In "Handbook of Toxicologic Pathology", (Haschek, W. M., and Rousseaux, C. G., eds), pp. 279-314, Academic Press, New York, 1991.

Ribelin, W. E. The effects of drugs and chemicals upon the structure of the adrenal gland. Fund. Appl. Toxicol. 4: 105-119, 1984.

Rogers, W. J., and The CAST Investigators. Preliminary report: effect of encainide and flecainide on mortality in a randomized trial of arrhythmia suppression after myocardial infarction. N. Engl. J. Med. 321: 406-412, 1989.

Rogers, W. J., and The CAST Investigators. Mortality and morbidity in patients receiving encainide, flecainide, or placebo. N. Engl. J. Med. 324: 781-788, 1991.

Smith, G. S., Thompson, M. D., Berlin, K. D., Holt, E. M., Scherlag, B. J., Patterson, E., and Lazzara, R. A study of the synthesis and antiarrhythmic properties of selected 3,7-diheterobicyclo[3.3.1]nonanes with substituents at the 2,4-positions and at the 9-position. European Journal of Medicinal Chemistry 25: 1-8, 1990.

Stryer, L. (ed.). Biochemistry, 3rd edition, pp 547-574, W. H. Freeman & Company,

New York, 1988.

Tallarida, R.J. and Murray, R.B. Manual of pharmacologic calculations with computer programs, Springer-Verlag, New York, 1986.

Teo, K. K., Yusuf, S., and Furberg, C. D. Effects of prophylactic antiarrhythmic drug therapy in acute myocardial infarction. An overview of results from randomized controlled trials. J. Am. Med. Assoc. 270: 1589-1595, 1993.

Thompson, M. D., Smith, G. S., Berlin, K. D., Holt, E. M., Scherlag, B. J., Helm, D.V.D., Muchmore, S. W., Fidelis, K. A. Synthesis and antiarrhythmic properties of novel 3-selena-7-benzyl-3-selena-7-azabicyclo[3.3.1]nonane-9-one and 7-benzyl-3-selena-7-azabicyclo[3.3.1]nonane hydroperchlorate. J. Med. Chem. 30: 780-788, 1987.

Woodliff, H. J., and Herrmann, R. P. (ed). Concise haematology. The Williams & Wilkins Company, Baltimore, 1973.

Woosley, R. L. Antiarrhythmic drugs. Annu. Rev. Pharmacol. Toxicol. 31: 427-455, 1991.

Woosley, R. L., and Roden, D. M. Pharmacological causes of arrhythmogenic actions of antiarrhythmic drugs. Am. J. Cardiol. 59: 19E-25E, 1987.

Zisman, S. T., Berlin, K. D., and Scherlag, B. J. The preparation of amide derivatives of 3-azabicyclo[3.3.1]nonanes as new potential antiarrhythmic agents. Organic Preparations and Procedures Int. 22: 255-264, 1990.

Zisman, S. A. A study of stereochemical and substituent effects on antiarrhythmic activity of selected 3-azabicyclo[3.3.1]nonanes and derivatives. Ph.D. Dissertation. Oklahoma State University, 1989.

TABLE 1

ORAL LETHAL DOSES OF GLG-V-13 IN MICE

Dose (mg/kg)	Male (Died/Used)	Female (Died/Used)
307.1	1/10	3/10
361.3	3/10	4/10
425	7/10	6/10
500	6/10	7/10
588	9/10	10/10
LD_{16} (mg/kg)	319 (311-326)	278 (270-286)
LD_{50} (mg/kg)	419 (365-148)	383 (333-441)
LD_{84} (mg/kg)	551 (538-564)	529 (513-545)

The data are presented as acute lethal dose (95% confidence limit).

TABLE 2

BODY WEIGHT, GLG-V-13, FOOD AND WATER CONSUMPTION OF CD-1 MICE (N = 5) FED GLG-V-13 FOR 45 DAYS

	Control group	Treatment groups		
		Low dose	Middle dose	High dose
Male				
Approximate GLG-V-13 consumption (mg/kg/day)	0	15.6±2.33	39.0±3.71	106.8±8.61
Initial body wt (g)	28.1±1.4	26.9±2.3	27.3±0.7	28.3±0.6
B.wt at 45 days (g)	34.4±1.4	33.3±3.6	32.7±2.8	34.7±1.0
B.wt gain (g)	6.3±1.2	6.4±1.7	5.4±2.4	6.5±0.9
Feed Consumption (g/day/mouse)	5.97±0.50	4.93±0.49*	4.74±0.31**	5.30±0.29*
Water Consumption (g/day/mouse)	6.93±0.59	6.89±0.72	7.21±0.90	8.16±1.37
Female				
Approximate GLG-V-13 consumption (mg/kg/day)	0	20.3±2.2	49.4±5.8	123.4±19.9
Initial body wt (g)	26.0±1.1	25.4±1.4	24.2±2.0	24.5±1.3
B.wt at 45 days (g)	32.2±1.1	30.8±1.5	30.0±1.7*	30.7±1.3
B.wt gain (g)	6.2±1.0	5.5±1.9	5.8±1.3	6.2±0.8
Food Consumption (g/day/mouse)	6.39±0.38	6.09±0.44	5.79±0.51	5.50±0.62*
Water Consumption (g/day/mouse)	7.96±0.60	8.41±0.75	8.33±0.71	8.33±0.64

** $p<0.05$; **$p<0.01$

TABLE 3

BODY WEIGHT, GLG-V-13, FOOD AND WATER CONSUMPTION OF CD-1 MICE (N = 15) FED GLG-V-13 FOR 90 DAYS

	Control group	Treatment groups		
		Low dose	Middle dose	High dose
Male				
Approximate GLG-V-13 consumption (mg/kg/day)	0	21.6±7.1	50.5±13.8	121.3±19.1
Initial body wt (g)	26.9±1.3	27.1±2.0	26.7±1.6	27.8±2.1
B.wt at 90 days (g)	36.1±1.9	36.6±2.2	34.7±2.9	36.7±3.9
B.wt gain (g)	9.2±2.1	9.4±2.8	7.9±3.2	9.6±3.4
Feed Consumption (g/day/mouse)	5.82±0.49	5.01±0.48**	4.75±0.36**	5.39±0.44*
Water Consumption (g/day/mouse)	6.99±0.51	7.30±0.77	7.47±0.78	8.78±1.35**
Female				
Approximate GLG-V-13 consumption (mg/kg/day)	0	26.7±7.9	59.9±13.6	136.3±22.2
Initial body wt (g)	25.7±1.4	24.7±2.0	24.8±1.6	24.8±1.3
B.wt at 90 days (g)	35.2±2.4	36.0±3.8	34.5±2.2	34.0±2.9
B.wt gain (g)	9.6±2.3	11.5±3.2	9.4±2.1	9.2±3.2
Food Consumption (g/day/mouse)	6.25±0.60	6.02±0.59	5.57±0.55**	5.50±0.53**
Water Consumption (g/day/mouse)	7.83±0.61	8.34±0.72*	8.69±0.74**	8.34±0.69*

*$p<0.05$; **$p<0.01$

TABLE 4

HEMATOLOGICAL VALUES (MEAN±SD) IN MALE CD-1 MICE FED GLG-V-13 FOR 45 DAYS (N = 5)

Dose (mg/kg/day)	0	15.6	39.0	106.8
WBC (10^3/mm^3)	10.6±3.76	9.90±1.33	8.98±3.08	10.0±1.96
RBC (10^6/mm^3)	8.70±0.47	8.60±1.36	9.31±0.76	9.13±0.97
HGB (g/100ml)	14.5±0.50	13.9±2.26	15.5±0.81*	15.2±0.74
HCT (%)	42.5±2.30	42.0±6.45	45.4±3.25	44.9±3.95
MCV (fl)	48.9±1.76	48.9±1.19	48.9±1.65	49.2±1.31
MCH (pg)	16.6±0.79	16.2±0.51	16.7±0.81	16.8±1.19
MCHC (g/100 ml)	34.0±1.34	33.1±0.43	34.1±1.38	34.1±2.32
PLT (10^5/mm^3)	11.3±1.80	13.3±1.62	11.9±1.35	12.2±0.71
TP (g/100 ml)	6.12±0.36	6.18±0.32	6.26±0.38	6.26±0.36
Differential				
SEG (%)	22.8±20.6	22.4±7.83	18.2±1.92	18.8±4.87
(Absolute #)	2433±2238	2248±987	1656±701	1830±373
Lymph (%)	73.6±18.3	74.0±7.97	77.2±2.59	76.4±5.46
(Absolute #)	7753±3521	7280±937	6902±2243	7675±1809
Mono (%)	2.2±3.4	2.0±1.4	3.0±1.2	3.6±1.5
(Absolute #)	221±279	208±164	272±153	369±183
Eosin (%)	1.4±1.7	1.6±0.9	1.2±0.4	1.2±0.8
(Absolute #)	153±235	163±103	106±45	125±82
Band (%)	0	0	0.4±0.5	0
(Absolute)	0	0	43±61	0
Baso (%)	0	0	0	0
(Absolute #)	0	0	0	0

*$p<0.05$

Abbreviation: WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular hemoglobin volume; MCH, mean cell hemoglobin; MCHC, mean cell corpuscular hemoglobin concentration; PLT, platelet; TP, total protein; SEG, segmented neutrophils; Lymph, lymphocytes; Mono, monocytes; Eosin, eosinophils; Band, neutrophilic band; Baso, basophil.

