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VERIFICATION OF MICROBIAL ETHANOL FORMATION IN HUMANS BY EXAMINATION OF POSTMORTEM INDOLEAMINE METABOLITES

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY

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"You've got to be very careful if you don't know where you're going, because you might not get there."

Yogi Berra

Table of Contents

Chapter 1: Ethanol and the Human Body

I. Introduction.....1

II. Ethanol Metabolism.....2

III. Postmortem Ethanol and Its Analysis.....7

A. Antemortem Ethanol Consumption and Associated Societal Problems.....7

B. Postmortem Ethanol Analysis.....8

C. Problems in Correlating Antemortem to Postmortem Ethanol Levels.....9

1. Antemortem and Postmortem Ethanol Distribution in the Body.....9

2. Postmortem Ethanol Degradation and Formation.....11

3. Case Examples.....20

D. Correlations Between Postmortem Ethanol and Antemortem Consumption...21

1. Ethanol Distribution.....22

2. Presence of Other Volatiles.....23

3. Ester Formation.....24

4. Other Methods.....25

IV. Serotonin Biochemistry and Relation to Ethanol.....25

A. Biosynthesis and Metabolism of Serotonin.....26

B. Alcohol Consumption Effect on Serotonin Metabolic Pathways.....30

C. 5-HTOL and 5-HIAA Analysis.....32

V. Current Study.....33

A. Examination of Microbial Ethanol Formation in Postmortem Tissue Specimens.....33

B. Analytical Method Development for Simultaneous Quantification of 5-HTOL and 5-HIAA.....34

C. Assignment of Postmortem Ethanol to Antemortem Consumption or Postmortem Microbial Formation.....34

Chapter 2: The Microbial Formation of Ethanol in Postmortem Tissue Specimens

I. Introduction.....35

II. Background.....37

III. Experimental Design and Methods.....40

A. Chemicals and Solutions.....40

1. Chemicals.....40

2. Sodium Fluoride Solution.....41

3. Preparation of Stock Test Solution.....41

4. Blood Calibrator Preparation.....42

5. Preparation of Internal Standard.....44

B. Sample Preparation.....44

1. Tissue Homogenization.....44

a. Specimen Preparation.....44

b. Homogenizer.....45

c. Homogenizer Cleaning.....45

d. Tissue Homogenization.....46

2. Initial Set-up.....46

3. Aliquoting for Analysis.....46

4. Sample Order.....47

C. GC Methodology.....48

1. Instrumentation.....48

2. Autosampler Parameters.....49

3. GC Parameters.....50

4. Integration Parameters.....50

5. Calibration.....51

6. Analytical Parameters.....52

D. Experimental Procedure.....54
1. Specimen Selections and Treatment.....54
2. Volatile Organic Analysis.....55
IV. Results and Discussion.....55
A. Results.....55
B. Discussion.....60
V. Conclusions.....67

Chapter 3: Liquid Chromatography Coupled with Atmospheric Pressure Chemical Ionization Mass Spectrometry

I. Introduction.....69
II. High Performance Liquid Chromatography.....71
A. Introduction.....71
B. Analyte Migration.....72
C. Column Efficiency.....75
D. Analyte Resolution.....76
E. Liquid Chromatography Summary.....77
III. Mass Spectrometry.....77
A. Introduction.....77
B. Atmospheric Pressure Chemical Ionization (APCI).....79
1. APCI Probe Assembly.....79
2. APCI Stack.....81
C. Ion Transmission and Detection.....83
1. Ion Optics.....83
2. Mass Analyzer.....85
3. Ion Detection.....88
IV. Conclusion.....90

Chapter 4: Establishment of an LC/MS Method for the Simultaneous Determination of 5-Hydroxyindole-3-acetic acid and 5-Hydroxytryptophol

I. Introduction.....91

II. Materials and Methods.....93

A. Chemicals and Solutions.....93

B. Metabolite Stock Solutions.....94

C. Mobile Phase Preparation.....95

D. β-glucuronidase Solution.....95

E. Internal Standard Stock Solution.....96

F. LC/MS Instrumentation.....96

G. LC/MS/MS and LC/MS/MS/MS Method Development.....97

H. Calibration Curves and Controls.....105

I. Specimen Extraction.....105

J. Extraction Efficiency.....107

K. Extraction Accuracy, Precision and Metabolite Stability.....107

L. Long-Term Metabolite Stability.....108

III. Results and Discussion.....108

A. Method Validation.....108

IV. Conclusion.....125

Chapter 5: LC/MS Determination of the 5-HTOL/5-HIAA Ratio in Postmortem Urine Specimens

I. Introduction.....126

II. Materials and Methods.....130

A. Specimen Selection.....130

B. Chemicals and Solutions.....132

C. Metabolite Stock Solutions.....132

D. Mobile Phase Preparation.....132

E. β-glucuronidase Solution.....132

F. Internal Standard Stock Solution.....132
G. LC/MS Instrumentation.....132
H. Calibration Curves and Controls.....132
I. Postmortem Specimen Preparation and Extraction.....133
III. Results and Discussion.....133
A. Forensic Urine Analysis.....133
B. Forensic Cases Containing Postmortem Ethanol of Unknown Origin.....145
1. Case #1.....145
2. Case #2.....146
3. Case #3.....147
4. Case #4.....148
5. Case #5.....149
6. Case #6.....150
7. Case #7.....151
IV. Conclusion.....153
References.....154

List of Tables

Table 2-1. Chemicals used and final concentrations of a Stock Test Solution.....42
Table 2-2. Concentrations of volatiles in a working stock and a blood calibrator solution.....43
Table 2-3. Typical analysis group sequence and vial positions for a determination of volatile organics.....48
Table 2-4. Specimen types used and initial ethanol concentrations determined.....56
Table 2-5. The effect of temperature on the amount of ethanol formed in specimens homogenized in 1.00% sodium fluoride.....58
Table 2-6. The effect of temperature on the amount of ethanol formed in specimens homogenized in the absence of any preservative.....59
Table 4-1. Percent Recovery ± s.d. for 5-HTOL & 5-HIAA.....119
Table 4-2. LOD, LOQ and LDR values determined for 5-HTOL & 5-HIAA.....120
Table 4-3. Intra-day accuracy and precision for repeated determinations over 8 days.....123
Table 4-4. Long-term stability of 5-HTOL and 5-HIAA.....124
Table 5-1. Distribution of cases obtained by year.....129
Table 5-2. Corroborating evidence accumulated for the 23 ethanol-positive specimens.....134
Table 5-3. Corroborating evidence accumulated for the 21 ethanol-negative specimens.....136
Table 5-4. 5-HTOL and 5-HIAA data collected for the 21 ethanol-negative specimens.....138
Table 5-5. 5-HTOL and 5-HIAA data collected for the 23 ethanol-positive specimens.....139

List of Figures

Figure 1-1. Ethanol metabolism to acetic acid.....3
Figure 1-2. Mechanism for the microbial formation of ethanol.....17
Figure 1-3. The synthesis of serotonin from tryptophan.....27
Figure 1-4. The metabolism of serotonin.....29
Figure 2-1. A representative headspace-GC chromatogram for the analysis of volatile organic compounds in a calibrator.....53
Figure 2-2. Ethanol concentration vs. time for specimen # 1.....62
Figure 2-3. Ethanol concentration vs. time for specimen # 2.....62
Figure 2-4. Ethanol concentration vs. time for specimen # 3.....62
Figure 2-5. Ethanol concentration vs. time for specimen # 4.....63
Figure 2-6. Ethanol concentration vs. time for specimen # 5.....63
Figure 2-7. Ethanol concentration vs. time for specimen # 6.....63
Figure 2-8. Ethanol concentration vs. time for specimen # 7.....64
Figure 2-9. Ethanol concentration vs. time for specimen # 8.....64
Figure 2-10. Ethanol concentration vs. time for specimen # 9.....64
Figure 2-11. Distribution of ethanol concentrations obtained for each set of forensic tissue samples homogenized in either NaF or water and stored at different temperatures over the elapsed time.....65
Figure 3-1. Representative LC system.....71
Figure 3-2. Representative LC chromatogram.....73
Figure 3-3. APCI probe assembly in the LCQ®.....81
Figure 3-4. LCQ® APCI stack components.....83
Figure 3-5. LCQ® ion optics.....85
Figure 3-6. LCQ® ion trap components.....87
Figure 3-7. LCQ® ion detection components.....89

Figure 4-1 Initial analysis of 5-HTOL and 5-HIAA pure standards incorporating a pH 6.00 mobile phase optimized for 5-HTOL ionization.....99
Figure 4-2. Initial analysis of 5-HTOL and 5-HIAA pure standards incorporating a pH 3.00 mobile phase optimized for 5-HIAA ionization.....100
Figure 4-3. Initial analysis of 5-HTOL and 5-HIAA derivatized pure standards.....102
Figure 4-4. MS/MS spectrum of 5-HTOL (*m/z* 250.1 → spectrum).....110
Figure 4-5. MS/MS/MS spectrum of 5-HTOL (*m/z* 250.1 → *m/z* 232.1 → spectrum)..111
Figure 4-6. MS/MS spectrum of 5-HIAA (*m/z* 264.1 → spectrum).....112
Figure 4-7. MS/MS/MS spectrum of 5-HIAA (*m/z* 264.1 → *m/z* 218.1 → spectrum)..113
Figure 4-8. MS/MS spectrum of 5-MMIA (*m/z* 220.1 → spectrum).....114
Figure 4-9. Probable APCI-MS fragmentation mechanism yielding the 5-HTOL-TMS MS/MS/MS quantification ion.....115
Figure 4-10. Probable APCI-MS fragmentation mechanism yielding the 5-HIAA-TMS MS/MS quantification ion.....116
Figure 4-11. Representative concatenated chromatogram of 5-MMIA, 5-HTOL and 5-HIAA.....118
Figure 5-1. 5-HTOL/5-HIAA ratio as a function of postmortem urine ethanol levels.....141
Figure 5-2. Average ethanol negative 5-HTOL/5-HIAA ratio (+ 4 s.d.) vs. range of ethanol positive ratios.....143
Figure 5-3. 5-HTOL/5-HIAA ratios for both ethanol-negative and ethanol-positive specimens and their relationship to the established 15 pmol/nmol cutoff...144

List of Abbreviations

ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
APCI	atmospheric pressure chemical ionization
BAC	blood alcohol concentration
BSTFA	N,O-bis[trimethylsilyl]trifluoroacetamide
C	Celsius
CAMI	Civil Aerospace Medical Institute
CAP	College of American Pathologists
CDT	carbohydrate deficient transferrin
CFR	code of federal regulations
CID	collision induced dissociation
conc	concentration
3-D	3 dimensional
dc	direct current
DDW	double distilled water
dL	deciliter
DNA	deoxyribonucleic acid
EC	electrochemical
ETG	ethyl glucuronide
FAEE	fatty acid ethyl ester
FAME	fatty acid methyl ester
FAA	Federal Aviation Administration
FID	flame ionization detector
g	gram
GC	gas chromatography
GGT	gamma glutamyl transpeptidase
h	hour(s)
hg	hectagram
5-HIAA	5-hydroxyindole-3-acetic acid

5-HIAL	5-hydroxyindole-3-acetaldehyde
HPLC	high performance liquid chromatography
5-HT	5-hydroxytryptamine
5-HTOL	5-hydroxytryptophol
5-HTP	5-hydroxytryptophan
i.d.	inner diameter
i.e.	for example
in	inch
kg	kilogram
kV	kilovolt
L	liter
LDH	lactate dehydrogenase
LDR	linear dynamic range
LLC	limited liability company
LOD	limit of detection
LOQ	limit of quantification
M	molar
m/z	mass-to-charge ratio
MS	mass spectrometry
MCV	mean corpuscular volume
MHz	megahertz
5-MMIA	5-methoxy-2-methyl-3-indoleacetic acid
µA	microampere
µg	microgram
µL	microliter
µm	micrometer
µM	micromolar
MEOS	microsomal ethanol-oxidizing system
mg	milligram
mL	milliliter
mm	millimeter

mM	millimolar
msec	millisecond
mtorr	millitorr
min	minute(s)
n	normal
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced)
NaF	sodium fluoride
NCI	negative chemical ionization
nmol	nanomole
PCI	positive chemical ionization
PCR	polymerase chain reaction
PDC	pyruvate decarboxylase
pmol	picomole
p.s.i.	pounds per square inch
QA	quality assurance
RF	radio frequency
RPM	revolutions per minute
RSD	relative standard deviation
sec	second(s)
sec-	secondary
SSRI	selective serotonin reuptake inhibitor
S/N	signal-to-noise ratio
s.d.	standard deviation
t-	tertiary
t	time
TMCS	trimethylchlorosilane
t_r	retention time
UAC	urinary alcohol concentration
USS	United States Ship
V	volts

Abstract

Toxicological examination of fatal aviation accident victims routinely includes analysis of ethanol levels. Two distinct questions immediately arise when considering such analyses. The first is, can we stop ethanol production immediately upon receipt of specimens. Previous research suggests such ethanol formation may be substantially diminished by addition of sodium fluoride to the specimen in question. This work, however, was only accomplished in antemortem blood and urine matrices. The current report clearly demonstrates the blockade of ethanol formation in postmortem tissue specimens by sodium fluoride at both 4°C and 25°C. The second question is if ethanol exists at the time of specimen receipt, can we differentiate between antemortem ingestion and postmortem microbial formation. Development of a single analytical approach to determine concentrations of 5-hydroxytryptophol (5-HTOL) and 5-hydroxyindole-3-acetic acid (5-HIAA), two well-known metabolites of serotonin, has provided a convenient, rapid and reliable solution to this problem. Antemortem ethanol leads to an elevation in the 5-HTOL/5-HIAA ratio for 11-19 hrs after acute ingestion. The liquid/liquid extracts of postmortem urine samples were derivatized and subjected to LC/MS for the simultaneous quantification of these two analytes, yielding detection limits of 0.10 ng/mL for each. Examination of the 5-HTOL/5-HIAA ratio was undertaken for 44 urine samples previously known to be antemortem ethanol-positive or antemortem ethanol-negative. A 5-HTOL/5-HIAA ratio of 15 pmol/nmol was shown to distinguish between these groups. Thus, we recommend the employment of this cutoff value to confirm or deny recent antemortem ethanol ingestion in postmortem urine samples.

Ethanol and the Human Body

Chapter 1

I. Introduction

It is thought that humans have had some form of ethanol available for consumption for almost as long as we have existed. There are reports that Egyptians, for example, first produced wine as early as 3500 B.C.[1] Ethanol is typically produced by fermentation, a process mediated by many different species of bacteria and yeast[2]. Fermentation is currently used by people around the world to produce alcoholic beverages for consumption.

Ethanol, one of the simplest of all alcohol molecules, is thought by many to be the cause of more societal problems in the world than any other single chemical species. Ethanol abuse is the most widespread addiction in the world, and deaths directly and indirectly attributable to ethanol increase worldwide every year.[3-5]

Our laboratory has long been interested in the metabolism of various neurochemicals, as well as the interaction of these compounds with exogenous drugs like ethanol. In combination with the Federal Aviation Administration's (FAA) Civil Aerospace Medical Institute (CAMI), we thus set out to examine the relationship between the metabolism of the neurotransmitter serotonin and ingested ethanol. This chapter provides a brief introduction to (1) ethanol distribution and metabolism in the human body following consumption, (2) ethanol analysis, (3) organisms that produce ethanol in the body after death, i.e., postmortem ethanol, (4) serotonin and its metabolism, and (5) the role serotonin metabolism may play in the assessment of ethanol origin in postmortem specimens. All of the work described within this dissertation has been associated with the general concern over postmortem occurrence of ethanol in various accidents. This entire effort, to date, has resulted in two separate papers, which have been accepted for publication, and will be published shortly.[6, 7] The reader is referred to these for more condensed presentations of the individual topics.

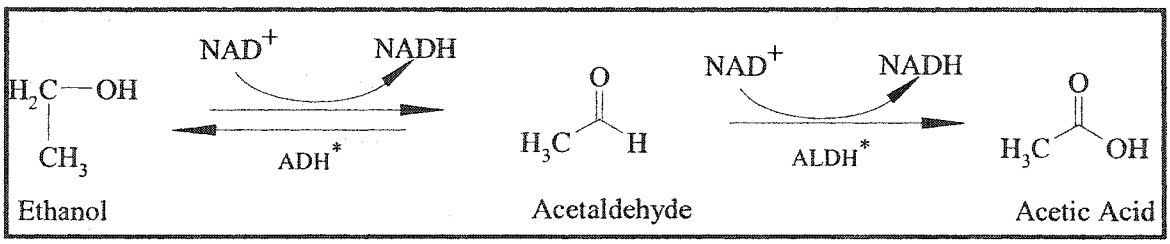
II. Ethanol Metabolism

Studies concerning the metabolism of ethanol first appeared in the literature in the 1860's. These studies reported that ethanol could be qualitatively retrieved from a subject's urine following oral consumption.[8] This fact, combined with the detection of ethanol on the subject's breath, led these early researchers to propose that ethanol was not actually metabolized in the body, but passed through to the urine and the breath unchanged. We now know that ethanol is readily absorbed into the blood following

consumption. Once in the blood, ethanol is rapidly distributed throughout the various fluids and tissues in the body almost directly in proportion to the fractional water content of each such fluid or tissue.[9-11] The rate of equilibration of ethanol between the blood and a tissue depends on the permeability of the tissue, the rate of blood flow through the tissue and the mass of the tissue.[12-14] Ethanol can freely permeate biological membranes but is practically insoluble in fats and oils. However, within only twenty minutes following consumption, ethanol is extensively distributed in an equilibrium fashion from the stomach to the brain, lung, spleen, kidney and liver.[15]

Approximately 90% of the ethanol that a person consumes is normally metabolized in the liver.[16] As can be seen in Figure 1-1, acetaldehyde is the initial product of ethanol metabolism and is produced through oxidation by alcohol dehydrogenase using oxidized nicotinamide adenine dinucleotide, NAD^+, as the cofactor.[17-22] Acetaldehyde is subsequently converted to acetic acid, by aldehyde dehydrogenase, which also employs NAD^+ as a cofactor. Acetic acid may be further metabolized to carbon dioxide and water.[8]

Figure 1-1. Ethanol metabolism to acetic acid.



* ADH: Alcohol dehydrogenase, ALDH: Aldehyde dehydrogenase

Activities for the two fundamental enzymes required for ethanol metabolism have been reported in the liver, stomach, colon, esophagus, kidney, small intestine and lung.[23-26] The small fraction of ethanol that is not metabolized in the liver or other tissues is typically excreted unchanged in the urine, in sweat glands, or as a volatile component of expired breath.[8, 27-32]

Many different countries have set limitations on the amount of ethanol that can be legally present in a person's system while in the public domain and, particularly while driving a motorized vehicle. In the United States legal levels of ethanol for drivers in blood, urine and expired breath vary from state to state. But, typical legal limits are: a urine alcohol concentration (UAC) above 100 mg/dL, a blood alcohol concentration (BAC) above 80 mg/dL, and a breath-alcohol concentration above 35 μg/dL.

Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), as noted above, are responsible for the majority of ethanol metabolism that occurs *in vivo*.[17-19] Jacobsen *et al.* first described the possible role of ADH in the oxidation of ethanol in 1951.[8] Alcohol dehydrogenase constitutes approximately 3% of the protein mass in the average human liver.[26] The elucidation of the role ADH plays in alcohol metabolism has been complicated by the more than twenty different isozymes discovered for this enzyme.[33] For simplification, the numerous different isozymes of this enzyme have been separated into five general ADH classes.[24] These classes of enzymes are differentiated according to their functional components, their K_m values for interaction with substrate and susceptibility to inhibition by 4-methylpyrazole.[33] Class I ADH is the most common form of ADH found in humans. This class typically consists of isozymes composed of any two of the α, β_1, β_2, γ_1 and γ_2 subunits, and is completely inhibited by

4-methylpyrazole (K_i = 0.2 μM).[33] Class I ADH is unique because it is the only class of ADH enzymes that can catalyze the oxidation of methanol in addition to the usual oxidation of ethanol by ADH.[33] Class I ADH is also the primary agent for liver metabolism of ethanol in humans. It has a K_m for ethanol of 4 mM, the lowest of any ADH class.[24] Class II ADH, discovered in 1977, consists of a single isozyme representative which contains two *Π* subunits. This isozyme is often referred to as simply, *Π*-ADH.[34, 35] *Π*-ADH has a K_m for ethanol that is approximately 4x times larger than that of class I ADH (K_m = 34 mM). Class II ADH is also less susceptible to inhibition by 4-methylpyrazole (K_i = 2 mM) than is class I ADH.[36] In the liver of some people, particularly alcoholics, up to 40% of all ethanol oxidation occurs via Class II ADH, whereas class II ADH is typically missing in oriental populations.[34] Like class II, class III ADH consists of only one isozyme. However, in this case, the isozyme is labeled as χ-ADH. The isozyme for χ-ADH has two χ subunits and exhibits a K_m of 3 M. χ-ADH is unique among ADH isozymes because it is not inhibited at all by 4-methylpyrazole.[33] Class IV ADH is commonly referred to as μ-ADH has a K_m = 18 mM and, like class II, is less sensitive to 4-methylpyrazole inhibition (K_i = 2mM) than is class I ADH.[24] It is also interesting to note that μ-ADH has only been reported in the stomach. While little is currently known about class V ADH, it has been partially characterized and is known to have a K_m for ethanol of approximately 30 mM and a K_i of 320 μM. The fraction of each of these five classes of ADH in the human liver varies greatly from person to person. The percentage of each ADH class depends on both genetics and the current state of health.[37] It is also common for the fractional content of the five different classes of ADH to change in an individual over time. Such changes

have been shown to occur as a result of an increased exposure to alcohol or a change in the status of the person's health. In addition to the oxidation of ethanol, class I ADH can catalyze oxidation of other alcohols such as methanol and 5-hydroxytrytophol,[38] and human class I ADH can catalyze the reduction of aldehydes.[39]

Aldehyde dehydrogenase catalyzes the oxidation of acetaldehyde to acetic acid. This metabolic reaction is vital in humans due to the severe toxicity of acetaldehyde.[40] ALDH activity is highest in the liver.[41] This enzyme has, however, been detected in blood and many other tissues including stomach, esophagus, lung and kidney.[42] Like the ADH group of enzymes, ALDH can also be broken up into 5 different classes due to the numerous different isozymes that have been characterized. The ALDH isozyme fractional composition of each individual varies by ethnicity, environment and gender.[24] An ALDH isozyme is classified according to its K_m for acetaldehyde. ALDH classes I, II and V have K_m values for acetaldehyde in the low μM range and are known as the low-K_m forms. Class I, class II and class V ALDH have K_m values for acetaldehyde of 30 μM, 3 μM and 50 μM respectively.[24] High-K_m forms of ALDH, which includes classes III and IV, have K_m values for acetaldehyde of 83 mM and 5 mM respectively.[24]

Two other hepatic enzyme systems, the microsomal ethanol-oxidizing system (MEOS) and catalase, are known to interact with ethanol.[43] However, under typical conditions the interaction of both of these systems is relatively minor compared to the ADH and ALDH systems. Thus, these are not discussed further here.

III. Postmortem Ethanol and Its Analysis

A. Antemortem Ethanol Consumption and Associated Societal Problems

The field of forensic toxicology is primarily concerned with the analytical identification and quantification of exogenous chemicals in postmortem specimens and correlating the compounds and levels to related antemortem consumption(s). Many such exogenous chemicals are not inherently harmful to the subject, and could be as relatively harmless as the common aspirin. The analyst does, however, pay particular attention to substances that can, and do, cause impairment and/or death when misused. Ethanol determination is the single most commonly requested test at the time of autopsy.[44] Small amounts of ethanol are routinely consumed on a daily basis without the knowledge of the consumer. In fact, ethanol has been reported to be present, although in very small amounts, in most bottled carbonated beverages, including bottled sparkling water.[45] However, the more common means of ethanol introduction involves direct and intentional consumption of alcoholic beverages. And, unfortunately, the most significant drug problem in the United States today is alcohol abuse.[46] In the United States, approximately "50% of fatal automobile accidents involve drinking drivers, 60% of the pedestrians killed in accidents have significant levels of alcohol in their systems, 50% of murder victims test positive for ethanol and 35% of suicide victims test positive (for ethanol)."[47] Other studies have shown that 50% of all violence is associated with alcohol misuse.[48] Experiments have also shown that human errors in aircraft simulators increase dramatically when ethanol is introduced, even at blood levels as low as 25 mg/dL.[49, 50] With these well-known undesirable affects of alcohol, it is no surprise that postmortem

ethanol determinations are the most commonly used chemical results in litigation of both criminal cases and civil lawsuits involving wrongful death.[51]

B. Postmortem Ethanol Analysis

The most common technique employed in the measurement of ethanol in postmortem specimens combines a headspace gas chromatograph with a flame ionization detector (GC-FID).[52, 53] This approach provides a low limit of quantification, typically 0.02-0.05 mg/dL, for ethanol and many other volatile organic compounds. Additionally, GC-FID typically requires little sample, which is beneficial when dealing with postmortem specimens, which may be available only in limited quantities.

Many different specimen types have been shown to be applicable to postmortem ethanol analysis. This is fortunate, particularly when the circumstances leading to death may provide samples limited both in size and diversity. Fatalities due to violent automobile and aviation accidents, for example, often lead to severely limited sample availability. Blood and urine are the two most commonly analyzed postmortem specimens with blood initially being the most sought after sample type.[12, 51, 54] If neither blood nor urine are available, however, a variety of other fluids and/or tissues may be subjected to analysis. The other fluids and/or tissues most frequently analyzed for ethanol concentration are vitreous humor, kidney, liver, brain, skeletal muscle and lung.[55-57] Bile is another body fluid that has been occasionally used for ethanol analysis.[52] And, more recent publications have shown that postmortem ethanol concentrations may even be measured in hair[58], bone-marrow[59, 60] and synovial fluid.[61]

C. Problems in Correlating Antemortem to Postmortem Ethanol Levels

1. Antemortem and Postmortem Ethanol Distribution in the Body

Even when ingested with substantial amounts of solid foods, the blood ethanol concentration reaches maximal levels within approximately two hours following ingestion. A subsequent decline to approximately 50% of the maximal values requires an additional 2-4 hours. Decay to virtually undetectable levels typically occurs approximately 8-10 hours after consumption.

The ethanol level increases seen in blood following ingestion occur in a correlated fashion in all other tissues and fluids in the body. Thus, all tissues and fluids experience virtually identical patterns of rises and falls in ethanol levels following ingestion. This leads to an expectation of correlated values for any and all such tissue and/or fluid samples from a single person at the same point in time as a result of antemortem ingestion only. The actual values in individual tissues and fluids are associated with the water content of each. However, as an example, it has been shown that a blood alcohol content of 100 mg/dL would be correlated to the following ranges of values for other tissues and fluids: urine and vitreous humor, 110-130 mg/dL; liver and kidney, 65-80 mg/hg; skeletal muscle and lung, 60-75 mg/hg; and brain 50-65 mg/hg.[15] Thus, multiple individual samples from a single body having ethanol contents (mg/dL for fluids and mg/hg for tissues), which varied by factors greater than 5 or 10 would be quite unusual under normal circumstances and would indicate some problems with the analyses and/or other effects.

The re-distribution of ingested ethanol following death is another factor that can influence the interpretation of ethanol concentrations obtained from postmortem samples.

It has previously been shown that postmortem diffusion of ethanol from the stomach can greatly increase the blood alcohol level of an individual and, therefore, lead to elevated blood results.[62-64] It was once thought that blood sampled from intact chambers of the heart or from the femoral vein was not susceptible to contamination due to postmortem diffusion and could reliably indicate antemortem ethanol. Pounder *et al.*, however, showed a large degree of variation in ethanol diffusion which has invalidated this former assumption.[62] Pounder *et al.* particularly showed that, in cases involving a high concentration of ethanol in the stomach, ethanol concentrations determined via blood analysis were simply not reliable, regardless of where the blood was sampled.[62] Postmortem diffusion of ethanol from the stomach to other organs is another factor that must be considered. This phenomenon occurs because of the high solubility of ethanol in water. The organs of the body are generally composed of between 70% and 80% water. Therefore, ethanol dissolved in water can readily diffuse from the stomach, esophagus or mouth to other organs after death.[65] The organs most affected by diffusion from the stomach, esophagus or mouth are obviously those of the nearby abdomen and the thoracic cavity. In cases where advanced stages of putrefaction exist postmortem, organ to organ ethanol diffusion has been shown to exhibit time, temperature and distance dependencies.[65]

These antemortem and postmortem distribution factors alone raise concerns when attempting to correlate ethanol measurements from a single fluid or tissue sample from a person with the associated antemortem ingestion levels. But, as seen in the next section, those are not the only difficulties encountered in establishing this correlation.

2. Postmortem Ethanol Degradation and Formation

Ethanol can be both created and destroyed in human tissue and fluid samples postmortem. While the degradation is typically viewed as contributing very little to postmortem alterations in this chemical, biosynthesis by microorganisms can and does greatly effect the levels of postmortem ethanol. We will briefly discuss postmortem ethanol degradation followed by a more extensive discussion of microbial ethanol formation postmortem.

Ethanol concentrations in the body have been shown under selected conditions to be capable of decreasing between the time of death and autopsy. Two different mechanisms have been identified which lead to the postmortem loss of ethanol: (1) ethanol oxidation by oxyhemoglobin and (2) consumption of ethanol by microbes and yeast. Oxidation of ethanol by oxyhemoglobin is known to be relatively insignificant, typically involving less than 1% of the ethanol present at the time of death.[66] However, some microbial species have been shown to be capable of eliciting substantial ethanol oxidation. These microbes include *P. putida*, *S. marcescens* and some individuals from the *Enterobacteriaceae* family.[21, 66-68] It has been shown, for example, that a combination of *P. putida* and *S. marcescens* decreased the ethanol concentration in a sample of blood from 51 mg/dL to 0 mg/dL in 28 days when stored at 4°C without any preservative.[66] However, Dick *et al.* showed that postmortem loss of ethanol caused by *P. putida* could be easily prevented by the simple addition of a 2.00% sodium fluoride solution to the sample upon collection.[66] This preservative addition was effective for samples stored at 4°C or room temperature for up to 49 days. The same report indicated that sodium fluoride prevented the action of *S. marcescens*, but in this case the sample had to be

stored at 4°C.[66] In short, proper handling of postmortem fluid and tissue samples can effectively eliminate the loss of ethanol during storage following sampling.

The microbial formation of ethanol in postmortem specimens is potentially the most complicating factor that has been encountered when attempting to correlate postmortem levels to antemortem ingestion. The first report demonstrating the presence of microbes in deceased individuals was published in 1895.[69] In 1936 M. Nicloux, a French scientist, reported the then unexpected postmortem formation of ethanol in corpses.[70, 71] In this study Nicloux examined five bodies at 0.5 to 3.5 months after death. At least three of the deceased individuals, due to age, personal habits and other data, were reasonably established to have consumed no alcohol prior to death; however, all of the corpses ultimately exhibited substantial amounts of ethanol in each of the specimens examined with an average ethanol tissue concentration of 80 mg/hg.[71] Subsequent reports in the late 1960's through mid 1970's became concerned with identifying the source(s) of this postmortem ethanol formation.[72-74]

D.J. Blackmore reported in 1968 on an aviation accident, which resulted in the death of two pilots.[72] While the likelihood that the pilots had consumed ethanol in the 24 hours prior to their death was established to be extremely small, he showed that both pilots unquestionably tested positive for ethanol in each of the blood specimens examined with levels ranging from 96 to 153 mg/dL.[72] Blackmore cultured the blood of the two pilots and discovered that the samples were contaminated with at least five different species of bacteria.[72] Each species of bacteria was then incubated individually with muscle tissue from the pilots, and even greater amounts of ethanol were produced in these incubated tissues.

In 1970 Bogusz *et al.* performed experiments similar to the incubations previously performed by Blackmore. However, these investigations carefully examined the postmortem concentration changes for compounds known or suspected to be either precursors to, or metabolites of, ethanol.[73] From their experiments Bogusz *et al.* determined that peak ethanol concentrations were observed between five and fifteen days after death, depending on the storage conditions of the specimen. These workers also reported that a primary, if not the most important, factor in the amount of ethanol formed was the amount of glucose present in the specimen at the time of death.[73] This observation linked the microbes present in the sample with the most probable primary substrate required for the production of postmortem ethanol.

In 1973 Blume *et al.* demonstrated that a putrefied postmortem blood specimen was capable of microbial production even while being stored at 4°C following collection.[74] Prior to publication of this manuscript, it was widely accepted in the forensic community that simple storage at 4°C for any postmortem specimen would completely prevent microbial formation of ethanol. Also up to this time, it was widely accepted that microbes could only be found in the intestinal tract and the urethra.[75] However, in 1974, an article by Koneman *et al.* clearly demonstrated the existence of many different microorganisms found in many different regions of the body.[75] Many of the microbes found by Koneman *et al.* had not been previously identified, and it was not immediately known whether these species could contribute to postmortem ethanol production or not. These two papers both led to a reconsideration of storage conditions and greatly increased the number of microbes which could be considered responsible for postmortem ethanol formation.

From all the above reports, published over a period of six years, four important points were established: 1) Ethanol can be produced in human tissues and fluids after death, 2) microorganisms were the agents most likely responsible for such postmortem ethanol production, 3) glucose is the substrate most probably used by the microbes for postmortem production of ethanol, and 4) storage of a specimen at 4°C does not necessarily eliminate postmortem ethanol formation.

Due to the above reports, others began to investigate exactly which microorganisms could be implicated in postmortem ethanol formation. According to a study published in 1996 by O'Neal *et al.* there are at least 99 species of mold, bacteria and yeast that are capable of producing ethanol.[47] *Candida albicans* is the microbe most often cited as being primarily responsible for the postmortem production of ethanol in humans.[76-78] This species of yeast is commonly found throughout the body with the highest concentrations located in the mouth and on the skin.[79] The human colon alone typically contains hundreds of different species of bacteria and can house up to one hundred trillion individual bacteria at any given time.[80, 81] *Escherichia coli* and *Proteus vulgaris* represent at least two species of bacteria found in the intestinal tract of humans that are capable of producing significant amounts of ethanol under the proper conditions.[79] Three other relatively common microbes shown to be capable of producing ethanol are *Enterococcus*, *Candida tropicalis* and *Klebsiella oxytoca*.[82] It has been demonstrated that microbes can redistribute themselves within the body after death.[83] Microbe penetration into the blood postmortem considerably increases the ability of that microbe to contaminate virtually every other part of the body. The migration of microbes throughout the body has been shown to occur very quickly. In fact, Robertson *et al.*

found that bacteria could travel throughout the entire body within hours following death if the body was not immediately refrigerated.[83]

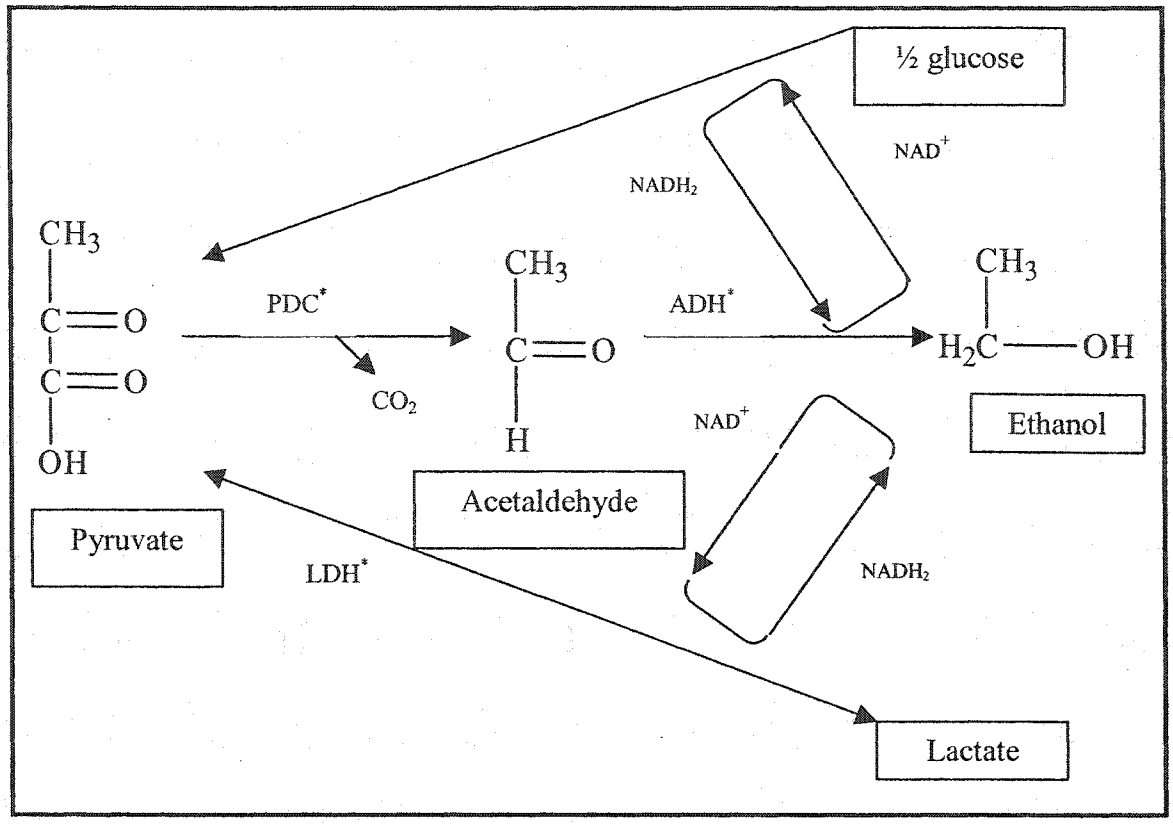
The quantity of postmortem ethanol that can be produced by microorganisms has been found to be substantial by comparison to pre-existing antemortem levels. For example, the *Mucor* species of mold is commonly found in decaying organic matter and human waste, and is a likely contaminant of a deceased body. This mold, when cultured under optimum conditions, was found to produce up to 4000 mg/dL of ethanol, 50 times the legal limit in blood for drivers.[84] Other ethanol production experiments have demonstrated that *C. albicans* has the ability to produce ethanol at concentrations up to 788 mg/dL, nearly ten times the legal limit in blood for drivers.[13, 85] These experiments demonstrate that the upper limit of ethanol production by microbes is indeed extremely high.

The number of individual microbes necessary to produce enough ethanol to significantly alter the results of a postmortem ethanol analysis has also been previously examined. The amount of ethanol that may be produced is limited by the amount of substrate available for microbes to consume. Harper *et al.* reported that, with sufficient substrate, a microbial concentration of 1×10^3 microbes/mL of body fluid could increase ethanol concentration by as much as 400 mg/dL.[86] Corry suggested that 1×10^5 microbes/mL would be required to increase the ethanol concentration by 400 mg/dL.[71] In a study undertaken to determine the number of microbes contained in a typical deceased body, Rose and Hockett found an average of 3×10^6 microbes/mL of body fluid at twelve hours after death, a reasonable time frame for the collection of specimens after an aviation accident.[69]

It is clear that microbes are present in the body prior to death, and the populations of the microbe species of concern increase after death. It is also clear from the studies discussed above that microbes have the ability to move throughout the body. And, many of the species present have the ability to produce ethanol. The mechanism by which this ethanol is produced, the time required for production, the substrates necessary for production and the distribution of these substrates are topics that have each been addressed and are now considered to be reasonably well resolved.