TABLE 5

HEMATOLOGICAL VALUES (MEAN±SD) IN FEMALE CD-1 MICE FED GLG-V-13 FOR 45 DAYS (N = 5)

Dose (mg/kg/day)	0	20.3	49.4	123.4
WBC (10^3/mm^3)	9.64±1.38	9.96±4.43	8.86±3.02	10.6±3.25
RBC (10^6/mm^3)	8.53±0.66	9.11±1.64	8.13±0.70	8.13±1.13
HGB (g/100ml)	14.7±0.96	15.5±3.62	14.8±1.13	14.9±1.98
HCT (%)	41.8±3.47	43.8±8.59	41.0±4.23	39.3±7.50
MCV (fl)	49.0±1.51	48.0±2.38	50.3±1.49	48.1±3.08
MCH (pg)	17.3±1.07	17.1±2.21	18.2±0.60	18.3±0.80
MCHC (g/100 ml)	35.2±1.51	35.0±4.95	36.2±2.09	38.3±3.75
PLT (10^5/mm^3)	11.6±1.36	11.2±0.71	11.8±1.35	11.7±0.88
TP (g/100 ml)	6.26±0.27	6.46±0.30	6.14±0.17	6.14±0.34
Differential				
SEG (%)	13.4±5.5	11.6±5.7	20.2±2.7*	17.8±6.1
(Absolute #)	1281±600	1294±1222	1829±809	2006±1155
Lymph (%)	82.8±8.1	83.2±4.40	73.4±2.60	77.4±4.83
(Absolute #)	7989±1470	8162±3180	6475±2133	8144±2243
Mono (%)	2.6±2.7	3.4±3.2	3.4±3.1	3.4±2.7
(Absolute #)	260±303	341±307	300±297	319±166
Eosin (%)	1.0±0.7	1.6±1.5	2.8±1.3*	1.4±0.89
(Absolute #)	91±60	129±82	242±121*	131±37
Band (%)	0.2±0.4	0.2±0.4	0.2±0.4	0
(Absolute)	19±43	34±76	13.6±30.4	0
Baso (%)	0	0	0	0
(Absolute #)	0	0	0	0

*$p<0.05$

Abbreviation: WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular hemoglobin volume; MCH, mean cell hemoglobin; MCHC, mean cell corpuscular hemoglobin concentration; PLT, platelet; TP, total protein; SEG, segmented neutrophils; Lymph, lymphocytes; Mono, monocytes; Eosin, eosinophils; Band, neutrophilic band; Baso, basophil.

TABLE 6

HEMATOLOGICAL VALUES (MEAN±SD) IN MALE CD-1 MICE FED GLG-V-13 FOR 90 DAYS (N = 15)

Dose (mg/kg/day)	0	21.6	50.5	121.3
WBC (10^3/mm^3)	8.15±1.92	9.46±2.36	8.04±2.83	7.86±1.64
RBC (10^6/mm^3)	9.66±0.84	9.42±0.53	9.57±1.33	9.48±0.84
HGB (g/100ml)	15.4±1.22	15.7±0.87	15.6±1.82	15.9±1.15
HCT (%)	46.7±3.56	45.5±2.60	45.7±6.56	45.9±4.60
MCV (fl)	48.4±1.58	48.3±0.65	47.8±1.53	48.5±1.65
MCH (pg)	16.0±0.70	16.6±0.82*	16.3±0.61	16.8±0.81**
MCHC (g/100 ml)	33.1±0.67	34.4±0.87**	34.2±1.55*	34.6±1.97*
PLT (10^5/mm^3)	13.5±2.04	13.4±1.07	11.8±2.95	12.6±1.60
TP (g/100 ml)	6.61±0.36	6.72±0.30	6.39±0.50	6.47±0.33
Differential				
SEG (%)	21.1±6.67	20.3±2.73	19.5±7.60	15.9±4.66
(Absolute #)	1729±778	2012±1127	1609±1012	1163±372*
Lymph (%)	75.3±6.93	73.4±6.91	76.7±8.18	78.6±6.82
(Absolute #)	6123±1528	6871±1522	6136±2177	6139±1186
Mono (%)	2.8±2.0	4.1±2.1	3.0±1.7	4.2±3.6
(Absolute #)	231±187	396±255	236±178	352±335
Eosin (%)	0.8±0.92	2.2±2.1*	0.8±1.4	1.2±1.2
(Absolute #)	71±77	176±159*	61±99	93±84
Band (%)	0	0	0	0.1±0.1
(Absolute)	0	0	0	6±20
Baso (%)	0	0	0	0
(Absolute #)	0	0	0	0

*$p<0.05$; **$p<0.01$

Abbreviation: WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular hemoglobin volume; MCH, mean cell hemoglobin; MCHC, mean cell corpuscular hemoglobin concentration; PLT, platelet; TP, total protein; SEG, segmented neutrophils; Lymph, lymphocytes; Mono, monocytes; Eosin, eosinophils; Band, neutrophilic band; Baso, basophil.

TABLE 7

HEMATOLOGICAL VALUES (MEAN±SD) IN FEMALE CD-1 MICE FED GLG-V-13 FOR 90 DAYS (N = 15)

Dose (mg/kg/day)	0	26.7	59.9	136.3
WBC (10^3/mm^3)	6.83±2.20	7.65±2.97	6.60±2.32	7.77±2.88
RBC (10^6/mm^3)	8.80±0.85	8.33±0.77	8.51±0.85	8.17±0.87
HGB (g/100ml)	15.5±1.46	15.5±1.17	15.5±1.23	11.9±1.47**
HCT (%)	44.3±4.00	41.4±4.61	42.4±4.31	40.9±4.73*
MCV (fl)	50.3±1.70	49.7±1.74	49.8±1.73	49.9±1.92
MCH (pg)	17.7±1.19	18.7±1.05*	18.2±0.70	18.6±1.12*
MCHC (g/100 ml)	35.1±1.53	37.6±2.07**	36.6±1.63*	37.2±2.42**
PLT (10^5/mm^3)	13.1±2.05	13.1±2.04	12.3±1.91	11.1±1.92**
TP (g/100 ml)	6.47±0.28	6.49±0.38	6.40±0.33	6.23±0.33*
Differential				
SEG (%)	19.9±11.3	13.4±6.00	14.4±6.17	13.9±4.02
(Absolute #)	1371±1031	1008±571	906±352	1033±380
Lymph (%)	77.5±10.8	82.3±7.46	81.9±6.37	82.4±4.65
(Absolute #)	5273±1763	6363±2678	5455±2090	6478±2623
Mono (%)	1.2±1.4	2.9±2.3*	2.7±1.9*	2.1±1.6
(Absolute #)	84±104	195±153*	176±139*	139±78
Eosin (%)	1.4±1.4	1.3±1.4	1.0±1.3	1.6±1.5
(Absolute #)	105±148	85±92	57±74	115±103
Band (%)	0	0.1±0.3	0	0
(Absolute)	0	3.0±13	0	0
Baso (%)	0	0	0	0
(Absolute #)	0	0	0	0

*$p<0.05$; **$p<0.01$

Abbreviation: WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular hemoglobin volume; MCH, mean cell hemoglobin; MCHC, mean cell corpuscular hemoglobin concentration; PLT, platelet; TP, total protein; SEG, segmented neutrophils; Lymph, lymphocytes; Mono, monocytes; Eosin, eosinophils; Band, neutrophilic band; Baso, basophil.

TABLE 8

BIOCHEMICAL VALUES (MEAN±SD, N = 5) FOR CD-1 MICE FED GLG-V-13 FOR 45 DAYS

Dose (mg/kg/d)	BUN (mg/dl)	ALP (IU/L)	LDH (IU/L)	GOT/AST (IU/L)	GPT/ALT (IU/L)	Na (meq/L)	K (meq/L)
Male							
0	28.2±5.8	48.6±15.5	1013.8±581.1	231.6±51.3	81.4±53.1	152.2±1.3	8.0±0.5
15.6	26.0±3.9	35.6±39.2	673.0±402.2	173.6±79.1	48.0±16.1	155.0±3.5	11.8±5.1
39.0	28.4±3.9	39.4±23.1	1019.4±476.7	180.0±65.1	43.0±7.81	151.2±1.9	7.1±0.9
106.8	31.8±4.7	51.8±19.2	1189.6±368.1	203.4±74.5	48.2±10.7	153.2±1.9	7.8±0.4
Female							
0	28.4±6.1	51.8±12.2	1489.2±354.5	222.4±71.1	42.2±10.1	153.0±2.7	7.8±1.1
20.3	34.4±8.0	54.0±8.21	1173.5±289.7	223.0±70.7	40.2±6.50	152.3±1.3	7.8±1.0
49.4	34.4±7.9	46.2±11.7	980.8±295.7*	185.8±113.5	43.2±11.1	151.6±1.1	6.9±0.7
123.4	31.2±6.5	49.6±12.8	1063.4±364.8	160.8±39.3	61.0±34.6	153.2±0.8	7.9±0.9

Abbreviation: BUN, blood urea nitrogen; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; AST, asparate aminotransferase; ALT, alanine aminotransferase.

TABLE 9

BIOCHEMICAL VALUES (MEAN±SD, N = 15) FOR CD-1 MICE FED GLG-V-13 FOR 90 DAYS

Dose (mg/kg/d)	BUN (mg/dl)	ALP (IU/L)	LDH (IU/L)	GOT/AST (IU/L)	GPT/ALT IU/L)	Na (meq/L)	K (meq/L)	Alb (g/dl)	Chole (mg/dl)
Male									
0	28.0±3.6	37.2±8.43	1400.1±434.4	237.1±94.3	88.8±101.8	155.9±3.51	7.5±0.7	3.4±0.2	140±16
21.6	30.2±5.4	42.1±12.7	1482.8±321.5	246.0±94.2	66.4±29.0	154.3±3.32	8.8±2.7	3.5±0.2	139±18
50.5	27.5±3.9	37.5±8.71	1244.7±529.7	217.9±28.1	64.6±40.1	152.2±1.8**	8.1±1.5	3.3±0.2	122±19**
121.3	28.9±3.7	45.3±24.2	1449.9±289.7	233.8±59.0	82.9±83.2	152.6±3.75*	7.6±0.9	3.3±0.2	134±20
Female									
0	25.3±3.6	54.2±17.6	1398.4±207.2	216.5±9.90	44.5±9.90	153.3±3.6	6.8±0.6	3.4±0.2	96±17
26.7	28.4±3.2*	42.3±10.8*	1421.6±372.0	203.3±51.7	55.6±13.6*	152.2±7.38	6.4±0.9	3.5±0.2	98±21
59.9	30.3±5.3**	46.6±17.5	1305.4±228.8	195.5±42.6	72.8±41.0*	152.1±2.63	7.0±0.7	3.4±0.2	89±25
136.3	28.7±4.4*	46.2±11.6	1368.8±261.1	212.2±42.2	52.2±16.4	152.5±3.08	7.2±0.4*	3.4±0.2	80±16*

*p<0.05; **p<0.01

Abbreviation: BUN, blood urea nitrogen; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; AST, asparate aminotransferase; ALT, alanine aminotransferase; Alb, albumin; Chole, cholesterol.

TABLE 10

ABSOLUTE AND RELATIVE ORGAN WEIGHTS (MEAN±SD) OF MALE CD-1 MICE FED GLG-V-13 FOR 45 DAYS (N = 5)

Dose (mg/kg/day)	0	15.6	39.0	106.8
Absolute organ weight (mg)				
Brain	463.4±38.2	500.0±33.1	487.8±20.0	489.7±45.5
Heart	179.6±22.9	176.3±29.4	180.1±18.4	208.1±23.9
Liver	2032.4±136.0	1861.8±225.4	1974.8±193.3	2113.0±187.4
Kidney	523.1±11.4	519.1±76.5	486.6±28.5*	594.4±45.5*
Spleen	93.5±12.2	97.9±23.0	108.6±28.2	101.1±7.0
Testis	229.7±30.0	224.1±23.4	234.7±23.9	247.1±25.7
Epididymis	114.1±35.8	102.2±19.1	105.2±8.5	109.4±5.4
Adrenal	9.0±1.6	11.8±3.3	9.8±2.1	15.0±6.4
Lung	230.6±49.4	229.4±47.4	327.5±65.6*	285.5±58.1
Relative organ weight (mg/g brain)				
Heart	390.9±68.7	352.6±51.8	370.3±48.7	432.5±21.0
Liver	4289.8±332.1	3724.8±362.9*	4063.7±543.9	4358.9±612.0
kidney	1135.5±106.6	1035.4±102.8	999.1±76.8*	1211.8±168.9
Spleen	201.7±21.3	194.8±36.8	224.6±68.0	204.7±16.5
Testis	499.5±85.5	451.3±69.0	482.2±58.5	522.0±91.1
Epididymis	244.2±73.8	206.4±47.4	215.9±18.5	225.4±24.2
Adrenal	19.5±3.1	23.3±5.5	20.2±4.67	31.1±18.0
Lung	502.8±133.1	460.2±85.0	671.3±131.7	537.7±77.6
Relative organ weight (mg/g body weight)				
Brain	13.5±1.5	15.1±1.6	15.4±0.9*	14.2±1.7
Heart	5.2±0.6	5.3±0.6	5.7±0.5	6.0±0.8
Liver	59.3±5.8	55.8±1.8	62.2±4.9	60.8±5.2
Kidney	15.2±0.6	15.6±1.2	15.3±0.9	17.1±1.0**
Spleen	2.7±0.4	2.9±0.6	3.4±0.8	2.9±0.2
Testis	6.7±0.9	6.8±0.8	7.4±0.7	7.1±0.8
Epididymis	3.3±1.2	3.1±0.8	3.3±0.3	3.2±0.2
Adrenal	0.26±0.05	0.35±0.10	0.31±0.07	0.43±0.17
Lung	6.7±1.4	6.9±1.9	10.0±2.1*	8.2±1.6

* $p<0.05$; ** $p<0.01$

TABLE 11

ABSOLUTE AND RELATIVE ORGAN WEIGHTS (MEAN±SD) OF FEMALE CD-1 MICE FED GLG-V-13 FOR 45 DAYS (N = 5)

Dose (mg/kg/day)	0	20.3	49.4	123.4
Absolute organ weight (mg)				
Brain	514.7±11.3	496.0±42.1	502.1±20.4	514.3±21.1
Heart	167.8±14.7	160.5±19.7	192.0±79.6	167.9±17.4
Liver	1901.3±179.6	1641.9±252.9	1585.6±134*	1705.8±180.8
Kidney	405.5±19.7	350.9±38.4*	403.9±28.4	414.0±43.5
Spleen	123.1±2.3	136.9±17.0	111.0±7.1*	130.0±25.4
Ovary + Uterus	246.4±58.3	237.4±74.2	238.6±49.4	187.9±35.6
Adrenal	12.7±3.63	13.3±1.26	12.8±1.60	12.8±2.40
Lung	234.9±40.0	277.6±71.8	229.4±50.5	223.0±108.7
Relative organ weight (mg/g brain)				
Heart	326.3±32.6	323.4±27.1	385.8±170.6	326.1±25.3
Liver	3697.3±375.7	3301.0±312.0	3165.8±341*	3315.9±315.6
kidney	788.4±47.3	705.1±39.3*	806.4±78.2	804.2±68.2
Spleen	239.3±8.4	275.6±19.7**	221.7±22.7	252.2±45.3
Ovary + Uterus	478.7±110.8	485.8±171.5	474.7±94.2	365.5±70.3
Adrenal	24.5±6.80	26.9±3.76	25.5±3.43	25.0±4.42
Lung	456.8±80.8	555.3±110.1	455.9±89.2	430.2±193.4
Relative organ weight (mg/g body weight)				
Brain	16.0±0.5	16.1±0.8	16.8±1.0	16.8±0.6
Heart	5.2±0.5	5.2±0.5	6.5±3.2	5.5±0.4
Liver	59.1±5.1	53.1±6.9	52.8±3.7	55.5±3.7
Kidney	12.6±0.8	11.3±0.8*	13.5±1.4	13.5±1.1
Spleen	3.8±0.2	4.4±0.4*	3.7±0.3	4.2±0.7
Ovary + Uterus	7.7±1.9	7.7±2.4	8.0±2.0	6.2±1.3
Adrenal	0.39±0.10	0.43±0.05	0.43±0.03	0.42±0.07
Lung	7.3±1.4	9.0±2.1	7.7±1.7	7.3±3.5

* $p<0.05$; ** $p<0.01$

TABLE 12

ABSOLUTE AND RELATIVE ORGAN WEIGHTS (MEAN±SD) OF MALE CD-1 MICE FED GLG-V-13 FOR 90 DAYS (N = 15)

Dose (mg/kg/day)	0	21.6	50.5	121.3
Absolute organ weight (mg)				
Brain	481.8±45.3	492.4±59.7	480.4±38.9	535.1±32.8**
Heart	206.3±33.8	205.6±35.9	223.9±56.5	235.2±31.6*
Liver	2027.2±229.1	2100.9±166.6	1973.3±233.1	2295.1±275**
Kidney	590.9±77.2	585.5±60.7	585.2±78.6	689.1±140.9*
Spleen	101.8±18.6	116.2±29.0	99.8±17.6	146.8±130.1
Testis	251.8±49.8	268.9±51.5	268.0±40.6	274.2±55.1
Epididymis	136.8±35.5	157.1±47.8	150.0±49.0	182.1±56.0*
Adrenal	12.7±5.3	16.0±6.9	12.1±5.4	19.2±6.7**
Lung	292.8±68.7	368.4±83.4*	334.9±88.4	371.5±86.4**
Relative organ weight (mg/g brain)				
Heart	430.3±67.7	423.6±74.8	467.3±113.5	440.5±60.6
Liver	4148.1±755.3	4333.8±454.8	4115.1±421.8	4305.0±603.6
kidney	1230.4±166.7	1203.7±109.0	1221.0±145.7	1286.3±245.3
Spleen	212.0±33.6	238.9±54.3	208.2±33.8	274.8±246.2
Testis	511.9±113.0	557.2±129.3	557.8±68.6	511.1±88.3
Epididymis	299.2±89.2	320.3±87.3	310.4±90.5	341.3±107.6
Adrenal	27.7±9.9	32.7±13.4	25.0±10.3	36.0±12.4*
Lung	606.8±122.5	757.8±156.8**	698.8±178.1	692.9±154.2
Relative organ weight (mg/g body weight)				
Brain	13.4±1.3	13.4±1.5	13.9±1.3	14.7±1.4*
Heart	5.7±0.8	5.6±0.8	6.4±1.0*	6.4±0.7*
Liver	56.2±5.5	57.4±5.0	56.8±4.8	62.6±4.0**
Kidney	16.3±1.5	16.0±1.7	16.9±1.9	18.7±2.6**
Spleen	2.8±0.5	3.2±0.7	2.9±0.5	4.1±3.9
Testis	7.0±1.3	7.4±1.5	7.7±0.8	7.5±1.4
Epididymis	3.8±0.9	4.3±1.2	4.3±1.5	5.0±1.5*
Adrenal	0.35±0.14	0.44±0.20	0.34±0.15	0.53±0.18**
Lung	8.1±1.8	10.0±1.8**	9.7±2.4*	10.1±2.1**