The most common pathway currently accepted to be used by bacteria to produce ethanol is shown in Figure 1-2.[87]

Figure 1-2. Mechanism for the microbial formation of ethanol



* PDC: pyruvate decarboxylase, ADH: alcohol dehydrogenase, LDH: lactate dehydrogenase

As can be seen in Figure 1-2, glucose and/or lactate is converted to pyruvate, which is then converted to acetaldehyde, and finally to ethanol. This is known as the Embden-Meyerhoff pathway[71]. Many different species of bacteria and yeasts are known to contain enzymes with alcohol dehydrogenase activity, which are utilized for the conversion of acetaldehyde to ethanol.[88] Nanikawa *et al.*, in a study published in 1988, showed that inhibiting alcohol dehydrogenase with 4-methylpyrazole inhibited the formation of ethanol by bacteria in postmortem blood samples, providing further

evidence for the use of the Embden-Meyerhoff pathway in ethanol formation by microbes.[89]

The conversion of pyruvate to ethanol can begin within hours of death[71]. Most of the pyruvate used for the production of endogenous ethanol comes from glucose[69,71]. It has been shown, however, that even in the absence of glucose ethanol can still be formed.[73] Lactate, as shown in Figure 1-2, as well as select amino acids, such as alanine and serine can be converted to pyruvate and, thus, they too can be considered precursors to endogenous ethanol in the absence of glucose.[73] Mannitol, a water soluble carbohydrate routinely used as a diuretic in Norway and Sweden, has been shown, as an alternative substrate, to yield higher concentrations of microbial produced ethanol than glucose.[90] Ethanol has also been produced using the sugars galactose, maltose, sucrose and lactose.[72, 91] Total sugar concentrations in blood can be as high as 1.00 g/dL.[72] This concentration of sugar provides a typical ethanol-producing microbe with more than a sufficient amount of substrate to form very substantial amounts of postmortem ethanol.

There were reports promoting the effectiveness of utilizing the somewhat isolated body fluids such as urine and vitreous humor for the determination of ethanol concentration due to the belief that these two fluids were relatively immune to postmortem ethanol production.[86, 92-94]

Urine, primarily because of the way that it is stored in the body, was widely considered resistant to microbial contamination, and therefore, a good candidate for ethanol analysis. The bladder is a closed system, receiving input from the kidneys and expelling urine externally as required. There is no substantial contact between the interior of the bladder and blood under normal circumstances. After death, unless the

bladder is ruptured due to the trauma, it was argued to remain a closed system relatively free from contamination. Following this logic, urine appeared to be a desired sample source for ethanol screening in postmortem cases. It is, however, known that glucose levels in urine can be elevated dramatically due to several different ailments such as diabetes, Cushing's syndrome, gastrectomy, pancreatic diseases, alimentary glycosuria, hyperthyroidism, infection, asphyxia, general anesthesia, brain tumors, myocardial infarction, cerebral hemorrhage, obesity and glycogen storage disease.[91] It is also known that glucose is excreted into urine when plasma concentrations exceed what the kidneys are capable of processing.[95] Under normal circumstances urine contained in the bladder is sterile, but microbial contamination of the bladder can occur under other circumstances through migration of microbes from the urethra or periurethral tissue.[91] A simple urinary tract infection, for example, can introduce staphylococci, streptococci and/or enteric bacilli into the bladder.[82] If an infection occurs while the individual suffers from one of the glucose producing ailments listed above, ethanol may be produced antemortem through urinary fermentation.[96] Thus, it is obvious that if either bacteria or yeast has colonized the bladder and/or glucose is present, urine would not be a "preferred" or "superior" sample source for postmortem ethanol screening.[97]

A second bodily fluid that has been promoted for ethanol analysis in postmortem specimens, and one that may be even more resistant to microbial contamination, is vitreous humor.[86, 93, 94, 98] Vitreous humor is the water-based fluid that fills the eye. The interior of the eye is also a bloodless closed system under normal circumstances, and therefore, presumed to be resistant to microbial contamination. Vitreous humor does, however, normally contain a substantial concentration of glucose, which has been shown

to be an excellent substrate for ethanol production.[93] Harper published a study in 1989 detailing fifty-one postmortem cases that tested positive for blood ethanol and also had vitreous humor available for examination. The vitreous humor from seven of the fifty-one cases examined had bacteria concentrations above the 1×10^3 microbes/mL limit previously described, and also notably contained glucose.[86] The highest concentration of bacteria found in a vitreous humor specimen was 5×10^4 microbes/mL. And, all seven of the samples above the proposed microbial cut-off had ethanol at levels between 2 and 335 mg/dL.[86] So, while vitreous humor is possibly the best choice of tissue or fluid samples available to assess antemortem ethanol consumption on the basis of a simple postmortem ethanol measurement, it is unfortunately quite possible that even vitreous humor could be contaminated by postmortem ethanol produced through microbial action.

3. Case Examples

Due to the possible complications discussed above, it is sometimes simply not possible to readily assign the origin of any ethanol detected postmortem as being derived from antemortem consumption or postmortem microbial formation. For example, we might consider from the USS Iowa disaster, where 47 sailors died after a gun turret exploded in 1989. The sailors involved in the explosion were isolated on a ship, and there was no physical evidence or compelling reason to believe that any of the victims had consumed ethanol prior to their deaths.[99] However, of the 47 victims tested, 49% were shown to be positive for ethanol (20-190 mg/dL in blood) at autopsy, which occurred within 48 hours of death. The specimens examined included blood, tissues and other bodily fluids for each vivtim. The unusually high percentage of positive results and

low expectation of antemortem consumption led investigators to attribute the presence of postmortem ethanol to postmortem formation via microorganisms.[99] Similarly, other studies reported postmortem ethanol positives which clearly exceeded the expected values based on knowledge of antemortem ethanol consumption alone. These studies included a report by Gilliland *et al.* (286 total bodies 22% unknown ethanol origin)[100], a report by De Lima *et al.* (27 total bodies, 67% unknown ethanol origin)[101], and Canfield *et al.* (79 total bodies, 46% unknown ethanol origin)[102]. In short, a large fraction of postmortem samples are routinely identified to contain substantial amounts of ethanol. However, a considerable fraction of these ethanol-positive samples are clearly not necessarily connected with any kind of alcohol consumption prior to death.

D. Correlations Between Postmortem Ethanol and Antemortem Consumption

The existence of postmortem ethanol, unfortunately, is not a direct indication of ethanol ingestion prior to death. However, many attempts at strengthening this correlation have been undertaken and some approaches are consistently believed to bolster the relationship. The two most commonly employed indicators for the confirmation of postmortem ethanol are: 1) self-consistent ratios of ethanol concentration in two or more tissues and/or fluids and 2) the detection of volatile organic compounds at levels not normally seen *in vivo*.

1. Ethanol Distribution

As stated earlier, ingested ethanol distributes fairly rapidly throughout the body according to the water content of the fluid or tissue. Therefore, a normal distribution pattern for consumed ethanol would result in the lowest concentrations of ethanol in the brain, higher concentrations in other tissues, and still higher concentrations in blood, urine and vitreous humor.[15,103] However, the range of values (given in mg/dL for fluids and mg/hg for tissues) should not differ by a factor greater than three under normal circumstances. The condition of the victim following the accident should certainly be considered when choosing which tissue or fluid to analyze for the determination of ethanol.[14] If, for example, at the time of sample collection it is noted that the victim has suffered a severe cranial fracture, elevated ethanol concentrations found in the brain may be appropriately suspected to be unreliable due to probable postmortem microbial contamination.[100] Likewise, a violent aviation or other accident can result in the mutilation of a body and consequently a greater probability of postmortem microbial ethanol formation.[44] The medical history of a deceased victim should also be consulted in the interpretation of a positive ethanol postmortem determination. If known to suffer from one of the glucose enhancing ailments previously mentioned, a patient is far more likely to experience postmortem microbial ethanol formation. In such cases, care must be taken to sample more than one tissue or fluid, if possible, to determine if the ethanol distribution pattern appears normal.[14, 47]

2. Presence of Other Volatiles

A second commonly employed piece of data to differentiate between consumed antemortem and microbially produced postmortem ethanol is the levels of various organic volatile compounds. The presence of methanol, propanol, isopropanol, sec-butanol, acetone, t-butanol and/or acetaldehyde in the 0-5 mg/dL range in fluids (or mg/hg in tissues) are all quite commonly encountered. However, at levels of 15-30 mg/dL (or mg/hg), these volatile organic compounds (VOCs) have all been suggested to be reliable markers of postmortem microbial ethanol formation.[8, 56, 104-107] n-Propanol is the most commonly suggested VOC for use as an indicator of postmortem microbial ethanol formation.[56, 105, 106] n-Propanol is not present in substantial amounts in the body (i.e., less than 0.1 mg/dL or 0.1 mg/hg) under normal circumstances. However, n-propanol is commonly formed at levels > 15 mg/dL (mg/hg) by postmortem microbial action.[106] n-Propanol, unfortunately, has also been observed to be simply a byproduct of putrefaction and, thus, is also commonly present in cases when postmortem microbial ethanol is not involved.[47] Like n-propanol, microbes can also form other volatiles including but not limited to methanol, sec-butanol, t-butanol, isopropanol, acetone and acetaldehyde.[8, 105-107] Therefore, the detection of these molecules at modest levels (i.e. ≥ 15 mg/dL in fluids and/or ≥ 15 mg/hg in tissues) suggests postmortem microbial formation of ethanol. However, alcoholic beverages also often contain substantial amounts of other alcohols besides ethanol, including methanol, butanol and n-propanol.[104] And, the antemortem consumption of such beverages may alone account for such postmortem elevations in these VOCs. These complicating factors further limit the

effectiveness of VOCs in assigning the source of postmortem ethanol to clearly be antemortem consumption, postmortem microbial formation, or both.

3. Ester Formation

Fatty acid methyl esters have also been proposed as an indicator for the consumption of ethanol prior to death.[104] Ethanol normally is combined enzymatically with free fatty acids in the body to form fatty acid ethyl esters (FAEE's).[108-113] It has been suggested that these FAEE's may be a suitable marker for ethanol ingestion.[114, 115] While it is true that FAEE is present in large concentrations after ethanol ingestion, these may also be readily detected when no ethanol has been consumed. Further, FAEE's persist for very long periods of time (days) after ethanol consumption, which essentially eliminates the value of FAEE's in assessing alcohol consumption in the hours before death.[113] And, alcohol impairment is only possible if the consumption occurred within hours prior to death. Methanol also reacts with the same fatty acids forming fatty acid methyl esters (FAME's). As ethanol is consumed, the oxidation of methanol in the body virtually ceases,[116, 117] providing a build-up of methanol and an enhanced formation of FAME's. It has been suggested that if someone dies while acutely intoxicated there would be a corresponding increase in the concentration of FAME's found in the fluids and tissues of the victim.[104] Unfortunately, FAME levels in the absence of antemortem consumption are both small and considerably variable. Thus, detection of a small to modest increase in these initially small, variable levels is simply not feasible.

4. Other Methods

Other methods for the assessment of antemortem consumed ethanol according to postmortem conditions or chemical levels have also been briefly examined. These methods include the postmortem analysis of (1) ethyl glucuronide (ETG),[16, 111, 118-120] a fairly unique metabolite of ethanol; (2) gamma glutamyl transpeptidase (GGT),[3, 111, 121-124] an enzyme for which the activity increases as a result of long-term ethanol abuse; (3) carbohydrate deficient transferrin (CDT),[3, 5, 22, 111, 114, 125-131] a second indicator of long-term alcohol abuse; and (4) mean corpuscular volume (MCV),[16, 124] a third indicator of long-term alcohol abuse. GGT, CDT and MCV, unfortunately, give insight into only the chronic intake (weeks to years) of ethanol and are not measurably affected by casual, short-term or acute ethanol consumption.[132] Analysis of ETG, however, is currently employed in forensic toxicology to detect alcohol consumption in the not so distant (<5 days) past. But, persistence of this indicator for up to 5 days is too long for the short-term (hours) assessment of antemortem consumption in relationship to impairment. Additionally, the analysis of ETG is difficult because the postmortem concentration of this compound is moderately low.[16]

IV. Serotonin Biochemistry and Relation to Ethanol

The analysis of two serotonin metabolites, 5-hydroxyindole-3-acetic acid (5-HIAA) and 5-hydroxytryptophol (5-HTOL) has the potential to be the most effective postmortem method for the assessment of antemortem ethanol consumption.

A. Biosynthesis and Metabolism of Serotonin

In the late 1930's two scientists separately isolated the same compound. One named the compound enteramine and the other named it serotonin. After the purification of these two compounds, they were found to be structurally identical, and the name serotonin was applied to both. Serotonin, also known as 5-hydroxytryptamine (5-HT) is a heterocyclic, biogenic, indoleamine that is found throughout nature.[133, 134] It is found in edible plants, invertebrates and vertebrates alike.[135] In mammals, serotonin is found in nearly every fluid and tissue in the body.

Serotonin is normally synthesized in man from the amino acid tryptophan. As can be seen in Figure 1-3 the synthesis is a two-step process involving the hydroxylation of tryptophan by tryptophan hydroxylase using tetrahydrobiopterin and molecular oxygen as cofactors to produce 5-hydroxytryptophan (5-HTP). The subsequent decarboxylation of 5-HTP to form serotonin is catalyzed by aromatic-L-amino acid decarboxylase and employs pyridoxal-5-phosphate as a cofactor.

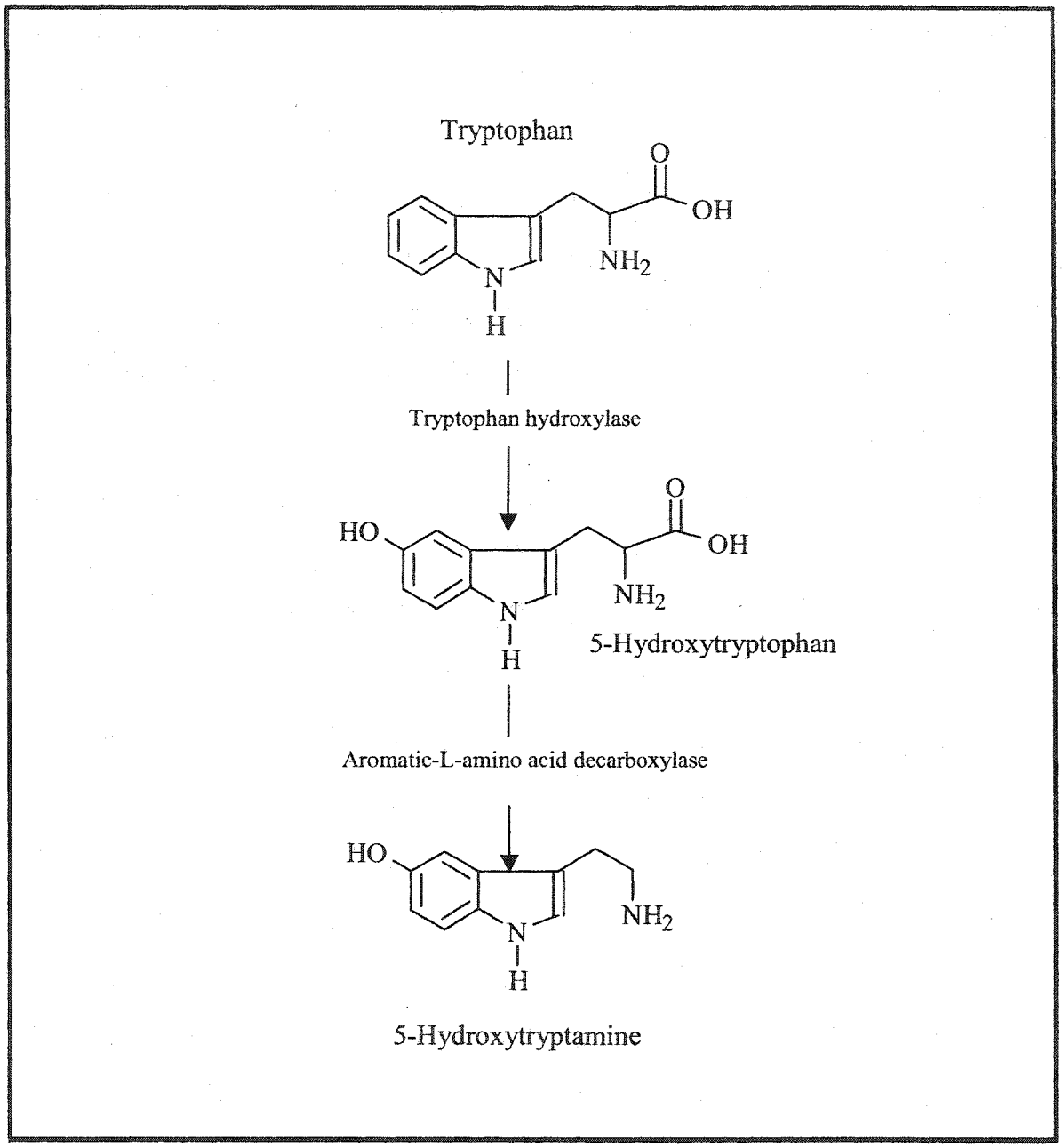


Figure 1-3. The synthesis of serotonin from tryptophan.

The synthesis of serotonin normally accounts for the consumption of less than two percent of the tryptophan present in the body, although synthesis is observed to occur in the brain, spinal cord, thymus, thyroid, pancreas, gastrointestinal tract and enterochromaffin cells.[136, 137] Under normal circumstances approximately 10 milligrams of serotonin is formed in a human per day, with the majority of this being produced in the gastrointestinal tract.[136] The body has been shown to employ serotonin for a variety of different purposes. These purposes include, or are thought to include, sleep regulation, neurotransmission, blood pressure regulation and smooth muscle contraction.[138, 139]

The usual metabolism of serotonin is initially catalyzed by monoamine oxidase. This first step involves oxidative deamination to form the intermediate 5-hydroxyindole-3-acetaldehyde (5-HIAL).[140, 141] As can be seen in Figure 1-4, following formation of the intermediate 5-HIAL, the metabolic pathway branches giving two possible products.