* $p<0.05$; ** $p<0.01$

TABLE 13

ABSOLUTE AND RELATIVE ORGAN WEIGHTS (MEAN±SD) OF FEMALE CD-1 MICE FED GLG-V-13 FOR 90 DAYS (N = 15)

Dose (mg/kg/day)	0	26.7	59.9	136.3
Absolute organ weight (mg)				
Brain	499.3±54.4	517.2±29.8	490.0±37.6	509.3±33.8
Heart	197.0±27.3	189.5±19.6	196.0±33.7	185.9±31.0
Liver	1929.1±230.1	2025.3±346.1	1915.2±297.6	1914.3±238.7
Kidney	486.1±46.1	484.9±78.0	460.2±54.9	471.4±55.2
Spleen	143.9±27.4	165.4±40.6	132.4±46.3	141.7±21.5
Ovary + Uterus	398.1±91.9	364.2±82.1	315.5±65.2**	307.4±79.0**
Adrenal	19.1±3.8	20.1±5.0	16.5±3.0*	19.7±4.8
Lung	322.9±79.3	312.5±65.6	289.2±48.3	303.3±53.0
Relative organ weight (mg/g brain)				
Heart	399.2±70.1	367.2±39.7	402.2±75.0	366.1±62.8
Liver	3925.4±769.0	3932.1±749.4	3926.3±655.2	3769.4±482.7
kidney	986.9±162.9	944.4±200.2	941.10±100.5	928.2±112.1
Spleen	290.0±55.5	323.1±96.4	286.1±77.4	278.2±37.9
Ovary + Uterus	805.4±198.4	705.8±157.4	642.9±117.2*	602.8±140**
Adrenal	38.5±8.83	39.1±10.7	33.6±5.31	38.8±9.22
Lung	642.4±136.3	607.7±144.4	595.5±120.5	597.2±106.5
Relative organ weight (mg/g body weight)				
Brain	14.2±1.6	14.5±1.6	14.3±1.4	15.0±1.3
Heart	5.6±0.7	5.3±0.8	5.7±0.9	5.5±0.9
Liver	55.0±6.3	56.1±7.3	55.3±5.8	56.3±5.6
Kidney	13.9±1.3	13.5±1.9	13.3±1.3	13.9±1.4
Spleen	4.1±0.7	4.6±0.9	4.0±0.8	4.2±0.5
Ovary + Uterus	11.4±2.8	9.9±2.3	9.21±2.3*	9.0±1.9**
Adrenal	0.54±0.13	0.56±0.13	0.48±0.08	0.58±0.14
Lung	9.2±2.2	8.7±1.4	8.4±1.5	9.0±1.7

* $p<0.05$; ** $p<0.01$

Figure Legends

Figure 1. Mean body weight change (a) and cumulative body weight gains (b) in male CD-1 mice fed GLG-V-13 at doses of 0, 15.6, 39.0, and 106.8 mg/kg/day for 45 days. Points are the mean for groups of 5 mice.

Figure 2. Mean body weight change (a) and cumulative body weight gain (b) in female CD-1 mice fed GLG-V-13 at doses of 0, 20.3, 49.4, and 123.4 mg/kg/day for 45 days. Points are the mean for groups of 5 mice.

Figure 3. Feed efficiency of male (a) and female (b) mice fed diets containing GLG-V-13 for 45 days. Points are the mean for groups of 5 mice.

Figure 4. Mean body weight change (a) and cumulative body weight gains (b) in male CD-1 mice fed GLG-V-13 at doses of 0, 21.6, 50.5, and 121.3 mg/kg/day for 90 days. Points are the mean for groups of 15 mice.

Figure 5. Mean body weight change (a) and cumulative body weight gains (b) in female CD-1 mice fed GLG-V-13 at doses of 0, 26.7, 59.9, and 136.3 mg/kg/day for 90 days. Points are the mean for groups of 15 mice.

Figure 6. Feed efficiency of male (a) and female (b) mice fed diets containing GLG-V-13 for 90 days. Points are the mean for groups of 15 mice.

Figure 7. Light micrograph of the liver of a male mouse given GLG-V-13 at the dose of 121.3 mg/kg/day for 90 days. Note multifocal, randomly scattered necrosis. Similar necrosis was observed in female mice administered GLG-V-13 at dose of 136.3 mg/kg/day for 90 days.

Figure 8. Light micrograph of adrenal glands of a female mice given GLG-V-13 at the dose of 134.5 mg/kg/day for 90 days. Note the vacuolation in the X zone of adrenal glands.

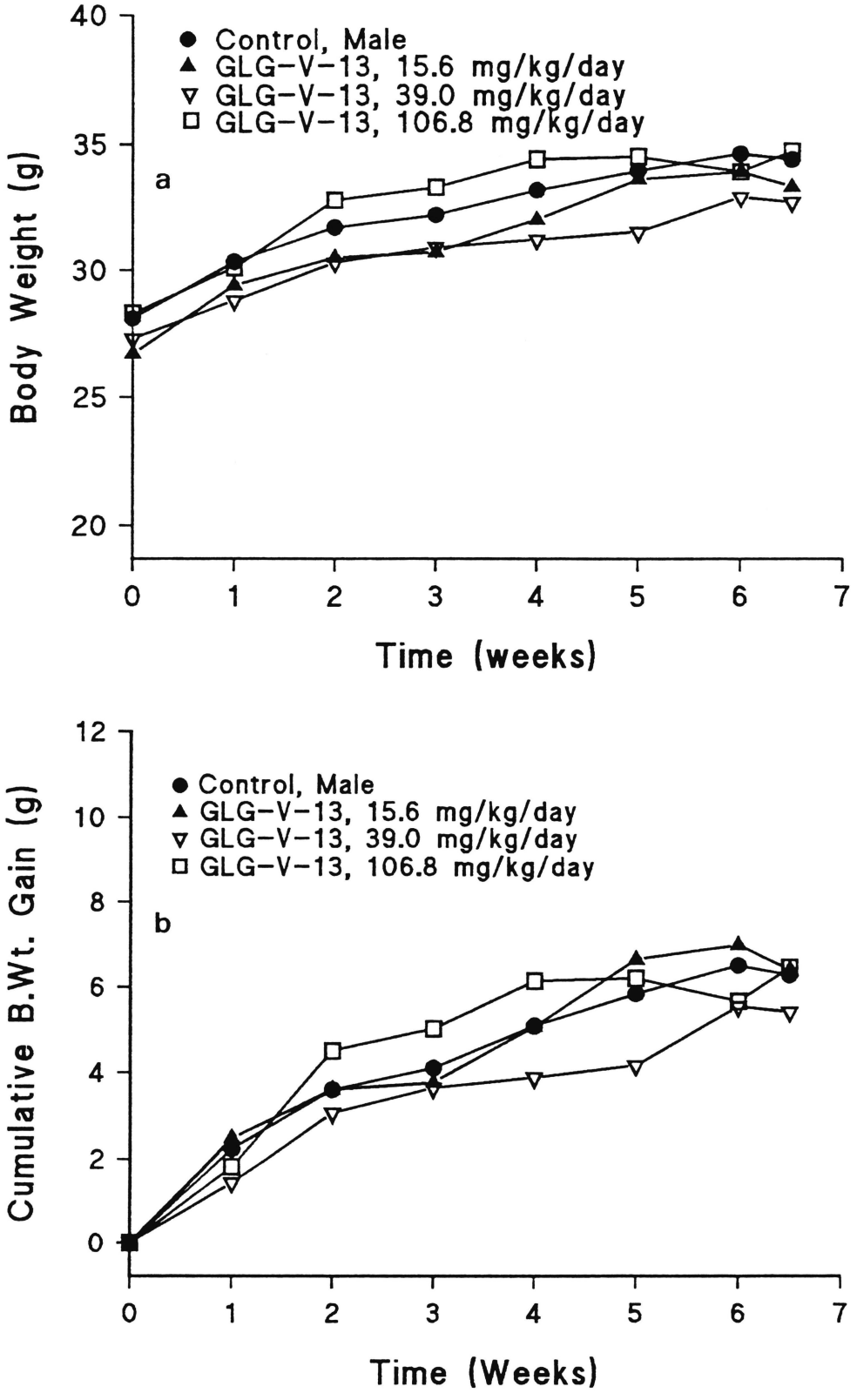

Control, Male
GLG-V-13, 15.6 mg/kg/day
GLG-V-13, 39.0 mg/kg/day
GLG-V-13, 106.8 mg/kg/day
a
Body Weight (g)
40
35
30
25
20
0
1
2
3
4
5
6
7
Time (weeks)
Control, Male
GLG-V-13, 15.6 mg/kg/day
GLG-V-13, 39.0 mg/kg/day
GLG-V-13, 106.8 mg/kg/day
b
Cumulative B.Wt. Gain (g)
12
10
8
6
4
2
0
0
1
2
3
4
5
6
7
Time (Weeks)

Figure 1

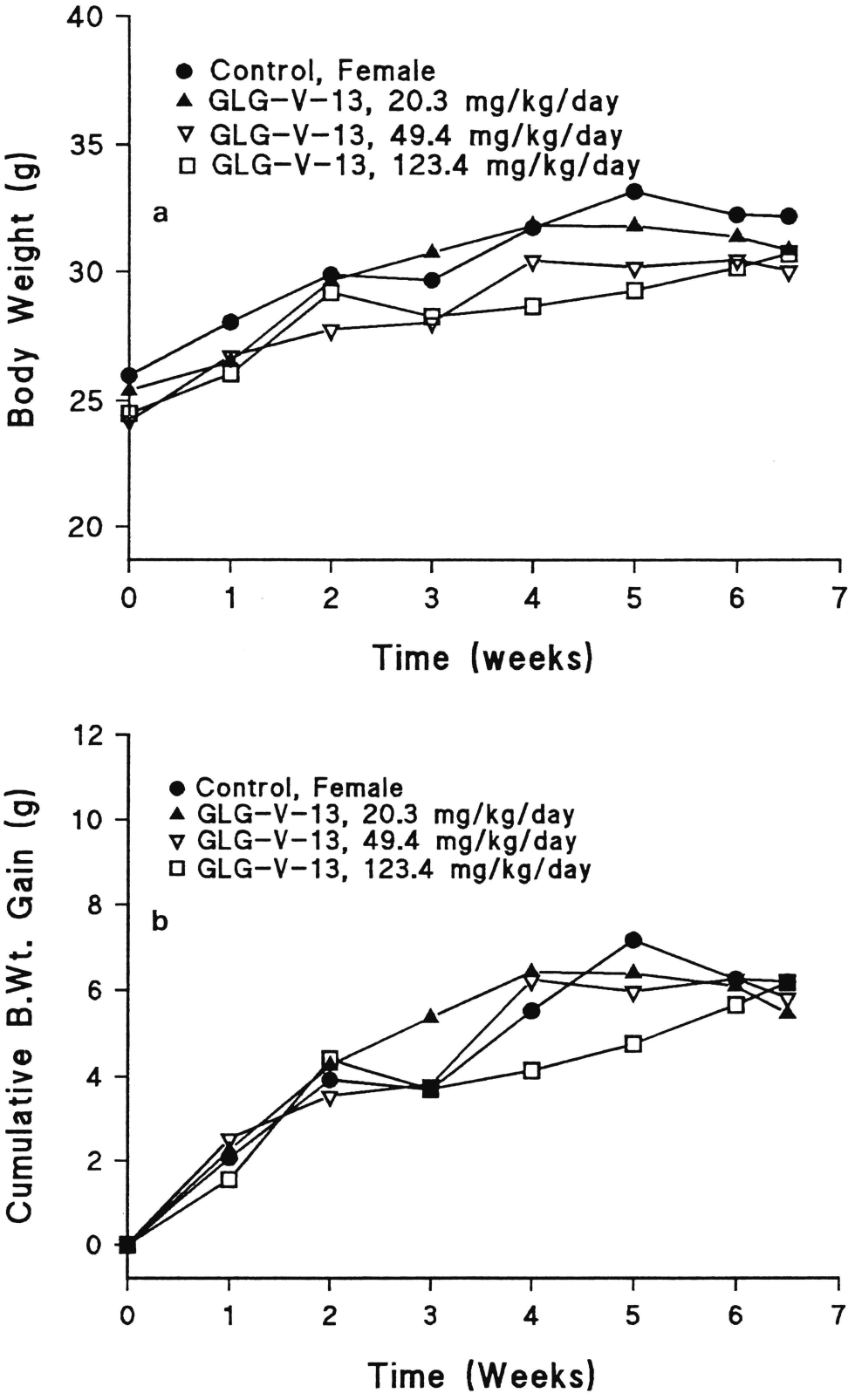
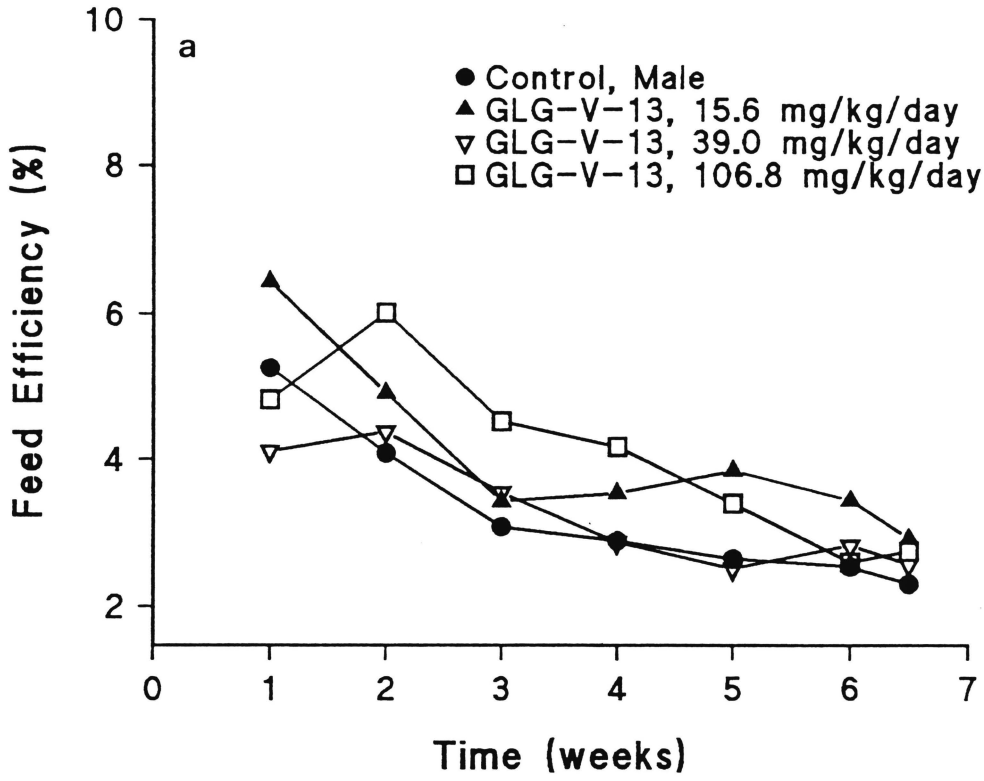

Body Weight (g)
40
35
30
25
20
● Control, Female
▲ GLG-V-13, 20.3 mg/kg/day
▽ GLG-V-13, 49.4 mg/kg/day
□ GLG-V-13, 123.4 mg/kg/day
a
0
1
2
3
4
5
6
7
Time (weeks)
Cumulative B.Wt. Gain (g)
12
10
8
6
4
2
0
● Control, Female
▲ GLG-V-13, 20.3 mg/kg/day
▽ GLG-V-13, 49.4 mg/kg/day
□ GLG-V-13, 123.4 mg/kg/day
b
0
1
2
3
4
5
6
7
Time (Weeks)

Figure 2


a
Control, Male
GLG-V-13, 15.6 mg/kg/day
GLG-V-13, 39.0 mg/kg/day
GLG-V-13, 106.8 mg/kg/day
Feed Efficiency (%)
Time (weeks)
10
8
6
4
2
0
1
2
3
4
5
6
7

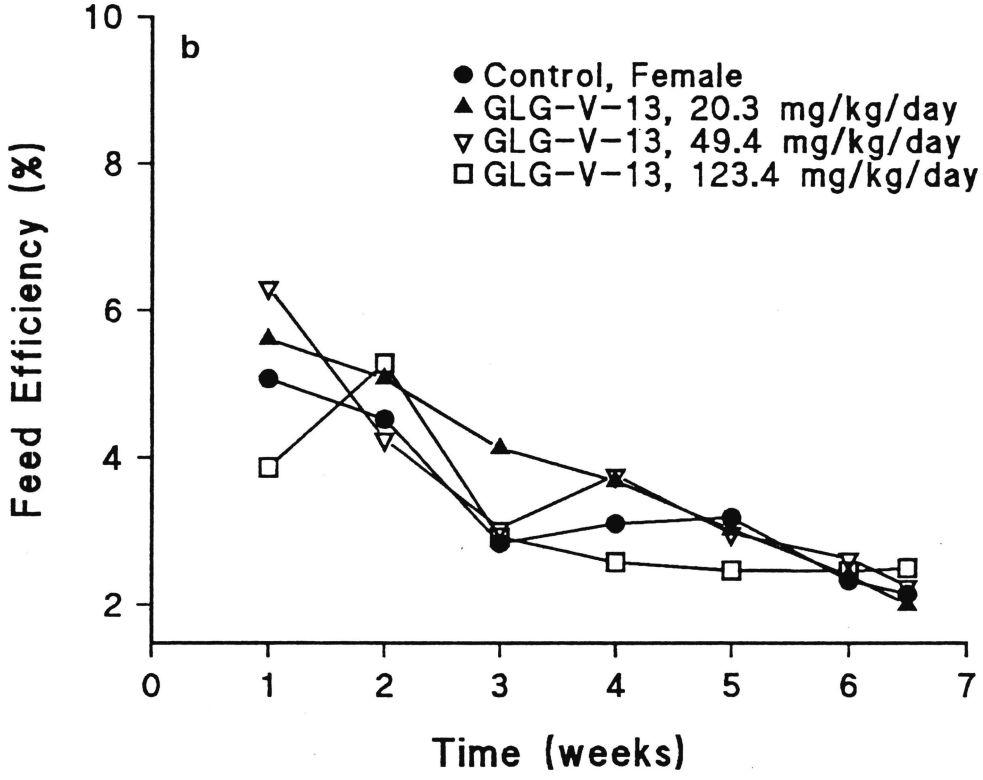

b
Control, Female
GLG-V-13, 20.3 mg/kg/day
GLG-V-13, 49.4 mg/kg/day
GLG-V-13, 123.4 mg/kg/day
Feed Efficiency (%)
Time (weeks)
10
8
6
4
2
0
1
2
3
4
5
6
7