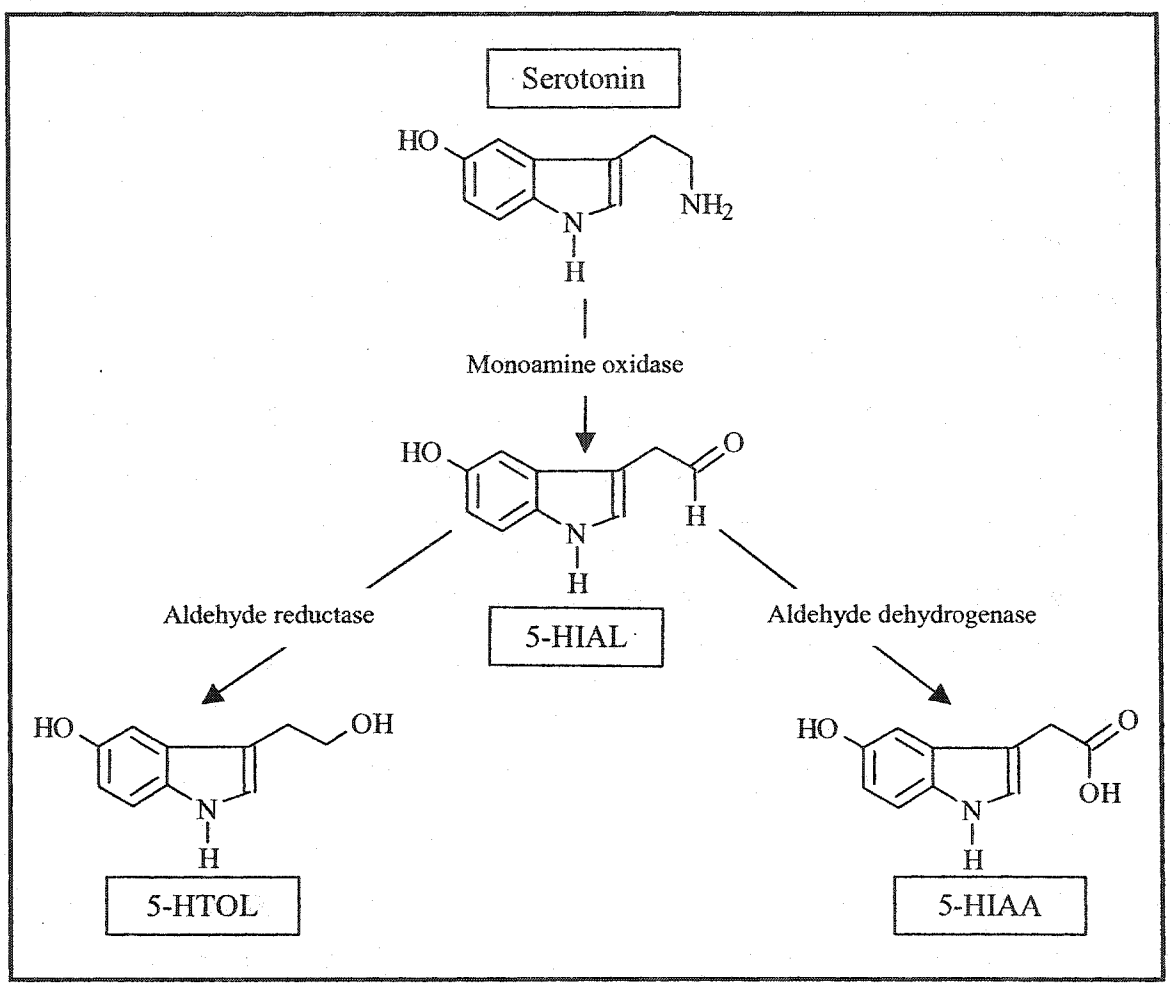


Figure 1-4. The metabolism of serotonin.

The oxidation of 5-HIAL is catalyzed by aldehyde dehydrogenase, which uses the oxidized form of nicotinamide adenine dinucleotide (NAD^+) as a coenzyme and produces 5-HIAA, which was first discovered in 1955.[39, 142-145] This represents the major pathway (90-99%) for serotonin metabolism under normal circumstances,[85] and 5-HIAA is presumed to have no physiological function.

The reduction of 5-HIAL is catalyzed by aldehyde reductase, which uses the reduced form of nicotinamide adenine dinucleotide (NADH) as a coenzyme and produces 5-HTOL.[138, 146] The remaining 1-10% of serotonin that is not oxidized to 5-HIAA is typically reduced to 5-HTOL. This process results in a normal ratio of 5-HTOL to 5-HIAA in the range of 1:100 to 1:10.[147] 5-HTOL was first discovered in 1962 by Kveder *et al.*[148] Any physiological role it may play in the body has not been clearly discerned to this day. However, 5-HTOL has been implicated as having some possible effects in temperature regulation, pituitary function and ovarian function.[149] Analysis of rat tissues and fluids showed 5-HTOL to be present in blood, plasma, urine and every organ examined with the highest concentrations found in the small intestine.[149-153]

B. Alcohol Consumption Effect on Serotonin Metabolic Pathways

An alteration in serotonin metabolism due to antemortem ethanol consumption was first reported in 1967.[154] The process of ethanol metabolism causes a cascade of events that can be seen to eventually realize an affect on the 5-HTOL/5-HIAA ratio.[155] For example, ethanol oxidation is accompanied by a reduction in the NAD^+ level and an increase in the NADH level. This alteration in the NAD^+/NADH results in a shift in the electrochemical balance, leading to a more "reduced" milieau.[156, 157] But, NAD^+ and

NADH also serve as cofactors for the enzymes that act upon 5-HIAL with NAD^+ required for 5-HIAA formation and NADH required for 5-HTOL formation. Therefore, as ethanol is metabolized and NAD^+ is converted to NADH, the metabolism of serotonin would be expected to be correspondingly altered.[154] The overwhelming majority of 5-HIAL normally follows the oxidative pathway when no ethanol is present, but requires NAD^+ as a cofactor. The minority pathway, however, employs NADH as a cofactor. Thus, in the presence of ethanol, we would certainly expect at least a partial shift in the metabolism of serotonin to the reductive pathway, leading to less formation of 5-HIAA and greater formation of 5-HTOL.[158-160]

A second factor that might directly contribute to an alteration of serotonin metabolism following ethanol consumption is the simple increase in the level of acetaldehyde derived from the action of alcohol dehydrogenase upon ethanol.[147] As acetaldehyde levels are elevated, this chemical would occupy more and more of the active site locations on any available aldehyde dehydrogenase. This interaction of acetaldehyde with aldehyde dehydrogenase would, of course, lead to the expected formation of acetic acid. However, increased occupancy of aldehyde dehydrogenase by acetaldehyde would also effectively decrease the availability of these sites for oxidation of 5-HIAL in the formation of 5-HIAA from 5-HT. Thus, the 5-HT metabolism would also be pushed by this mechanism in favor of 5-HTOL formation over 5-HIAA formation.[148]

Either or both of the two factors described above could be envisioned to cause the ratio of 5-HTOL to 5-HIAA to shift to a higher value.[161-163] In fact, following the consumption of ethanol, the 5-HTOL/5-HIAA ratio in urine begins to shift almost

immediately.[164] The molar ratio, which before ethanol ingestion was in the 0.01 to 0.10 range, reaches its peak of up to 1.50[165, 166] at approximately eight hours after consumption and, then, gradually returns to normal at 12-18 hours after consumption.[167] The 5-HTOL/5-HIAA ratio and its alteration following ethanol consumption appears to not be affected, or, at least, not greatly affected by the consumption of serotonin rich foods, the postmortem microbial formation of ethanol or the use of selective serotonin reuptake inhibitors (SSRI) antidepressants.[47, 85, 167, 168] Therefore, the analysis of these two compounds and the quantification of this ratio would hopefully serve as a potent tool for the precise assignment of postmortem ethanol in forensic samples as being due to antemortem consumption or postmortem microbial formation.

C. 5-HTOL and 5-HIAA Analysis

The analysis of 5-HTOL and 5-HIAA has historically been somewhat difficult and demanding. A lengthy extraction is required to isolate these compounds from the tissue/fluid matrix. Once isolated, two different analytical procedures have typically been employed in parallel, with two distinctly different methods of detection, for the quantification of each of these compounds. 5-HTOL analysis in such samples currently requires the use of gas chromatography in combination with a mass spectrometric detector (GC/MS) following a pentafluoropropionic anhydride derivitization.[39, 107, 111, 129, 146, 147, 155, 161, 165, 169] 5-HIAA is typically most effectively analyzed by high performance liquid chromatography (LC) in combination with an electrochemical detector (LC/EC).[170-179] The necessity of utilizing two unrelated techniques for the detection of these two compounds dramatically increases the complexity of the analysis, decreases the

precision associated with the ratio and increases the probability of encountering a substantial error in the attainment of the desired ratio. These analytical difficulties have been a primary obstruction to the widespread utilization of the 5-HTOL/5-HIAA ratio for routine assessment of antemortem ethanol consumption in the forensic toxicology field.

V. Current Study

As seen in the work to follow, it was our intent to develop a method for the simultaneous determination of 5-HTOL and 5-HIAA using only the single technique of high performance liquid chromatography combined with mass spectrometry (LC/MS). The ratio thus determined would hopefully be capable of providing a greater degree of certainty in discerning between antemortem ethanol consumption and postmortem ethanol formed by microbes.

A. Examination of Microbial Ethanol Formation in Postmortem Tissue Specimens

Postmortem microbial formation of ethanol in typical urine and blood samples as well as urine and blood samples spiked with substrate has now been well established. However, postmortem formation of ethanol in tissue samples had not previously been demonstrated. Likewise, the ability of 1% sodium fluoride and 4°C to act as a preservative had been previously well established for blood and urine samples, similar presentation had not been examined for tissue samples. Thus, our initial investigation focused on (1) demonstrating postmortem microbial ethanol formation in tissues and (2)

demonstrating the preservative effect of "blocking" such formation by addition of 1% sodium fluoride while examining storage at two different temperatures.

B. Analytical Method Development for Simultaneous Quantification of 5-HTOL and 5-HIAA

There were two distinct phases of this method development. The first phase was the design and implementation of an LC/MS method that (1) provided sufficient sensitivity, selectivity and detection limits for both 5-HTOL and 5-HIAA, (2) provided separation of the two analytes, and (3) was appropriate for easy selection and inclusion of a readily available internal standard for routine determinations. The second phase consisted of the design and development of an extraction procedure that was applicable to postmortem specimens that provided accuracy, precision and optimal recovery of these two compounds in preparation for the LC/MS analyses.

C. Assignment of Postmortem Ethanol to Antemortem Consumption or Postmortem Microbial Formation

Upon completion of the analytical method development portion of this work we attempted to apply this novel method to postmortem specimens obtained from aviation accidents. As will be explained in detail later, forty-four such postmortem specimens were analyzed for their concentrations of both 5-HTOL and 5-HIAA by LC/MS. After analysis, the 5-HTOL/5-HIAA ratio was determined for each specimen, and an attempt to correlate this ratio with the presence or absence of antemortem ethanol consumption was undertaken.

The Microbial Formation of Ethanol in Postmortem Tissue Specimens

Chapter 2

I. Introduction

Investigations seeking the source(s) of possible postmortem ethanol formation in human specimens began in earnest in the late 1960's and early 1970's. Many workers examined such formation under various environmental and/or storage conditions. These studies produced conflicting results concerning the effectiveness of preservatives, and proper specimen storage temperature in preventing postmortem ethanol formation. For example, earlier reports, which have now been substantially discounted, on the subject of sodium fluoride effectiveness by Chang and Kollman,[76] as well as Blume and Lakatua,[74] indicated sodium fluoride might be ineffective in preventing postmortem ethanol production in blood specimens. Later reports, which are now widely accepted as valid and appropriate[180, 181], however, by Lough *et al.*[182] and Jones *et al.*[183] indicated complete inhibition of postmortem ethanol production using sodium fluoride.

Unfortunately, the previous studies demonstrating the preservative effect of sodium fluoride were done on blood and urine samples alone. We, thus, decided to focus our attention on tissue sample preservation, which had not previously been investigated. However, results from the particular chemical preservative to be used, sodium fluoride, and the temperatures to be investigated, 4°C and near room temperature, would obviously provide data to directly compare to the previous fluid studies. For the tissue studies, we examined the potential formation of ethanol and other postmortem derived small organic molecules as a result of (1) presence and absence of sodium fluoride, (2) storage of the sample at 4°C or 25°C, and (3) storage at 0, 25, 48, 72 and/or 96 hours. The investigation of the stability of these samples with respect to these additives, temperatures and times was chosen to be relevant to our routine investigations at the FAA involving victims of aviation accidents. Such accidents, quite frequently do not provide urine, blood, vitreous humor, or other commonly analyzed fluid samples. Indeed, tissue fragments may be all that one has with which to work in such cases.

The experiments described in this chapter were specifically designed to measure the content of ethanol, acetaldehyde, methanol, acetone, isopropanol, n-propanol, sec-butanol, isobutanol and n-butanol in postmortem tissue samples.

II. Background

Microbial formation of ethanol in postmortem specimens is, by far, of most concern when attempting to relate postmortem ethanol to antemortem consumption. The first report of postmortem formation of ethanol in corpses appeared in 1936.[70, 71] Postmortem ethanol was found, surprisingly, to be present in a sample known to involve no prior antemortem ingestion. This and subsequent results established that simple postmortem existence of ethanol could clearly not be used as proof of antemortem ingestion. Today, it is known that many different microbes can contribute to postmortem formation of ethanol in animals.[71] Investigations have been performed to identify the particular species of bacteria, yeast and/or fungi predominately responsible for postmortem ethanol production in humans and the mechanism by which it is formed.[47, 82, 87, 88] *Candida albicans* has been the microbe most often ascribed to be responsible for postmortem production of ethanol in humans.[76, 77] This species of yeast is commonly found to be associated with humans.[79] Located ubiquitously throughout the body, the highest levels of *C. albicans* are typically found in the mouth and on the skin.[79] However, it should be noted that approximately 100 species of bacteria, yeast and fungi have been shown capable of producing postmortem ethanol.[47] Glucose is the most probable substrate in the human body used by these microbes to form ethanol.[73] Other endogenous compounds can also be utilized as substrates including, but not limited to, lactate, mannitol, galactose, maltose, sucrose and lactose.[72, 73, 90, 91]

In addition to the many microbes commonly found in/on humans, we must also be concerned with microbes not normally found *in vivo*, such as the *Mucor* species of mold commonly found in decaying organic matter and soil.[84] Such "uncommon" species may

well be introduced from the environment if the circumstances surrounding death are violent, as is often seen in aviation accidents. After death, endogenous and/or exogenous microbes may begin rapidly consuming the glucose and/or other nutrients present in the body and produce ethanol and/or other organic volatiles.[72, 73, 90, 91] Under selected conditions, substantial concentrations of ethanol may be formed within hours of death.[181] This relatively short time required for microbes to begin producing ethanol obviously complicates the interpretation of a positive ethanol result for postmortem specimens in relationship to antemortem consumption. Postmortem microbial ethanol formation could certainly invalidate a simple positive postmortem ethanol result and its associated implication of cause in an aviation accident.

Due to possible postmortem microbial formation of ethanol, the preservation of biological specimens has been a major concern for virtually all such studies. The most commonly utilized substance for the preservation of postmortem specimens is sodium fluoride. The fluoride ion prevents the formation of glucose-6-phosphate from glucose-1-phosphate by inhibiting the enzyme phosphoglucomutase. Inhibition of phosphoglucomutase in a typical microbe prevents further polysaccharide synthesis and, in turn, halts microbial growth.[182] Storage temperature and length of storage time clearly play an important role in possible microbial formation of ethanol. Numerous studies have examined microbial production of postmortem ethanol as a function of both storage time and storage temperature in blood and urine specimens.[181-183] Additionally, these studies have demonstrated the effectiveness of added sodium fluoride in preventing microbial action.

The vast majority of research publications involving microbial ethanol formation investigated blood and/or urine spiked with glucose and/or spiked with *C. albicans* or other microbes. Blood of reasonably assignable victim origin is submitted to the FAA laboratory in only approximately 70% of all aviation accident cases, and victim assignable urine samples occur even less frequently. Thus, tissue specimens are often the only victim specific sample type available for postmortem analyses. Furthermore, these tissue specimens have almost certainly been subjected to trauma as a result of the violent nature of the aviation accident. As a result, the tissues are almost certainly contaminated with both endogenous and exogenous microbes capable of producing significant amounts of ethanol. In a previous study using rDNA amplification, which examined 45 blood and tissue specimens obtained from fatal aviation accident victims, 95% were contaminated with enteric bacteria capable of ethanol production.[184] Therefore, we examined the formation of ethanol in untreated tissue specimens originally obtained from fatal aviation accident victims. All such samples were stored at -20°C following collection, and all such samples were < 4 years old These tissues were subsequently prepared for our studies by adding or not adding sodium fluoride as a preservative. We then examined production of various organics in these samples for up to 96 hours at either 4°C or 25°C.

We employed headspace gas chromatography with flame ionization detection (GC-FID), the most common analytical technique for this purpose, for the separation and quantification of volatile organic compounds (VOCs) from our tissue specimens. The sensitivity, selectivity and detection limits of headspace-GC-FID are more than sufficient for the detection and quantification of the minute amounts of volatile compounds, which may be present in such a specimen.[185-187]

III. Experimental Design and Methods

A. Chemicals and Solutions

1. Chemicals

All aqueous solutions were prepared using double deionized water (DDW), which was obtained using a Milli-QT_{plus} Ultra-Pure Reagent Water System (Millipore®, Continental Water Systems; El Paso, TX).

The following chemicals were purchased from Sigma Chemical Company (Sigma Chemical Co, St. Louis, MO) in high purity and used without any further purification.

Sodium Fluoride

t-Butanol

Acetaldehyde

Methanol

2-Propanol

Acetone

n-Propanol

Isobutanol

n-Butanol

sec-Butanol

Ethanol

Purchased ethanol controls were conveniently obtained as 25, 50, 100, 150 and 300 mg/dL ethanol standards from Restek Corporation (Restek Corp., Bellefonte, PA).

2. Sodium Fluoride Solution

Sodium fluoride was prepared in a volume of 500 mL having a final concentration of 1.00% (w/v). This solution was prepared by dissolving 5.00 g of sodium fluoride in approximately 100 mL of DDW in a 500 mL volumetric flask and then diluting to the mark with DDW.

3. Preparation of Stock Test Solution

A stock test solution was prepared by adding the following chemicals to a 100 mL volumetric flask that initially contained approximately 20 mL DDW. The compounds added were: acetaldehyde, methanol, 2-propanol, acetone, n-propanol, isobutanol, n-butanol and sec-butanol. The amounts of these compounds added and their final concentrations can be seen in Table 2-1. After the addition of all compounds, the flask was filled to the mark with DDW, tightly stoppered, and the solution was mixed by inversion. This solution was stored at 4°C and was never used after 1 month following preparation.

Table 2-1. Chemicals used and final concentrations in a stock test solution.

Compound	Volume Added (mL)	Final Concentration (mg/dL)
Ethanol	10.00	7907
Acetaldehyde	2.00	1576
Methanol	10.00	7920
2-Propanol	10.00	7800
Acetone	2.00	1592
n-Propanol	15.00	12060
Isobutanol	2.00	1620
n-Butanol	5.00	4050
sec-Butanol	5.00	4050

4. Blood Calibrator Preparation

Blood calibrators were prepared by transferring 20.00 mL of the stock test solution to a clean 100 mL volumetric flask via volumetric pipette. The flask was filled with DDW to the mark, capped and mixed well. This solution was known as a working stock solution.

A volumetric pipette was used to transfer 10.00 mL of the working stock solution into a clean 100 mL volumetric flask. This flask was filled to the mark with blood that

was previously certified to be negative for any volatile organic compounds. This flask was mixed well and labeled "Blood Calibrator Solution."

Two mL portions of the blood calibrator solution were pipetted into a series of forty-five 16 x 100 mm screw-cap culture tubes. These tubes were capped and labeled with the date prepared. The concentrations of the compounds in a working stock solution and in a blood calibrator solution can be seen in Table 2-2.

Table 2-2. Concentrations of volatiles in a working stock and a blood calibrator solution.

Compound	Working Stock Concentration (mg/dL)	Blood Calibrator Concentration (mg/dL)
Ethanol	1581	158
Acetaldehyde	315	31
Methanol	1584	158
2-Propanol	1560	156
Acetone	318	31
n-Propanol	2412	241
Isobutanol	324	32
n-Butanol	810	81
Sec-Butanol	810	81

5. Preparation of Internal Standard

The stock internal standard solution was prepared by the following method. A volumetric pipette was used to transfer 5.00 mL of t-butanol into a 1.00 L volumetric flask containing approximately 100 mL DDW. The volumetric flask was then filled to the mark with DDW and inverted repeatedly to mix. This flask contained 394 mg/dL t-butanol.