Figure 3

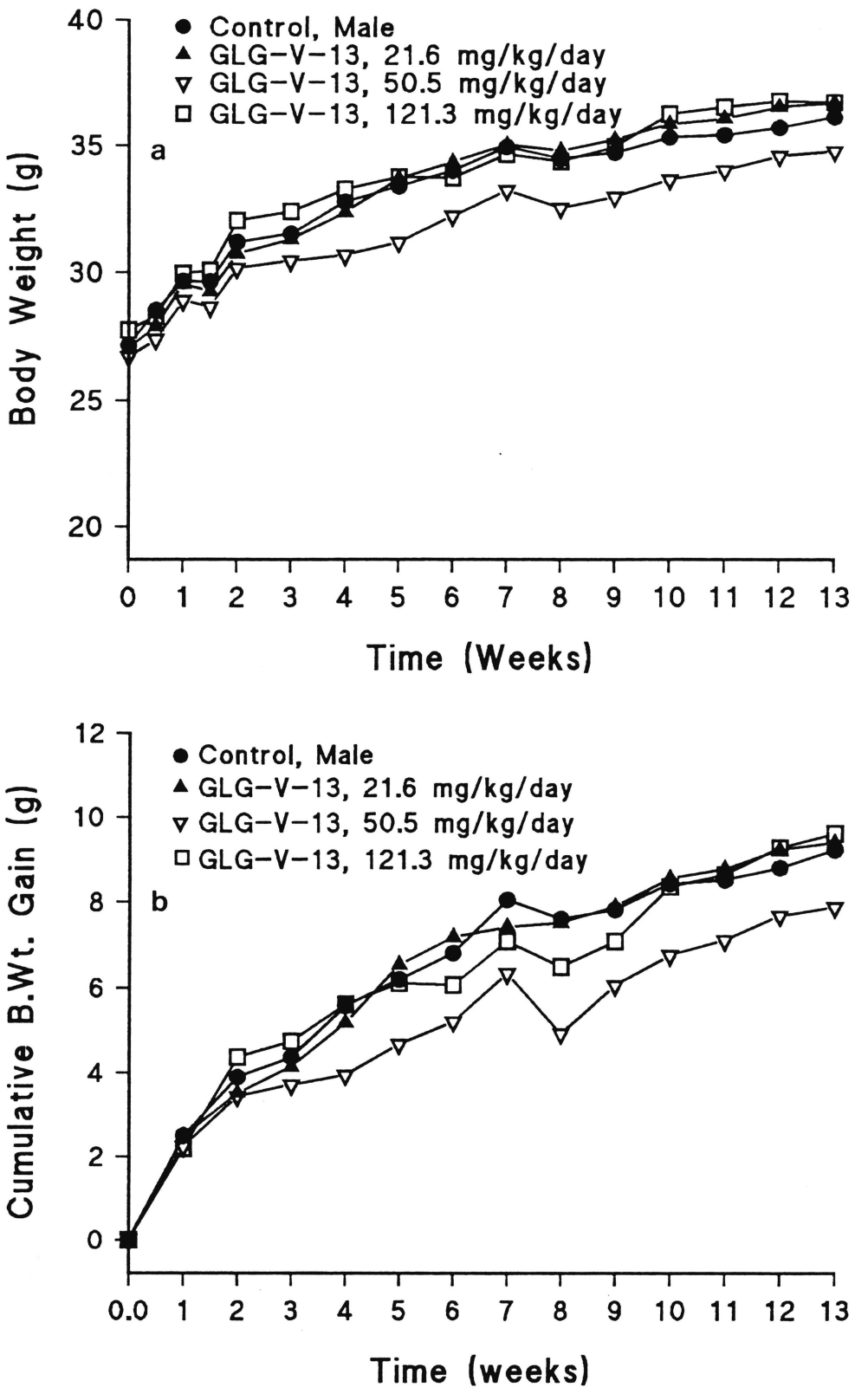

Control, Male
GLG-V-13, 21.6 mg/kg/day
GLG-V-13, 50.5 mg/kg/day
GLG-V-13, 121.3 mg/kg/day
a
Body Weight (g)
40
35
30
25
20
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Time (Weeks)
Control, Male
GLG-V-13, 21.6 mg/kg/day
GLG-V-13, 50.5 mg/kg/day
GLG-V-13, 121.3 mg/kg/day
b
Cumulative B.Wt. Gain (g)
12
10
8
6
4
2
0
0.0 1 2 3 4 5 6 7 8 9 10 11 12 13
Time (weeks)

Figure 4

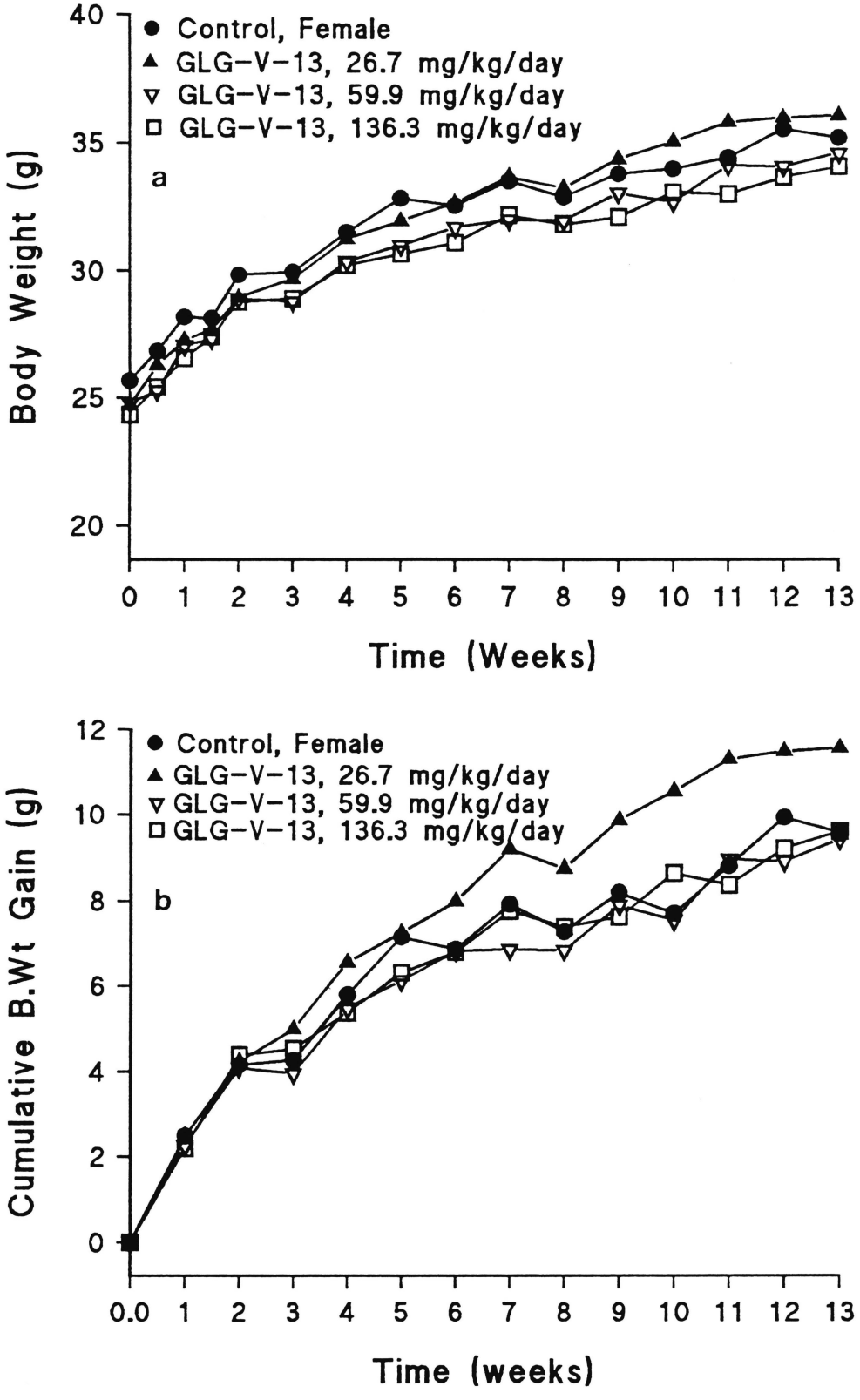

Control, Female
GLG-V-13, 26.7 mg/kg/day
GLG-V-13, 59.9 mg/kg/day
GLG-V-13, 136.3 mg/kg/day
a
Body Weight (g)
40
35
30
25
20
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Time (Weeks)
Control, Female
GLG-V-13, 26.7 mg/kg/day
GLG-V-13, 59.9 mg/kg/day
GLG-V-13, 136.3 mg/kg/day
b
Cumulative B.Wt Gain (g)
12
10
8
6
4
2
0
0.0 1 2 3 4 5 6 7 8 9 10 11 12 13
Time (weeks)