Twenty-five mLs of the stock internal standard solution was pipetted into a 250 mL volumetric flask, sodium fluoride (1.00 g) was added to the flask, the flask was filled to the mark with DDW, stoppered and then mixed by inversion. This flask working internal standard solution contained 39.4 mg/dL t-butanol and simultaneously contained 0.40% sodium fluoride. This working internal standard solution was capped tightly and stored at 4°C. Sodium fluoride was primarily added to the working internal standard solution to increase the ionic strength of this solution. The increased ionic strength of the aqueous phase causes a larger portion of the volatile organic compound to move into the headspace of the vial. This technique is commonly used in volatile analysis and is known as "salting out."[188]

B. Sample Preparation

1. Tissue Homogenization

a. Specimen Preparation

It was necessary to homogenize tissue specimens before analysis. Each tissue specimen was weighed. Once the weight of the specimen was determined a corresponding two volumes of 1.00% sodium fluoride (or water) in DDW was added.

For example, if 2.00 g of tissue was supplied, 4.00 mL of a 1.00% sodium fluoride solution (or 4.00 mL of water), was added to that specimen.

b. Homogenizer

The homogenizer used was a PRO250 Post Mounted Homogenizer (PRO Scientific, Oxford, CT). This homogenizer had a variable speed range from 10,000 to 30,000 RPM, and was set on 22,000 RPM for all tissues homogenized in this study. The homogenizing probe used with this homogenizer was 10 mm in diameter and had a saw-toothed bottom configuration.

c. Homogenizer Cleaning

To prevent carry-over contamination between samples, the homogenization generator was cleaned in the following manner (1) before any homogenization was undertaken, (2) between individual tissue samples, and (3) after all samples in a group had been completed.

The generator was disengaged from the homogenizer assembly. An Allen wrench was used to disassemble the generator by loosening the setscrew on the rotor shaft. The rotor knife was then removed from the generator assembly. All exposed surfaces of the knife, rotor shaft and main generator housing were thoroughly washed with hot, soapy water and then double rinsed first with distilled water then with DDW. Stiff wire brushes supplied by the manufacturer were used for the purpose of cleaning the inside of the main generator housing. These brushes were inserted into both ends of the housing with hot

soapy water. The interior was then double rinsed, once with distilled water followed by once with DDW. The generator was then dried with a paper towel and re-assembled.

d. Tissue Homogenization

With the homogenizer set up under a negative pressure ventilation hood, the generator was inserted into the sample, and the power was turned on. A typical sample consisted of 8–10 g of tissue and 16–20 mLs of either water or 1.00% sodium fluoride. Tissues were homogenized until an even consistency with no suspended pieces was achieved. The final consistency of the homogenate approximated that of blood.

2. Initial Set-up

Stored at 4°C prior to utilization, all calibrators, controls and homogenized specimens were brought to room temperature immediately prior to analysis by placing them on the desktop at least 30 min prior to beginning the experiment(s). After warming to room temperature all calibrators, controls and homogenized specimens were vortexed briefly to ensure that they were mixed thoroughly.

3. Aliquoting for Analysis

A typical group of vials subjected to analysis included 3 blood calibrators, 8 purchased controls and 36 unknown specimens. A 500 µL aliquot from each vial was pipetted into an individual 10 mL glass reaction vial (Microliter Analytical Supplies, Suwanee, GA) using a 1.00 mL disposable serological pipette. At this point, the samples were treated one at a time until the stage where a tight seal of the vial cap was achieved.

For a given vial, a 0.50 mL aliquot of the 39.4 mg/dL t-butanol internal standard solution was added to one reaction vial using a fixed volume Oxford Sampler P7000 Pipette (Oxford Labware, St. Louis, MO). Immediately after the addition of the internal standard, an inert silicone/Teflon® septa (Microliter Analytical Supplies, Suwanee, GA) was placed on each vial followed by a metal cap (Microliter Analytical Supplies, Suwanee, GA). The cap was sealed using a handheld crimping tool (Wheaton Scientific Products, Millville, NJ). The vial was checked for a tight seal by trying to rotate the metal cap around the top of the vial. If the cap rotated freely then the vial was re-crimped. After a good seal was achieved internal standard was added to the next vial and the process was repeated until all forty-seven samples in that group had been prepared.

4. Sample Order

All samples in a group were analyzed in duplicate. Each group also included three blood calibrators, one DDW blank (prepared independently of all other samples) and the series of purchased controls. The run sequence for a typical group can be seen in Table 2-3.

Table 2-3. Typical analysis group sequence and vial positions for a determination of volatile organics.

Vial Position	Sample Type
1-3	Blood calibrators
4*	DDW blank
5	25 mg/dL ethanol purchased control
6 – 15	Unknowns
16	50 mg/dL ethanol purchased control
17-26	Unknowns
27	100 mg/dL ethanol purchased control
28-37	Unknowns
38	150 mg/dL ethanol purchased control
39-44	Unknowns
45	300 mg/dL ethanol purchased control

*The DDW blank used in position 4 was injected to validate no carryover between injections.

C. *GC Methodology*

1. Instrumentation

Analyte separation was achieved using a Varian CP-3800 dual gas chromatograph (GC) (Varian, Inc., Palo Alto, CA). The GC was fitted with an automated Varian CombiPAL® (Varian, Inc., Palo Alto, CA) headspace GC injection system that allowed for automated sample pretreatment and injection. The CombiPAL® was utilized in

conjunction with a 1.00 mL SGE gas tight syringe (400 psi max pressure) obtained from SGE (SGE Chromatography Products, Austin, TX). The sample volume was set to 0.50 mL. The GC was equipped with dual fused silica capillary columns obtained from Restek Corporation (Restek Corp., Bellefonte, PA). The GC columns were an Rtx-BAC1 (0.53 mm i.d. x 3.00 μm x 30 m) and an Rtx-BAC2 (0.53 mm i.d. x 2.00 μm x 30 m) and were both employed for every analysis. The stationary phase for each column was composed of dimethyl polysiloxane. The fundamental difference between these two utilized columns was the stationary phase film thickness, which varied by 1.00 μm between the two columns. This allowed for the comparison of retention times and/or capacity factors on two columns for enhanced certainty in peak identification and quantification. Detection and quantification of the analytes of interest was accomplished using a flame ionization detector (FID). Control of the GC system, monitoring of FID signals, integration of chromatographic peaks and communication with the GC system was achieved using a Dell™ Optiplex GX 1 (Dell™, Round Rock, TX) personal computer system equipped with Varian Star© Chromatography Workstation software version 5.3.1 (Varian, Inc., Palo Alto, CA).

2. Autosampler Parameters

The CombiPAL® GC injection system provided incubation of the specimen prior to injection in order to promote the release of volatile molecules into the headspace of the vial. This feature was utilized for our experiment with the incubation temperature set to 50°C and the incubation time set to 13.30 minutes per sample. The injection syringe was also heated. In order to facilitate transfer of volatile organic molecules, the syringe

temperature was set to 50°C. The syringe fill speed was set to 100 µL/sec and the injection speed was set to 150 µL/sec. Following injection the syringe was flushed for 30 seconds with carrier gas to ensure no carryover of volatile molecules to the next injection.

3. GC Parameters

The GC injector and detector temperatures were set at 150°C. The GC oven was held isothermally at 40°C for the duration of the 10 minute run. The flow rate for the helium carrier gas was set at 20 mL/min and was held constant for all analyses. The head pressure exerted on the capillary column by this flow of helium was typically 8.7 p.s.i. Hydrogen and compressed air were added to the carrier gas after its exit from the column. The flow rate for hydrogen was set to 30 mL/min and the flow rate for compressed air was set to 300 mL/min.

4. Integration Parameters

The Varian Star© Chromatography Workstation software used for these experiments allowed for automated identification and integration of all observed peaks. Three successive minimal criteria were used for the identification of peaks in a chromatogram. These three criteria were signal to noise ratio, peak area and retention time. The signal to noise ratio necessary to consider a detector excursion to be a possible peak was set to a minimum value of five. The amount of noise present was determined during each run by examination of appropriate baseline region(s) near peaks of concern. Peak area was used both in the initial identification and, subsequently, in the quantitative

measurement of potential peaks. Any potential peak with an area less than 2000 counts was not integrated. Retention time was also used as a peak criterion. The retention time for ethanol and other pertinent volatiles was initially established on both columns using a prepared blood calibrator. Any peak having a retention time within ±2.00% of the value determined with the calibrator was considered acceptable. Retention times falling outside of this ±2.00% window were not used.

5. Calibration

Before the analysis of unknown specimens in a group, a series of blood calibrators were analyzed. The calibrators had an ethanol concentration of 158.2 mg/dL and were prepared as described in section A.4 above. Three calibrators were used in a group, each containing 0.50 mL of the blood calibrator solution and 0.50 mL of working internal standard. The first calibrator was injected and a one-point calibration curve was derived from the result. The calibration curve was forced through the origin, and a linear fit was established by these two points. The two additional calibrators were then injected to ensure the accuracy and precision of the initial concentration obtained. Both such calibrators were required to yield ethanol concentrations within ±2% of their known values when determined using the calibration curve. Purchased controls were run in order to further verify the accuracy of the calibration curve. The results obtained from all purchased controls had to fall within 10% of their known value for the curve to be accepted and used for unknown specimens.

6. Analytical Parameters

Both the limit of detection (LOD) and the limit of quantification (LOQ) were determined for the detection of ethanol using this technique. The LOD, as defined by our laboratory, is the lowest analyte concentration detected with a peak S/N ratio of at least five and a retention time within ± 2% of a known standard. The LOD for this method was determined to be 0.10 mg/dL with respect to ethanol. The LOQ was the lowest concentration detected that met the parameters described above and had a S/N of at least ten. The LOQ for this method was determined to be 0.20 mg/dL with respect to ethanol. However, as per the FAA laboratory guidelines, no VOC was quantified below a concentration of 1.00 mg/dL. A representative chromatogram for the analysis of ethanol and other volatiles using this method can be seen in Figure 2-1 below.

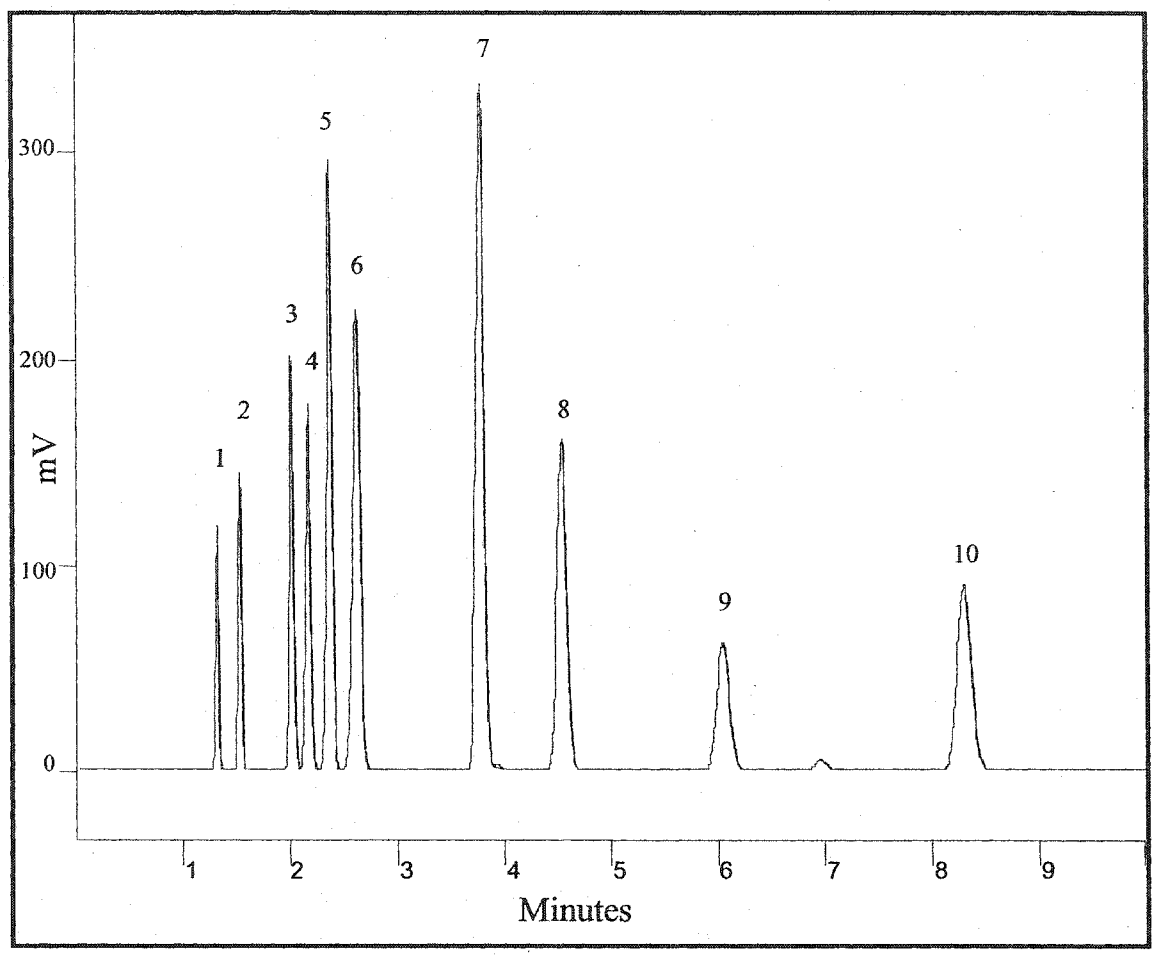


Figure 2-1. A representative headspace-GC chromatogram for the analysis of volatile organic compounds in a calibrator. Peaks of interest are: 1. acetaldehyde, 2. methanol, 3. ethanol, 4. acetone, 5. isopropanol, 6. t-butanol, 7. n-propanol, 8. sec-butanol, 9. isobutanol and 10. n-butanol.

D. Experimental Procedure

1. Specimen Selections and Treatment

Biological specimens from fatal aircraft accident victims are stored at -20°C at our laboratory for up to 5 years following their initial analysis. Using our laboratory database, specimens were selected from cases where microbial contamination was almost certainly involved in a significant manner (see Discussion below).

Tissue specimens of 8–10 g were thawed, divided into two approximately equal parts and weighed. The first part (4-5 g) of the specimen was diluted with 2 parts of DDW, while the second part (4-5 g) was diluted with 2 parts of 1.00% sodium fluoride. The tissue samples were then homogenized following the homogenization procedure detailed in section B.1 above. To prevent sample-to-sample contamination, the homogenizer was thoroughly cleaned between each homogenization as previously described. Following homogenization, both the water and 1.00% sodium fluoride tissue homogenates were divided again into two parts by transferring approximately half of the volume of each to clean 16 x 100 mm screw-capped culture tubes. All four samples from the original tissue specimen were labeled appropriately and placed on ice awaiting an initial volatile organics (VOCs) analysis. Following the initial analysis, each specimen was capped and placed either in a refrigerator at 4°C or on a shelf at 25°C. The homogenates remained either in the refrigerator or on the shelf for the remainder of the experiment.

2. Volatile Organic Analysis

The headspace GC-FID was calibrated using the previously described calibration solution. A calibration solution was also run separately as a test mixture each day prior to analysis to evaluate the operation of the instrument. Ethanol controls were run before, during and after sample analysis. Samples from each specimen were analyzed immediately after homogenization to establish a baseline concentration of volatiles. At various times following initial homogenization, a portion of each specimen was aliquoted into a headspace vial and subjected to analysis for volatile organic compounds (VOCs).

IV. Results and Discussion

A. Results

Due to the nature of aviation accidents, both the time required for the recovery of accident victims and the environmental conditions to which the victims were exposed postmortem are highly variable. As discussed below, the specimens selected for this study had strong indications of prior microbial activity. Table 2-4 outlines the different tissue specimen types chosen. It should be noted that each specimen was necessarily periodically uncovered for sampling and, therefore, potentially exposed to the microorganisms present in the local environment for brief periods of time. Thus, potential unwanted contamination could have occurred during these studies.

Table 2-4. Specimen types used and initial ethanol concentrations determined.

Specimen Number	Specimen Type	Ethanol Concentration (mg/hg)
1	Muscle	6
2	Kidney	18
3	Muscle	4
4	Kidney	4
5	Muscle	23
6	Kidney	5
7	Kidney	28
8	Kidney	1
9	Muscle	2

The nine tissue specimens selected for this study, and shown in Table 2-4, were each divided into two equal parts and homogenized after the addition of water or 1.00% sodium fluoride, respectively. Volatile analysis was immediately performed on a portion of these samples. The remaining portions of the homogenized specimens were then capped and stored at either 4°C or 25°C for subsequent analysis. Thus, a single original tissue sample provided four individual primary samples for subsequent analysis. Two of these four primary samples were stored at 4°C and examined for ethanol and other volatiles at t = 0, 24, 48, 72 and 96 hrs. The remaining two of the four primary samples were stored at 25°C and examined for ethanol and other volatiles at t = 0, 24 and 48 hrs.

An initial comparison of the nine specimens homogenized in both water and 1.00% sodium fluoride yielded the following results. The range (1-28 mg/hg) and average (10mg/hg) initial ethanol concentration (t = 0) for these specimens was the same for both groups. Tables 2-5 and 2-6 show the individual initial ethanol concentration present in each of these samples.

The samples homogenized in 1.00% sodium fluoride and stored at 4°C and 25°C demonstrated what was judged to be no significant difference in ethanol concentrations between the two storage temperatures throughout the entire duration of the experiment (96 hrs for 4°C; 48 hrs for 25°C samples). The largest individual increase in ethanol concentration observed for the sodium fluoride group occurred in a kidney sample (specimen #7) stored at 4°C. This specimen had an initial ethanol concentration of 28 mg/hg and at 96 hrs contained 33 mg/hg ethanol. This slight increase, however, was judged to be relatively insignificant.

Table 2-5. The effect of temperature on the amount of ethanol formed in specimens homogenized in 1.00% sodium fluoride.

Specimen #	Preservative	Temperature	Initial Ethanol (mg/hg)	Final Ethanol* (mg/hg)
1	NaF	4°C	6	6
		25°C		6
2	NaF	4°C	18	20
		25°C		16
3	NaF	4°C	4	3
		25°C		3
4	NaF	4°C	4	2
		25°C		4
5	NaF	4°C	23	23
		25°C		22
6	NaF	4°C	5	5
		25°C		6
7	NaF	4°C	28	33
		25°C		29
8	NaF	4°C	1	1
		25°C		1
9	NaF	4°C	2	1
		25°C		1

* Final ethanol concentrations were recorded after an incubation time of 48 hrs at 25°C and 96 hrs at 4°C.

The nine tissue specimens homogenized without preservative and subsequently stored at 4°C demonstrated significant and substantial ethanol formation over the course of the 96 hour experiment as seen in Table 2-6. The average ethanol increase for these nine specimens stored at 4°C was 42 mg/hg (1470%), with increases ranging from 22–75 mg/hg (107%–7500%). The largest individual increase in ethanol concentration observed occurred in a kidney sample (specimen #8). While this sample initially contained 1 mg/hg ethanol, at 96 h this specimen was found to contain 76 mg/hg ethanol. This

specimen had a net ethanol concentration increase of 75 mg/hg (7500%) over the course of the experiment.

Table 2-6. The effect of temperature on the amount of ethanol formed in specimens homogenized in the absence of any preservative.

Specimen #	Preservative	Temperature	Initial Ethanol (mg/hg)	Final Ethanol* (mg/hg)	% Increase
1	None	4°C	6	28	367
		25°C		25	317
2	None	4°C	18	45	150
		25°C		40	122
3	None	4°C	4	46	1050
		25°C		30	650
4	None	4°C	4	40	900
		25°C		88	2100
5	None	4°C	23	80	252
		25°C		48	109
6	None	4°C	5	49	880
		25°C		71	1320
7	None	4°C	28	58	107
		25°C		90	221
8	None	4°C	1	76	7500
		25°C		62	6100
9	None	4°C	2	43	2050
		25°C		41	1950

*Final ethanol concentrations were recorded after an incubation time of 48 hrs at 25°C and 96 hrs at 4°C.

The non-preserved specimens were also examined at 25°C after 48 h. As expected, all nine showed dramatic increases in ethanol concentrations. The average increase in ethanol concentration at 25°C for these nine specimens was 45 mg/hg (1432%) at 48 h, with increases ranging from 19–84 mg/hg (109%–6100%). The largest individual percent increase in ethanol concentration observed occurred in a kidney sample (specimen #8), with an initial ethanol value of 1 mg/hg and a final ethanol value of 62 mg/hg (6100%) at 48 h.

B. DISCUSSION

The primary purpose of this study was to determine postmortem ethanol in nine tissue specimens from aviation fatalities homogenized in either water or 1.00% sodium fluoride, and stored at both 4°C and 25°C. All tissue cases selected were chosen because they were strongly suspected to have experienced microbial contamination. This strong suspicion was due to either a substantially abnormal distribution of ethanol in various tissues and fluids examined from the victim or initially abnormal concentrations of examined volatile organic compounds. As has been well documented, under normal circumstances following ingestion, ethanol distributes throughout the entire body according to the water content of various tissues and fluids.[9, 12, 13, 55, 59, 65] Therefore, when substantially different ethanol distribution is observed between two or more tissues and/or fluids from one victim, postmortem microbial ethanol formation is strongly indicated. A second commonly used criterion to evaluate postmortem microbial ethanol formation is the presence of abnormally high levels of other organic volatiles in a specimen. Relatively high concentrations of organic volatiles such as acetaldehyde,

n-propanol, sec-butanol, isopropanol, acetone and iso-butanol, when discovered during routine ethanol analysis, also strongly suggest the possibility of postmortem microbial action.[8, 47, 56, 77, 101, 105, 106, 181]

One of the initial goals of this study was to make a direct comparison between the changes, if any, in ethanol concentrations in specimens stored in water and 1.00% sodium fluoride. The differences observed between these two groups were substantial. Out of the 36 individual experiments performed, i.e., 18 in water at 4°C or 25°C and 18 in 1.00% sodium fluoride at 4°C or 25°C, all 18 of the non-preserved samples showed a very substantial increase in ethanol concentration between the initial and the final measurement. And, both the 4°C and 25°C groups without preservative showed dramatic increases in ethanol concentration, the increases occurred at a slower rate in the refrigerated specimens. Conversely, the 18 specimens preserved with 1.00% sodium fluoride all demonstrated no significant increases in ethanol concentration. In Figures 2-2 through 2-10 below ethanol concentration is plotted versus time for all four conditions examined in each of the specimens. The data collected for each of the nine specimens is shown in Figure 2-11 as a distribution plot. Additionally, the initial and final ethanol concentrations for samples homogenized in either 1.00% sodium fluoride or water and subsequently stored at either 4°C or 25°C are summarized in the previously presented Tables 2-5 and 2-6. From observation of Figures 2-2 through 2-11 and a comparison of the data presented in Tables 2-5 and 2-6, it is obvious that sodium fluoride was extremely effective at inhibiting postmortem microbial ethanol formation. This agrees well with the previous reports of sample preservation for the cases of both blood and urine.[181-183]

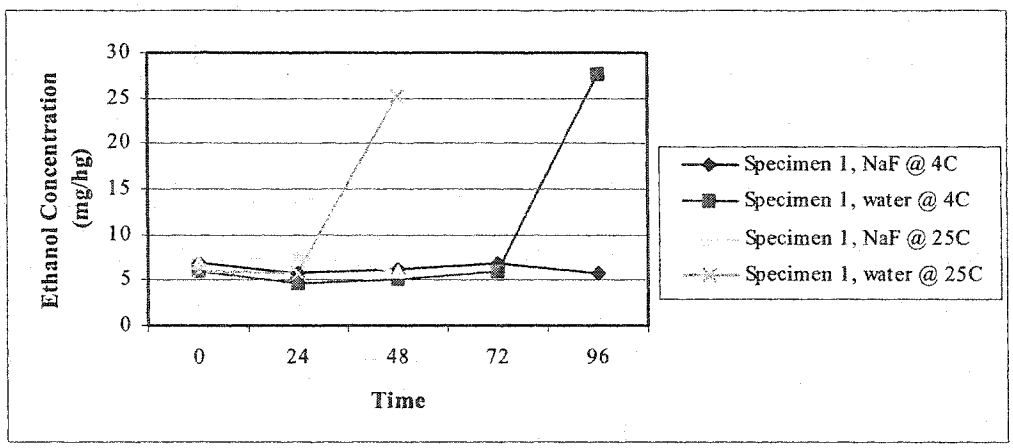


Figure 2-2. Ethanol concentration vs. time for specimen # 1.

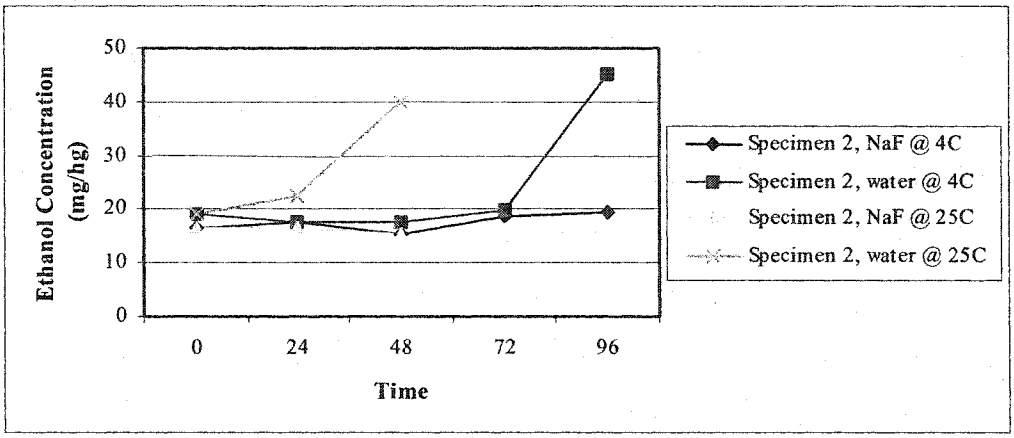


Figure 2-3. Ethanol concentration vs. time for specimen # 2.

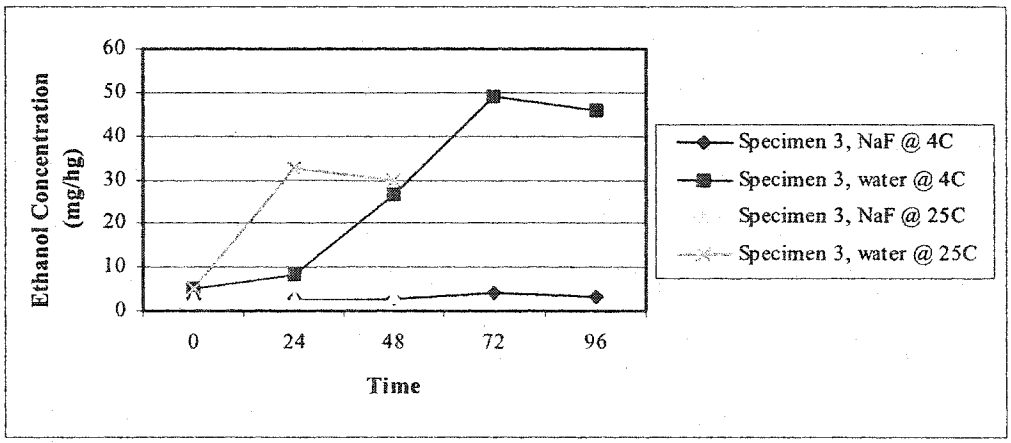


Figure 2-4. Ethanol concentration vs. time for specimen # 3.

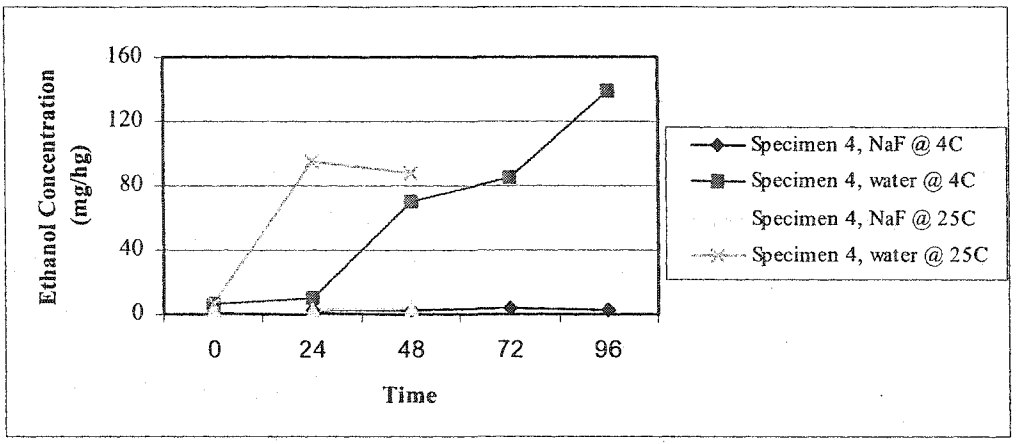


Figure 2-5. Ethanol concentration vs. time for specimen # 4.

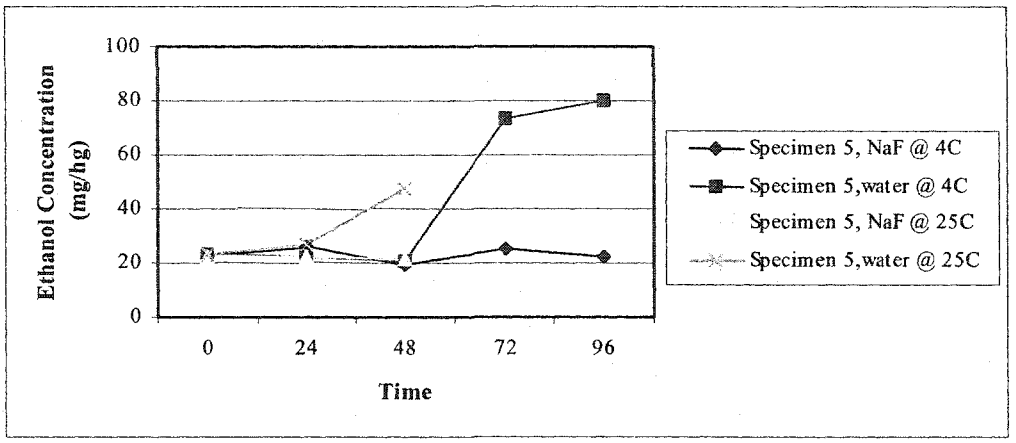


Figure 2-6. Ethanol concentration vs. time for specimen # 5.

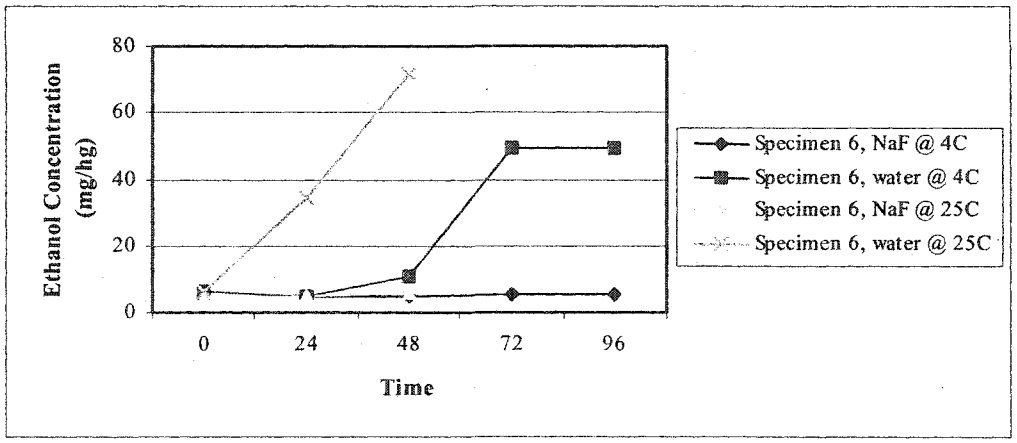


Figure 2-7. Ethanol concentration vs. time for specimen # 6.

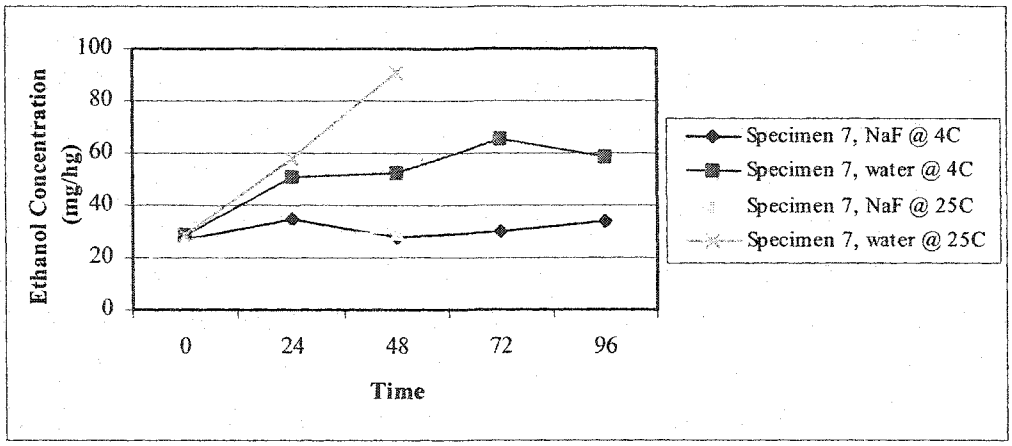


Figure 2-8. Ethanol concentration vs. time for specimen # 7.

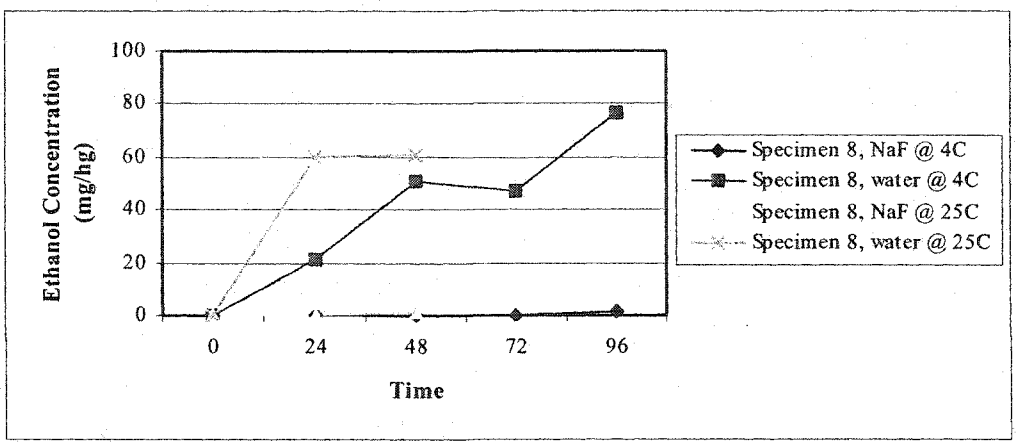


Figure 2-9. Ethanol concentration vs. time for specimen # 8.

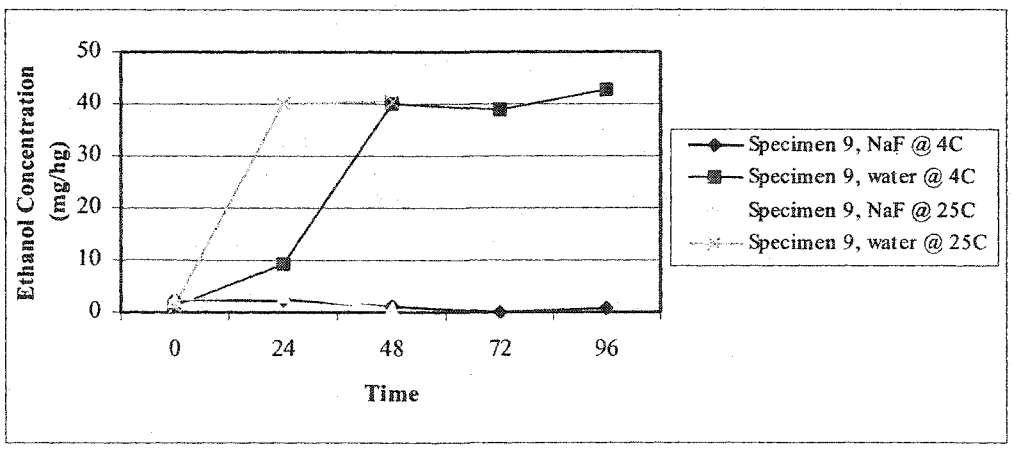


Figure 2-10. Ethanol concentration vs. time for specimen # 9.

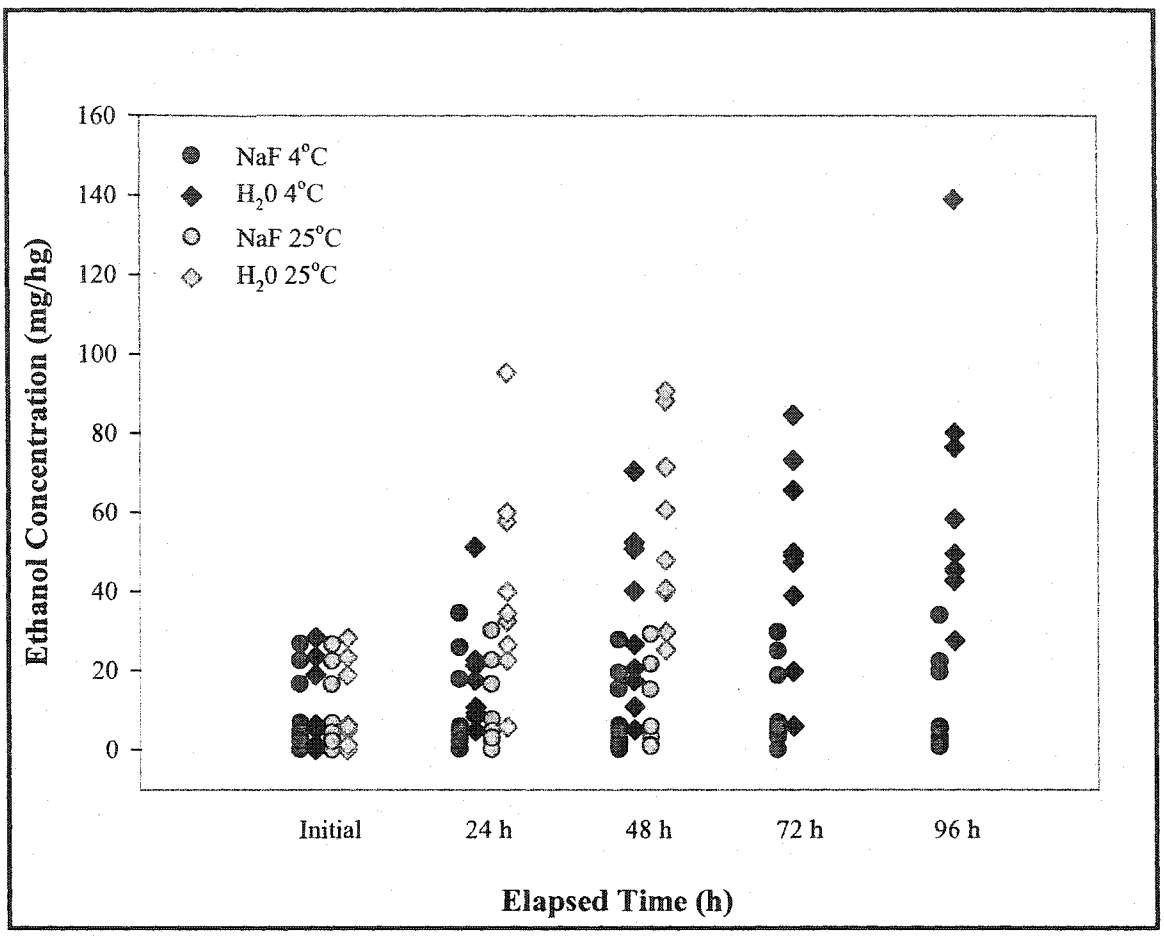


Figure 2-11. Distribution of ethanol concentrations obtained for each set of forensic tissue samples homogenized in either NaF or water and stored at different temperatures over the elapsed time.