Figure 5

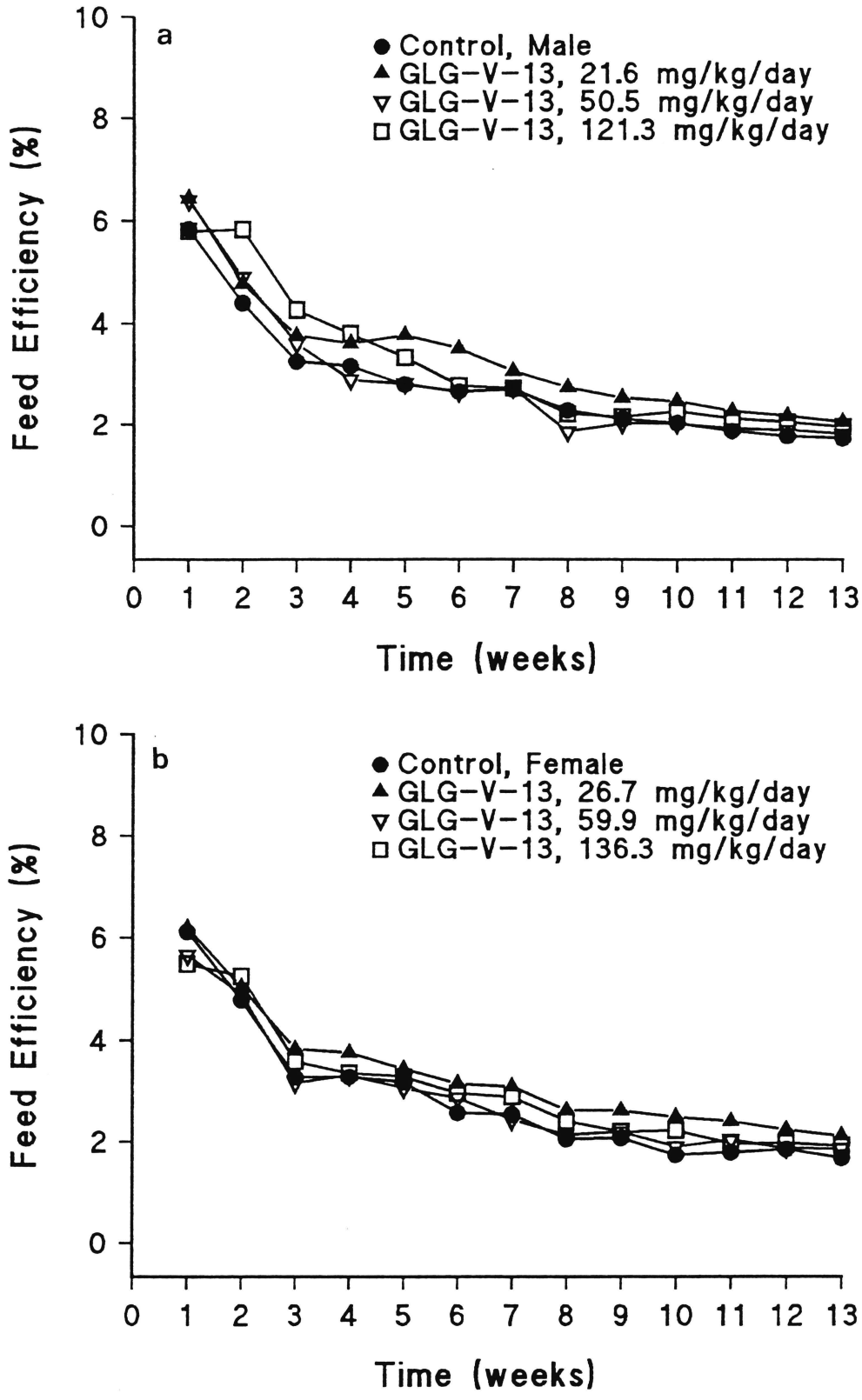

a
● Control, Male
▲ GLG-V-13, 21.6 mg/kg/day
▽ GLG-V-13, 50.5 mg/kg/day
□ GLG-V-13, 121.3 mg/kg/day
Feed Efficiency (%)
Time (weeks)
b
● Control, Female
▲ GLG-V-13, 26.7 mg/kg/day
▽ GLG-V-13, 59.9 mg/kg/day
□ GLG-V-13, 136.3 mg/kg/day
Feed Efficiency (%)
Time (weeks)

Figure 6

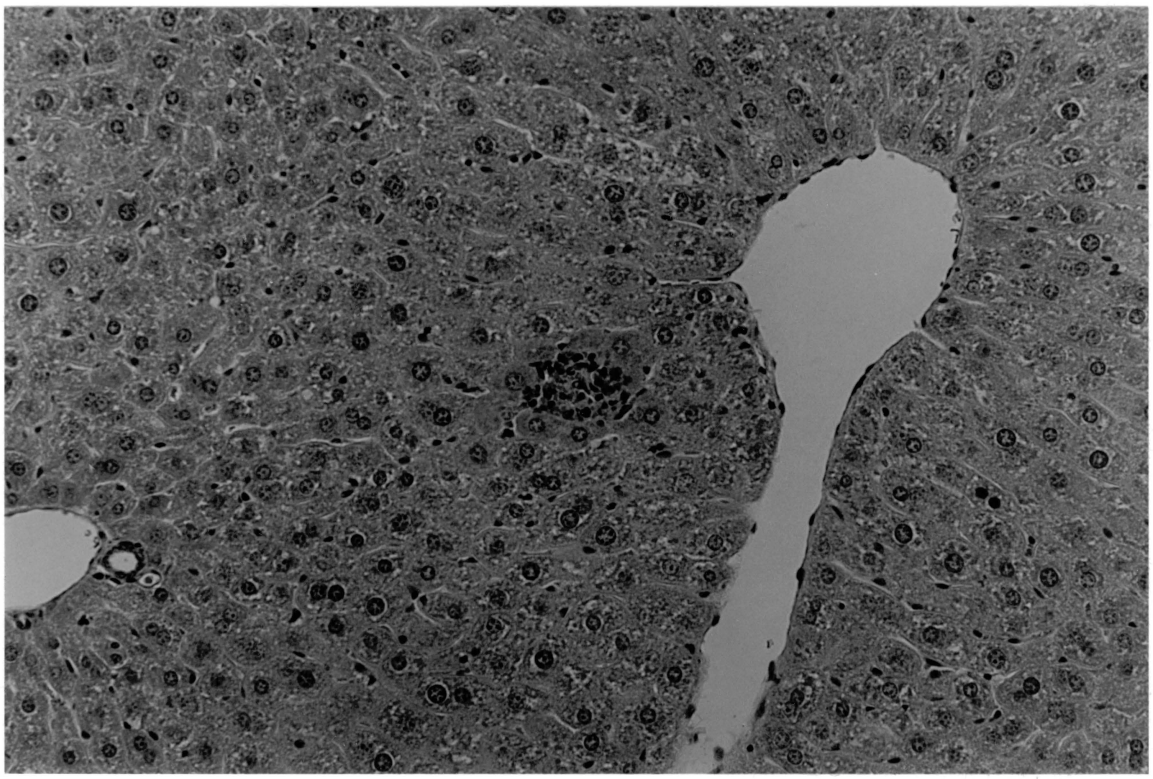


Figure 7

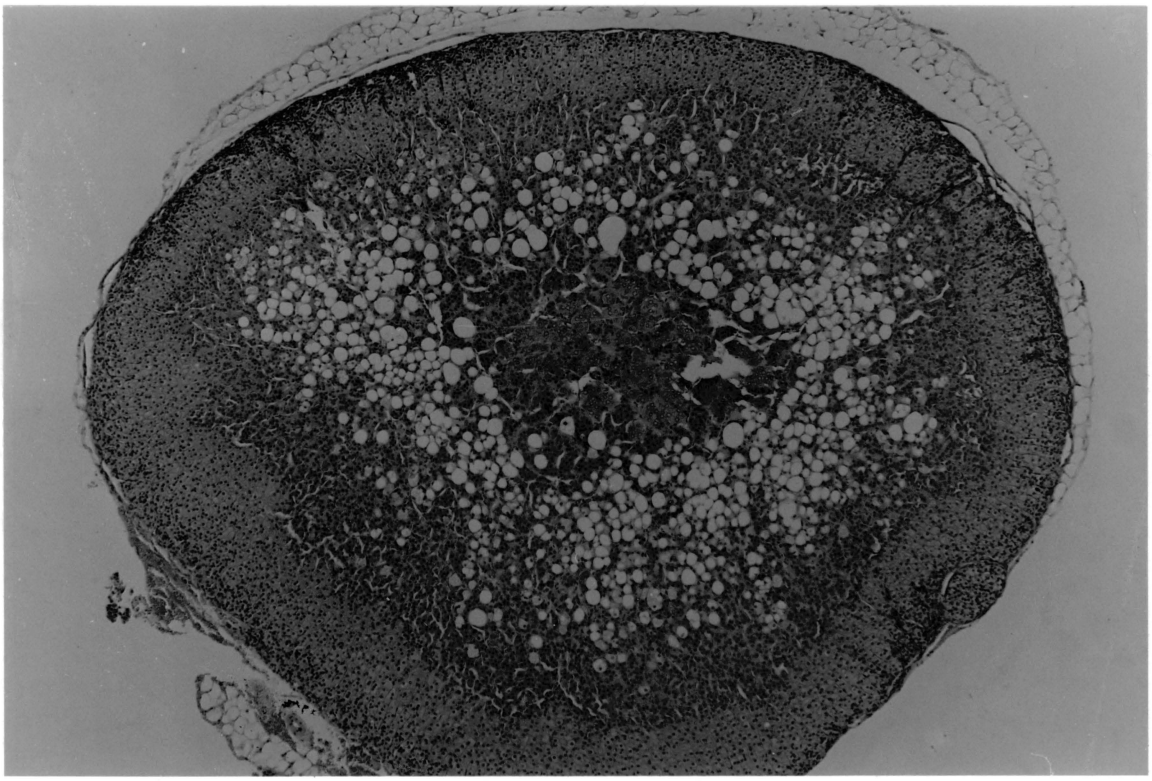


Figure 8

CHAPTER XII

SUMMARY AND CONCLUSIONS

Cardiac arrhythmia is the leading cause of sudden cardiac death. There are about 300,000-400,000 patients who die from sudden cardiac death each year in the USA. Therefore, control and prevention of arrhythmias have significant therapeutic importance. Pharmacotherapy with antiarrhythmic drugs is the most common mode of treatment. However, most currently used antiarrhythmic drugs have serious side effects and toxicities. Since the Cardiac Arrhythmias Suppression Trial (CAST) in which several antiarrhythmic drugs showed an increase rather than a decrease in mortality, reexamination of the clinical uses of most currently available antiarrhythmic drugs has revealed that most antiarrhythmic drugs are less than ideal in therapy for arrhythmias. Therefore, the search for new antiarrhythmic drugs is a continuing challenge for medicinal chemists, cardiovascular physiologists, pharmacologists, and cardiologists.