In a majority of the non-preserved specimens investigated, the final ethanol concentration after 96 h at 4°C was similar to the final value observed after 48 h at 25°C. These comparable final concentrations of ethanol achieved were somewhat expected and may be explained by examining three factors. First, at lower temperatures, one would reasonably expect the rate of ethanol formation by microbes to be slower than those at room temperature. Second, formation of acids from the fermentation of carbohydrates, amino acids and alcohols could be imagined to result in attainment of a pH value which essentially killed the microbes, providing an upper limit for ethanol formation before death. Lastly, there is a different, but finite amount of substrate available in each of these specimens for the microbes to consume. After the available substrate supply has been depleted, of course no further ethanol can be produced.

From the nine total specimens originally analyzed, six of the specimens were initially reported negative for ethanol content, as defined by the FAA criteria (<10 mg/hg). After 96 h of storage at 4°C with no preservative, all 6 specimens had ethanol concentrations above this base cutoff. Furthermore, 5 of the 6 were above 40 mg/hg, which is independently and legally recognized as the FAA's legal cutoff for ethanol intoxication/influence(14CFR91.17). At 25°C, all six previously reported negative specimens had values above 10 mg/hg after 48 h, with four of the six above 40 mg/hg.

The results of this study reveal several trends. First, at both storage temperatures, ethanol was formed in all of the specimens when sodium fluoride was absent. Second, ethanol formation rate was slower at reduced temperatures, but, with extended time, the refrigerated specimens produced ethanol concentrations comparable to those at room

temperature at lesser times. Third, ethanol formation was virtually eliminated when specimens were homogenized in 1.00% sodium fluoride, whether stored at either 4°C or 25°C.

It is notable that during the course of our experiments, we observed various volatile organic compounds being formed in these samples. However, we also observed the various volatiles which were initially present to sometimes decrease in concentration over time. Thus, changes in any of the volatile organics, including ethanol itself, were clearly shown to **not** be reliable indicators of postmortem microbial action.

V. Conclusions

It is clear from these experiments that all nine tissue specimens from actual aviation accident victims contained microbes capable of ethanol production. We have demonstrated that even at 4°C, significant amounts of postmortem microbial ethanol does form in the absence of a preservative. Additionally, we have demonstrated that the addition of sodium fluoride to postmortem tissue specimens during the homogenization process prevented the formation of ethanol at storage temperatures of both 4°C and 25°C. We recommend that all tissue specimens should be immediately homogenized in the presence of added sodium fluoride as a precaution to block any further postmortem microbial ethanol formation. Furthermore, we recommend these specimens not be stored at temperatures above 4°C to further ensure stability. However, caution must always be used when interpreting any ethanol results from postmortem samples since, even if these

precautions are followed, we cannot rule out the possibility of postmortem microbial ethanol production in specimens prior to receipt and processing by the laboratory.

Liquid Chromatography Coupled with Atmospheric Pressure Chemical Ionization Mass Spectrometry

Chapter 3

I. Introduction

The separation, and subsequent detection, of analytes of interest forms the backbone upon which the field of analytical forensic toxicology is based. A variety of separation techniques are available for use in such an analytical laboratory. These can be coupled with any one of numerous available detectors for the determination of analytes in biological samples. High performance liquid chromatography (LC) is one of the most common separation techniques used today. The first published report describing liquid chromatography appeared in 1906 when Mikhail Tswett, a botanist, used a glass tube filled with calcium carbonate and ether as the mobile phase to separate plant pigments. He detected the individual components visually as they eluted from the base of the column.[189, 190] Since that point liquid chromatography, and more recently, high performance liquid chromatography has grown considerably in its application to a wide variety of analytical problems.

Like LC, mass spectrometry has been employed in analytical laboratories for decades. By the early 1950's magnet based mass spectrometers had been designed, built, and shown to be reasonably functional. In 1953 Wolfgang Paul produced the first example of what is now the most common mass analyzer, the quadrupole mass spectrometer.[191]

The coupling of mass spectrometry with LC was initially difficult to achieve. This difficulty was due to the conflicting physical state requirements; the LC effluent was necessarily a liquid while the MS required a gas phase at very low pressures at the inlet for optimal operation. Much of the work that led to the discovery of a suitable interface for these two instruments was done in the late 1960's by Tal'roze *et al.*[192] The first interface capable of performing this difficult union was known as the moving wire interface, which was first demonstrated in 1974.[193] Since the initial combination of these two techniques nearly thirty years ago, this technology has dramatically improved in sensitivity, selectivity, detection limits and breadth of applicability. The use of LC/MS is rapidly becoming a mainstay in the field of analytical forensic toxicology.[194-201]

This chapter first discusses aspects of basic LC. Following a brief LC review, ion trap-mass spectrometry, as it applied to our investigations, will be discussed.

II. High Performance Liquid Chromatography

A. Introduction

A high performance liquid chromatography (LC) system is comprised of a mobile phase reservoir, a mechanical pump, an injector, an analytical column, a detector and a data recorder or a computer that translates the output data. A representative LC system can be seen in Figure 3-1 below.

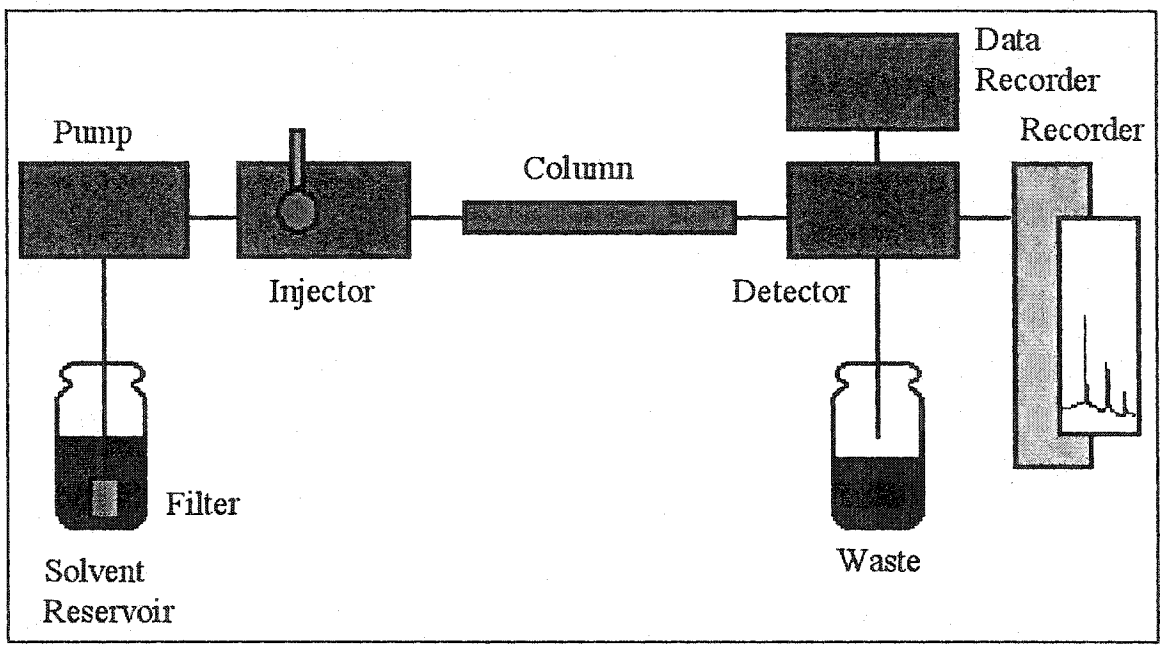


Figure 3-1. Representative LC system.

Many different detectors can be used to quantifiy analytes of interest after their separation. In our studies we employed a mass spectrometer for this purpose as will be discussed later. The analytical column in the LC system is perhaps the most studied and variable component of such a system. Today, a wide variety of LC columns are

available, providing the analyst with the capability to perform nearly any separation desired. The theory behind LC separations is discussed in the following sections.

B. Analyte Migration

The distribution of analytes of interest between a stationary phase and a mobile phase, typically liquid or gas, represents the basic principle of all chromatographic separations. This distribution directly affects the amount of time required for analytes to travel through the length of a packed column, which provides the basis for LC separation. The time required for the elution of an analyte is dependent on the amount of time spent by the analyte adsorbed on the stationary phase of the column versus the amount of time spent traveling along with the mobile phase. The ratio of the concentration of the analyte in the stationary phase, C_s, to the concentration of an analyte in the mobile phase, C_m, is defined as the partition coefficient, K, for that analyte and this equilibrium constantis given as:

$$K = \frac{C_s}{C_m} \qquad \textbf{Equation 3-1}$$

The fraction of analyte molecule present in the mobile phase at any one time is thus provided by:

$$\frac{C_m}{C_s + C_m} = \frac{1}{(1+K)} \qquad \textbf{Equation 3-2}$$

And, the fraction of analyte molecules present in the stationary phase at any one time is provided by:

$$\frac{C_s}{C_s + C_m} = \frac{K}{(1+K)} \qquad \textbf{Equation 3-3}$$

Figure 3-2 below represents the output of a typical LC experiment.

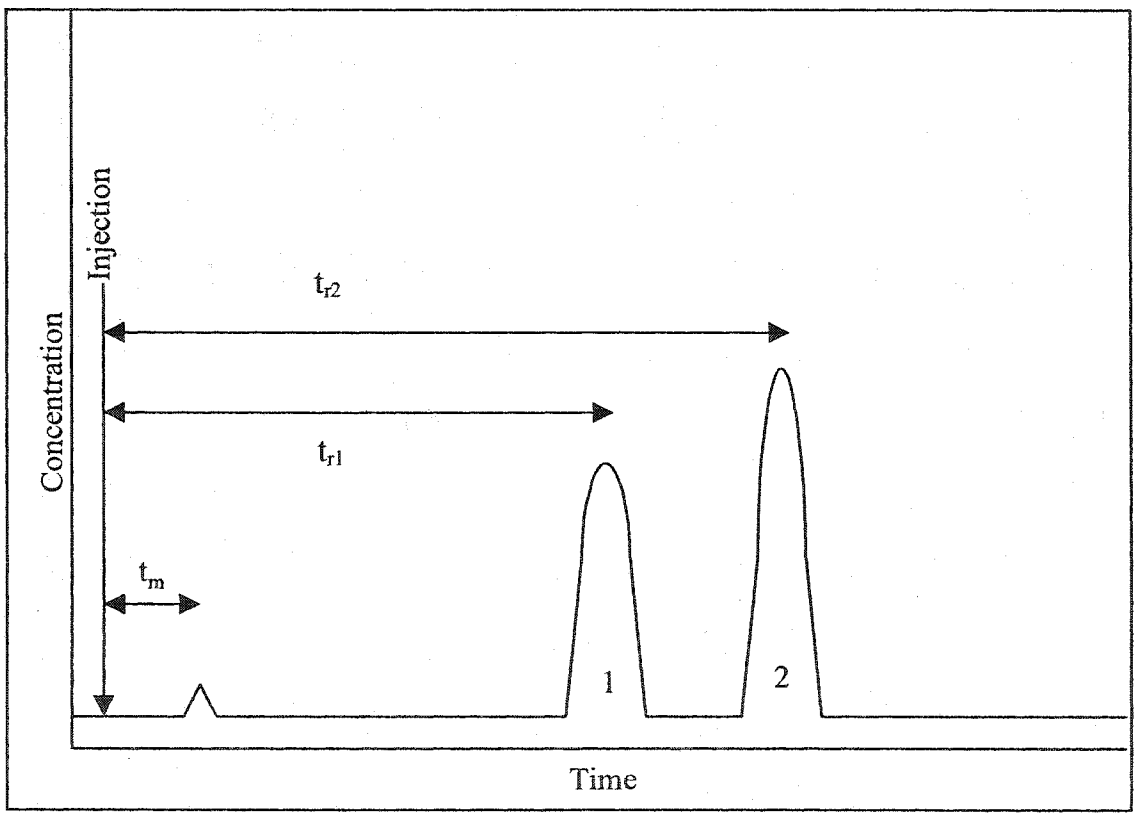


Figure 3-2. Representative LC chromatogram.

The amount of time required for an analyte to travel the entire length of an analytical column is known as the retention time (t_r). Retention time is one of the most important parameters obtained from a liquid chromatogram. Comparison of the retention time of an unknown analyte to that of a standard can be used to provide unknown identification. Two eluting components, 1 and 2, are indicated in Figure 3-2 to have retention times of t_{r1} and t_{r2}, respectively. The special retention time of t_m indicates a non-retained component. The adjusted retention time (t_r') for a given component is the time required for a compound to travel the length of an analytical column relative to the retention time of an non-retained component (t_m) as shown in Figure 3-2. This parameter can be calculated as:

$$t_r' = t_r - t_m \qquad \textbf{Equation 3-4}$$

Another equally important and alternative retention parameter related to analyte migration in LC is the capacity factor (k'). The capacity factor is defined as the ratio of the adjusted retention time to the elution time of a non-retained substance, as seen below:

$$k' = \frac{(t_R - t_m)}{(t_m)} \qquad \textbf{Equation 3-5}$$

The capacity factor is considered by many to be more important than either the retention time or the adjusted retention time because it should be constant from one LC system to the next, independent of flow rate, column length, and other variables. The constant nature of k' should only depend upon utilization of the same column material, liquid

elution material and temperature. Therefore, k' could also be advantageously used for the identification of an unknown analyte in an even wider variety of circumstances.

C. Column Efficiency

Unlike retention times and capacity factors, which are primarily related to individual analytes, the column efficiency is an important parameter which tells us something about the separation power of the entire chromatographic setup in an LC system. The column efficiency in an LC separation is primarily defined as the number of theoretical plates, with more theoretical plates indicating greater column efficiency. A theoretical plate is an imaginary region contained within the column where analyte partitioning achieves equilibrium between the stationary and mobile phases. An analyte can be perceived to move through the column in an incremental fashion, seemingly pausing at each theoretical plate, taking time to achieve a momentary equilibrium between the stationary and mobile phase within that theoretical plate. The mobile phase, in this scenario, then moves the analyte down the column to interact with the stationary phase in the next theoretical plate. This somewhat discontinuous process of analyte adsorption onto the stationary phase, mobile phase movement and subsequent desorption into the following mobile phase, repeats itself until the analyte exits the column. The more total time an analyte spends adsorbed onto the stationary phase in this process the longer it takes for that analyte to travel the length of the column, resulting in an increased retention time (t_r). An equation for calculation of the number of theoretical plates (N) for a given column is provided by:

$$N = \frac{L}{H} = 16\left(\frac{t_r}{s_t}\right)^2$$ **Equation 3-6**

where L represents the length of the column and H represents the height equivalent to a theoretical plate (HETP). Thus, greater efficiency for a column is indicated, in general, by a smaller value for H. As also seen in equation 3-6, the number of theoretical plates can be calculated as the square of the ratio of the retention time of an analyte, t_r, to the peak standard deviation, σ_t. The peak standard deviation, σ_t, is derived from assuming the peak to be gaussian in character. If gaussian, σ_t is simply the peak width measured at 0.607 times the peak height. The value of σ_t can also be determined as one-fourth of the width of the peak measured at the base. The higher the number of theoretical plates the more analytes that can be resolved in a given time and/or elution volume. Typical numbers of theoretical plates found in modern analytical LC columns range from 3,000–10,000. In some situations, even less than 3,000 plates is more than sufficient for successful analysis.

D. Analyte Resolution

Resolution between two eluting analytes is defined as the ratio of the distance between the centers of the two corresponding chromatography peaks, divided by the average width of those peaks. In general, increasing the length of the column increases the resolution of two adjacent analytes. In theory, any required separation can be achieved, given a column of sufficient length and availability of the associated increase in time required for the separation to occur. The resolution (R_s) is calculated as:

$$R_s = \frac{t_{r2} - t_{r1}}{0.5(w_1 + w_2)}$$ **Equation 3-7**

where t_{r1} and t_{r2} are the retention times of the two analytes in question, and w_1 and w_2 are the widths of their respective peaks measured at the baseline. Most chromatographers accept a resolution of 1.5 or greater to indicate that the two peaks are "baseline" separated. A resolution of 1.0 to 1.5 indicates the two peaks are sufficiently separated for most analytical applications, although such peaks may exhibit a slight overlap.

E. Liquid Chromatography Summary

It is clear that a higher degree of efficiency in an analytical column, or a larger number of smaller theoretical plates, results in sharper and/or narrower peaks that are more easily separated from other peaks and, thus, provides better resolution. As such, our efforts at achieving adequate separations primarily focused on achieving sufficient efficiency and resolution of the desired analytes.

III. Mass Spectrometry

A. Introduction

Mass spectrometry, by definition, is the separation of charged particles according to the mass-to-charge ratio of the particular atomic or molecular species investigated.

Analyte molecules are most easily introduced to the mass spectrometer via the source in the gas phase. Inside the source, some of these molecules are ionized by electron bombardment and/or other suitable means. Following ionization, the charged particles are accelerated by application of a large voltage and their direction of movement is generally controlled by ion optics composed of various charged deflector plates. After acceleration, the charged particles are separated according to their mass-to-charge (*m/z*), ratio in a mass analyzer portion of the spectrometer. Particles that survive passage through the mass analyzer are subsequently typically detected by an electron-multiplying device. The quadrupole mass analyzer consists of four metallic rods, through which the ions to be separated are passed. The individual rods have a fixed dc and an alternating RF voltage applied to them. Depending on the electric field, only ions of a particular mass-to-charge ratio, which is selected by the operator, will be stable and, thus, finally arrive at the detector. All other ions will be out of focus and will be deflected into the rods. By varying the strengths and frequencies of these electric fields, different ions can be caused to achieve stable passage through the rods and, thus, be detected. Continuous introduction of a given sample followed by scanning the selected *m/z* ratios provides one with an output signal as a function of the *m/z* ratio. Such a scan is commonly referred to as a mass spectrum.

The experiments described in the following chapters were performed on an ion trap mass spectrometer manufactured by Finnigan and forming the detector portion of the instrument known as the LCQ®. Ionization in these experiments occurred via an atmospheric pressure chemical ionization (APCI) apparatus that will be discussed in the next section. After ionization occurs, ions are directed toward the ion trap mass analyzer

through two consecutive octapoles. These octapoles are operated in a manner similar to a quadrupole, as described earlier. However, octapoles have eight metal rods as opposed to the four in a quadrupole. The greater number of rods allows for more precise *m/z* selection of the ions, which are then passed on to the ion trap. The ion trap mass analyzer uses a combination of dc and RF voltages to maintain selected ions in a stable circular orbit. After the stable orbit is achieved, ions can be selectively ejected out of the trap into the detector for quantification.

B. Atmospheric Pressure Chemical Ionization (APCI)

1. APCI Probe Assembly

Atmospheric pressure chemical ionization was first introduced in the late 1980's and is the second most common ionization technique employed today for LC/MS.[202] Many of the difficulties associated with the initial union of LC with MS were caused by the introduction of a relatively large volume of liquid from the LC (resulting in an even larger volume of gas when vaporized) into the high vacuum input environment of the mass spectrometer. In APCI this problem is solved by the evaporation and removal of most of the solvent introduced into the MS before the high vacuum region is encountered. The APCI can be considered to be a flash vaporizer connected to a plasma-generating ion source. In the LCQ®, the molecules present in the mobile phase are introduced from the LC into the APCI probe through a 102 x 0.19 mm silica sample tube that is heated by a coiled heating element, as shown in the APCI probe assembly diagram of Figure 3-3. The effluent from the silica sample tube then passes through a vaporizer tube, which is maintained at 450°C. Nearly all effluent leaving the vaporizer tube is in the gas phase.