During the last decade, Berlin and his co-workers have developed a unique synthetic methodology to obtain a series of compounds classified as 3,7-deheterabicyclo[3.3.1]nonanes (DHBCN). These DHBCN derivatives were systematically screened for their potential to suppress induced ventricular arrhythmias in anesthetized dogs by Scherlag and his co-workers. Several DHBCN derivatives exhibited effective inhibitory effects on induced ventricular arrhythmias in dog models

and therefore are potential candidates for further development as newer agents for the treatment of life-threatening arrhythmias. Among the various DHBCN derivatives screened, BRB-I-28 (7-benzyl-3-thia-7-azabicyclo[3,3,1]nonane·$HClO_4$), a soluble form of BRB-I-28 (7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane·HCl), and GLG-V-13 (3-[4-(1*H*-imidazol-1-yl)benzoyl]-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane 2 $HClO_4$) showed more effective antiarrhythmic properties than lidocaine. These three DHBCN derivatives have unique properties in that they have very low proarrhythmic and minimum cardiodepressant actions. All of these properties make them worthy of further development as potential antiarrhythmic drugs.

The objectives of this thesis were to study preclinical pharmacokinetic, pharmacodynamic and toxicological profiles *in vivo* in animals and *in vitro* biological systems. Since these studies are required regulatorily for investigational new drug submission (IND) and new drug application (NDA) by the Food and Drug Administration (FDA).

Several sensitive reversed-phase HPLC methods have been developed to determine BRB-I-28 and GLG-V-13 and their metabolites in biological fluids, including plasma and urine. These HPLC methods have been fully validated and successfully used in pharmacokinetic studies.

The pharmacokinetics of BRB-I-28 was studied in dogs. The agent BRB-I-28 exhibits pharmacokinetic profiles characteristic of one compartment and two compartment open models after oral and intravenous administration, respectively. BRB-I-28 has a relatively rapid absorption rate from the gastrointestinal tract with 44.5% bioavailability and extensive tissue distribution pattern. It has a slow elimination rate, with less than 3% of BRB-I-28 being excreted in dog urine as the

parent drug. The *in vitro* and *ex vivo* plasma protein binding of BRB-I-28 was less than 30%. 7-Benzyl-3-thia-7-azabicyclo[3.3.1]nonane-3-oxide, a major metabolite and 7-benzoyl-3-thia-7-azabicyclo[3.3.1]nonane, a minor metabolite have been identified in dogs and rats. Pharmacokinetics of the sulfoxide metabolite was characterized as a 1-compartmental model in dogs.

BRB-I-28 has been demonstrated to have concentration-dependent inhibitory effects on guinea pig myocardial Na^+,K^+-ATPase and Mg^{2+}-activated ATPase activities and rat mitochondrial respiratory chain. The site of inhibition of BRB-I-28 on respiratory chain was localized between flavoprotein (FP_n) and CoQ of Complex I. In addition, it also has significant inhibition on mitochondrial ATPase activity. Inhibitory effects on ATPase and the mitochondrial respiratory chain may account for some of the electrophysiological properties and inotropic effects of BRB-I-28.

The acute and subchronic toxic effects of BRB-I-28 were investigated in mice. The estimated LD_{16}, LD_{50}, and LD_{84} for BRB-I-28 given orally was 86, 128, and 189 mg/kg for male mice and 87, 131, and 196 mg/kg for female mice, respectively. The acute toxic signs, including tremors, convulsion, twitching followed by dyspnea and apnea leading to death were of CNS in origin.

All of the mice survived in the 45- and 90-day toxicity studies. No significant toxic signs were observed during the studies. Based on the histopathology, the target organs were liver, kidney and myocardial cells in the mice fed the high dose of BRB-I-28 (77.6 mg/kg/day for male mice and 90.3 mg/kg/day for female mice for 90 days). However, the lesions in liver, kidney, and lesions in myocardial cells were mild in nature. No significant microscopic lesions were observed in any dose groups of the 45-day toxicity study and two lower dose groups of the 90-day toxicity study. No-

effect dose levels of BRB-I-28 were 30.4 mg/kg/day for male mice and 36.1 mg/kg/day for female mice.

The pharmacokinetics of GLG-V-13 were studied in dogs and rabbits. The compound exhibits pharmacokinetic profiles characteristic of one compartment and two compartment open models after oral and intravenous administration, respectively. The compound has relatively rapid absorption from gastrointestinal tract with bioavailability of 53.2% in dogs and 66.7% in rabbits. Extensive distribution was found to be the characteristic of this compound. This may result from low protein binding, accumulation in some highly perfused organs, and rapid metabolism. Several possible metabolites have been isolated from dogs treated with GLG-V-13 by HPLC system, and characterization of these metabolites is in progress. This agent has slow elimination rate with about 8-10% of GLG-V-13 excreted in dog urine as parent drug. The *in vitro* and *ex vivo* plasma protein binding of GLG-V-13 was <30%.

Compound GLG-V-13 has concentration-dependent inhibitory effects on the mitochondrial respiratory chain. The site of inhibition of GLG-V-13 on the respiratory chain was localized between flavoprotein (FP_n) to CoQ of Complex I. In addition, it also produces significant inhibition on mitochondrial ATPase activity. The inhibitory effects on mitochondrial oxidative phosphorylation may result in the depletion of ATP. This effect could possibly produce an increase in cytosolic Ca^{2+} concentration. The inhibitory effects on mitochondrial respiration may account for some of the potential toxic effects of this compound.

The acute and subchronic toxic effects of GLG-V-13 were investigated in mice. The estimated LD_{16}, LD_{50}, and LD_{84} for GLG-V-13 given orally were 319, 419, and

551 mg/kg for male mice and 278, 383, and 528 mg/kg for female mice, respectively. The acute toxic signs were of CNS in origin.

All mice survived and no overt toxic signs were observed in the 45- and 90-day subchronic toxicity studies of GLG-V-13. Based on histopathology, the target organs were liver, kidney, and adrenal gland only in the mice fed the high dose of GLG-V-13 (121.3 mg/kg/day for male mice and 136.3 mg/kg/day for female mice for 90 days). However, these lesions were mild in nature. No significant microscopic lesions were observed in any dose groups of the 45-day toxicity study and in the two lower dose groups of the 90-day toxicity study. No-effect dose levels of GLG-V-13 were 50.5 mg/kg/day for male mice and 59.9 mg/kg/day for female mice. A significant reduction in cholesterol in high dose group may be a favorable pharmacological effect of GLG-V-13. Based on these data, GLG-V-13 has a low systemic toxicity potential.

Based on the present data, both BRB-I-28 and GLG-V-13, novel antiarrhythmic agents, have favorable good pharmacokinetic characteristics such as rapid absorption rate, intermediate elimination rate, and reasonable oral bioavailability. The data derived from acute and subchronic toxicity studies indicate that both compounds are less toxic than lidocaine, one of the most commonly used antiarrhythmic drugs. With low potential for systemic toxic effects, and proarrhythmic actions, these two compounds are very good candidates for further development as antiarrhythmic agents.

Additionally, the results of this thesis research will be the basis for further and more elaborate pre-clinical pharmacokinetic and toxicokinetic studies essential for submission of Investigational New Drugs (IND) to FDA. All the methods established can be used for future clinical investigation of these potential antiarrhythmic drugs.

The data also can also be used to guide pharmacokinetic and toxicological studies and development of dosage forms and formulations in humans.

2

VITA

Chun-Lin Chen

Candidate for the Degree of

Doctor of Philosophy

Thesis: PHARMACOKINETIC, PHARMACODYNAMIC, AND TOXICOLOGICAL STUDIES ON NEW ANTIARRHYTHMIC AGENTS FROM SELECTED 3,7-DIHETERABICYCLO[3.3.1]NONANE DERIVATIVES

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Professional Experience: Assistant Lecturer, Department of Biochemistry, China Pharmaceutical University, Nanjing, July, 1986, to June, 1988; Lecturer, Department of Biochemistry, China Pharmaceutical University, Nanjing, July, 1986, to April 1991; Graduate Research Assistant, Department of Physiological sciences, Oklahoma State University, May, 1991, to July, 1994.

Honors: A Travel Fellowship, Am. Soc. Pharmacol. Exp. Ther., 1992; Distinguished Teacher Award, China Pharmaceutical University, 1991; Research Excellence Award, Jiangsu Province's Biochem. Soc., 1990.

Professional Organization: American Society of Pharmacology and Experimental Therapy, International Society for the Studies on Xenobiotics, Society of Chinese Bioscientist in America, China Pharmaceutical Society, China Biochemical Society.