The vaporized molecules, forced through the heated tube by nitrogen gas flow, encounter a coronal discharge upon emerging from the outlet of the tube. A high potential, ± 3 kV to ± 5 kV, is applied to a metallic needle known as the "corona needle" producing a discharge current of 3-6 μA as it arcs to the spray shield portion of the APCI stack components (see below). When encountering this electrical discharge the solvent molecules form plasma ions. In the positive chemical ionization (PCI) mode, which was employed for all of our experiments, these newly formed solvent plasma ions transfer protons to neutral analyte molecules in a very soft ionization procedure to predominately form protonated molecular ions and/or modestly solvated molecular ions. Obviously, an appropriate LC solvent is required for the successful ionization of analyte molecules in this approach. The precise mechanism of formation of protonated molecular ions is not known to this day.

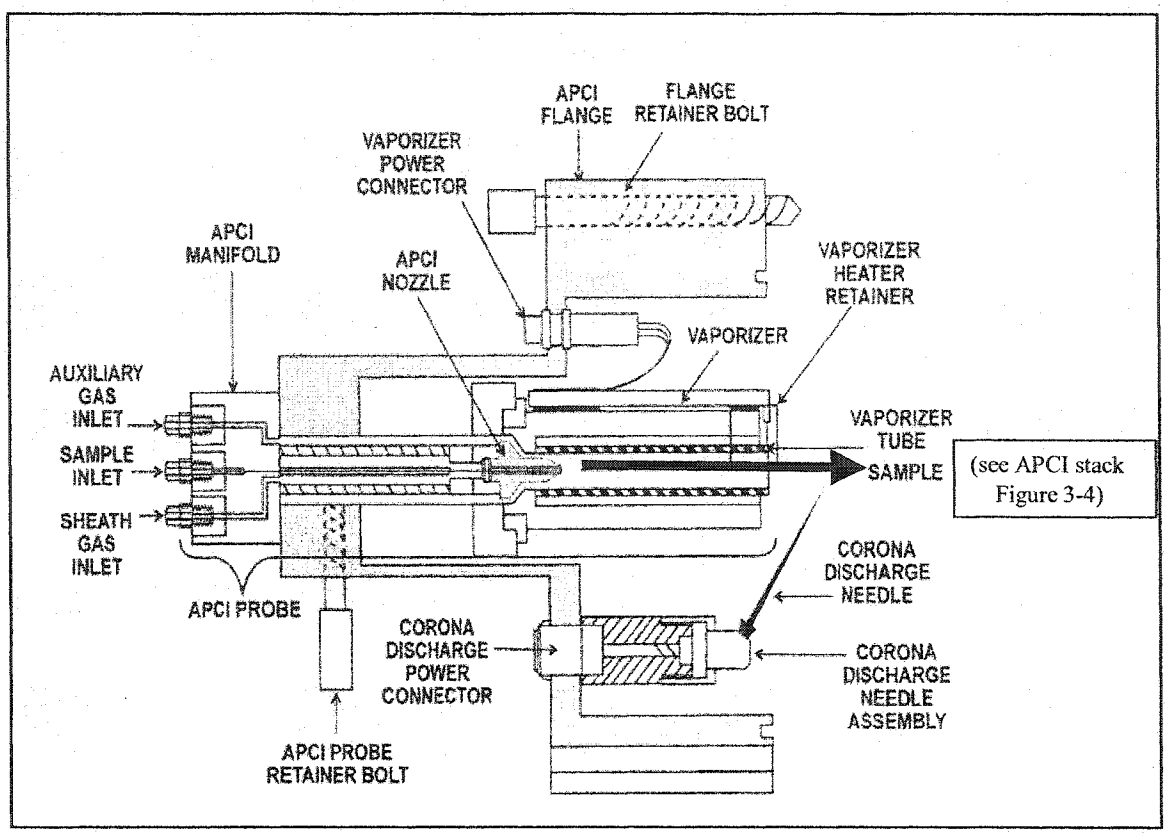


Figure 3-3. APCI probe assembly in the LCQ®.[203]

2. APCI Stack

The ions that are formed in the APCI probe assembly in the LCQ® migrate toward the stainless steel spray shield as they exit the capillary sample tube. This spray shield, shown in Figure 3-4 below, is held at ground potential. In the middle of the spray shield is a heated capillary, which is 0.020" in diameter, and is maintained at a slightly negative potential, -10 to -30 V, to attract the positive ions and a temperature between 150°C and 200°C. The newly formed ions and any solvent droplets that may remain after vaporization are pushed toward the spray shield by the flow of nitrogen gas. These particles enter and pass through the heated capillary where most of the remaining solvent

is driven off, leaving behind charged analyte ions and relatively few neutral compounds. As particles exit the heated capillary they pass through an object known as the tube lens. This lens begins to focus the ions that passed through the heated capillary. Following the tube lens, the ions and remaining neutral molecules encounter the skimmer. The skimmer serves one main purpose, to remove the majority of the remaining neutral particles. Removing such neutral particles helps minimize background noise from the resulting data. The skimmer is shaped like a cone, with the point of the cone facing toward the source of the particles. The very small hole in the center of the cone allows for a substantial differential vacuum to be placed on either side of the skimmer. The pressure on the ion source side of the skimmer is held at approximately 1 torr, while on the mass analyzer side the pressure is maintained at approximately 1×10^{-5} torr. The hole in the skimmer is notably placed slightly off center from the direct exit path of the heated capillary. A small potential, which is sufficient to attract the charged particles, is applied to a small ring that surrounds the hole of the skimmer. Neutral molecules, not affected by this electrical field, simply run into the side of the skimmer, preventing their transmission to the following mass analyzer.

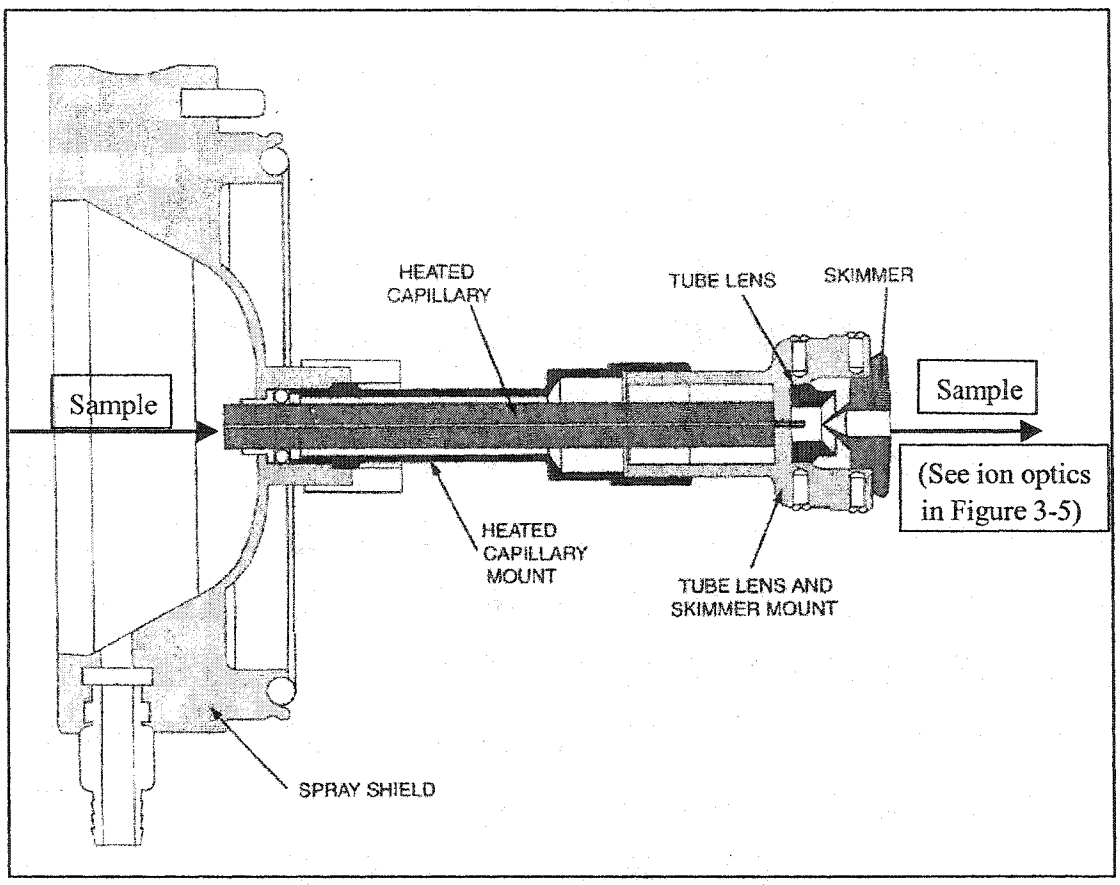


Figure 3-4. LCQ® APCI stack components.[203]

C. *Ion Transmission and Detection*

1. Ion Optics

The primary ion optics portion of the LCQ®, as seen in Figure 3-5, consists of an octapole, the interoctapole lens/gate and a second octapole. The entire region is under high vacuum, approximately 1 x 10^{-5} torr, and receives input ions from the skimmer in the previous section. The dc-offset voltage applied to the first octapole draws ions into its entrance and accelerates the ions sufficiently for passage through both octapoles. The

applied ac and dc voltages form an electric field within the octapole that guides the accelerated, ionized molecules along the longitudinal axis. The dc voltage applied to the metal rods is of opposite charge to the ions of interest. The inter-octapole lens connecting the first and second octapoles serves as an ion gate, allowing ions to pass or not pass at the command of the operator. A large potential, approximately 200 V and having the same sign as the ions of interest, is simply applied to the lens whenever one wants to prevent ions from passing through the lens and entering the second octapole. When the operator wants to introduce more ions into the trap, the potential is removed from the lens, and the ions pass freely into the second octapole. The controlling computer routinely performs this gating function for the operator as it alternately senses an empty and full ion trap. The second octapole is fundamentally identical to the first and serves as the final filter in the ion optics portion of the LCQ®. The second octapole guides the stream of ions having the desired m/z ratio directly into the entrance of the ion trap.

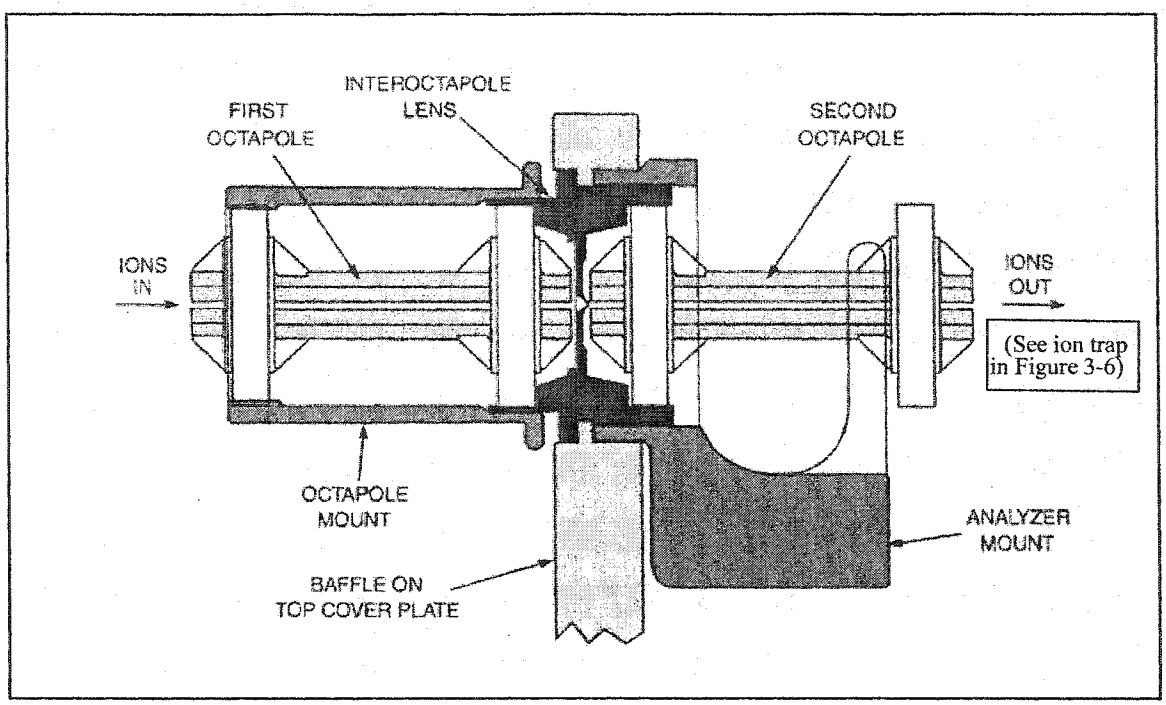


Figure 3-5. LCQ® ion optics.[203]

2. Mass Analyzer

The LCQ® incorporates an ion trap mass analyzer as shown in Figure 3-6, for the final step in the isolation and selection of ions to be detected. The ion trap as a unit is composed of three individual electrodes. All three electrodes are made of stainless steel. The inner surfaces of these electrodes are hyperbolic in shape. While a detailed discussion of the theory of the ion trap is beyond the scope of this chapter, a brief overview is given below. As can be seen in Figure 3-6 the ion trap consists of an entrance endcap electrode, a ring electrode, and an exit endcap electrode. To attract ions into the trap a 10 V dc voltage opposite in polarity to the ions of interest is applied to the entrance endcap electrode. The trap is filled with helium gas at pressure of approximately 1.5 mtorr. This helium gas acts as a damping agent for the ions in motion

within the trap. This damping slows the motion of the ions, which reduces the possibility of the analyte ions either colliding with one another or randomly colliding with the surface of the electrode. Once a sufficient number of analyte ions have been accumulated in the ion trap, an ac voltage of variable frequency (usually 0.76 MHz), and amplitude, 0 V to 8500 V, depending on the *m/z* ratio of the desired ion, is applied to the ring electrode. Since this voltage is in the radio frequency range it is referred to as the ring electrode RF voltage. This RF voltage applied to the ring electrode creates a three-dimensional field within the trap. This 3-D field causes the motion of the ions contained within the trap to conform to a circular or nearly circular path. The trapped ions can stray in the axial direction, toward one of the two endcap electrodes, or can stray in the radial direction, toward the ring electrode. In order for an ion to remain in the trap its motion in both directions must be stabilized in the desired circular path. The RF voltage applied to the ring electrode determines the mass-to-charge ratio for ions that are capable of adopting a stable orbit within the trap in a single experiment. The ions that are not able to develop a stable orbit within the trap collide with the electrodes and are neutralized. Ions emerge from the trap through the exit hole as a result of a selective increase in the voltage of the endcap electrode.

Once a given group of ions having a particular mass-to-charge ratio are contained in a stable orbit in the trap, it is possible to selectively fragment these trapped ions so that MS/MS (MS^2) and, subsequently, MS/MS/MS (MS^3) can be performed. The helium present in the trap provides a source of reasonably soft fragmentation through CID for each such fragmentation process. The LCQ® is thus, capable of isolating a product ion, fragmenting that ion and collecting the resulting daughter ion(s), and fragmenting the

daughter ion and collect the granddaughter ion(s). This sequence of fragmentation and collection can occur up to ten successive times for a specific analyte of interest. When the desired ions have been isolated within the trap after successive rounds of fragmentation, they are ejected from the trap through a hole in the endcap electrode by application of a resonance ejection RF voltage applied to the endcap electrodes. The ions are then directed to the detection element.

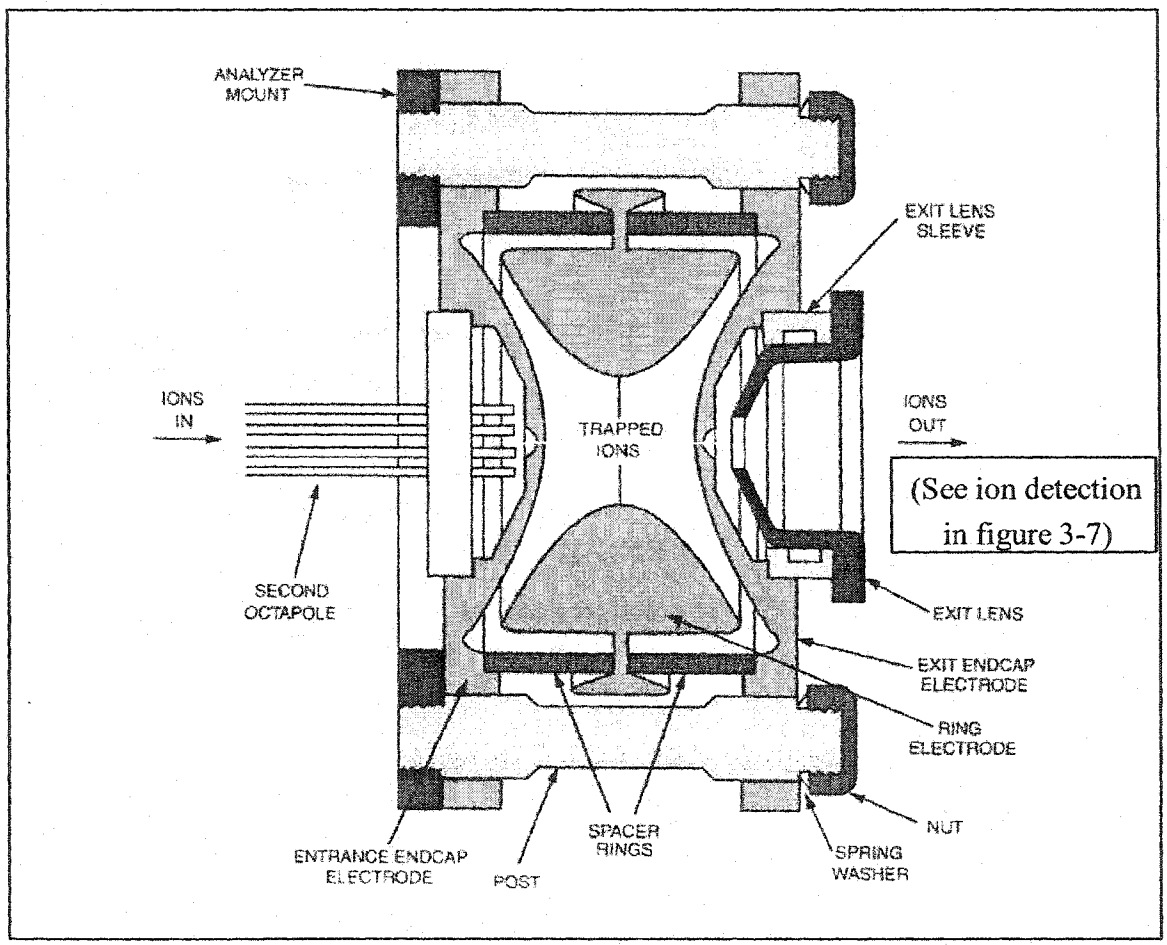


Figure 3-6. LCQ® ion trap components.[203]

3. Ion Detection

As ions are ejected from the ion trap they leave through a hole in the exit endcap electrode. An exit lens serves to focus the ions immediately after they leave the trap. A conversion dynode, seen in Figure 3-7, attracts the exiting ions so they can be subsequently detected and quantified. The dynode has a potential of 15 kV (of opposite charge to that of the analyte ions) applied to it, which attracts ions of opposite charge. When the rapidly accelerated ions collide with the surface of the conversion dynode multiple electrons are ejected. The electrons are directed into the opening of an electron multiplier. The electron multiplier converts the electrons detected into a significantly amplified output signal that is converted by the associated computer software into a mass spectrum.

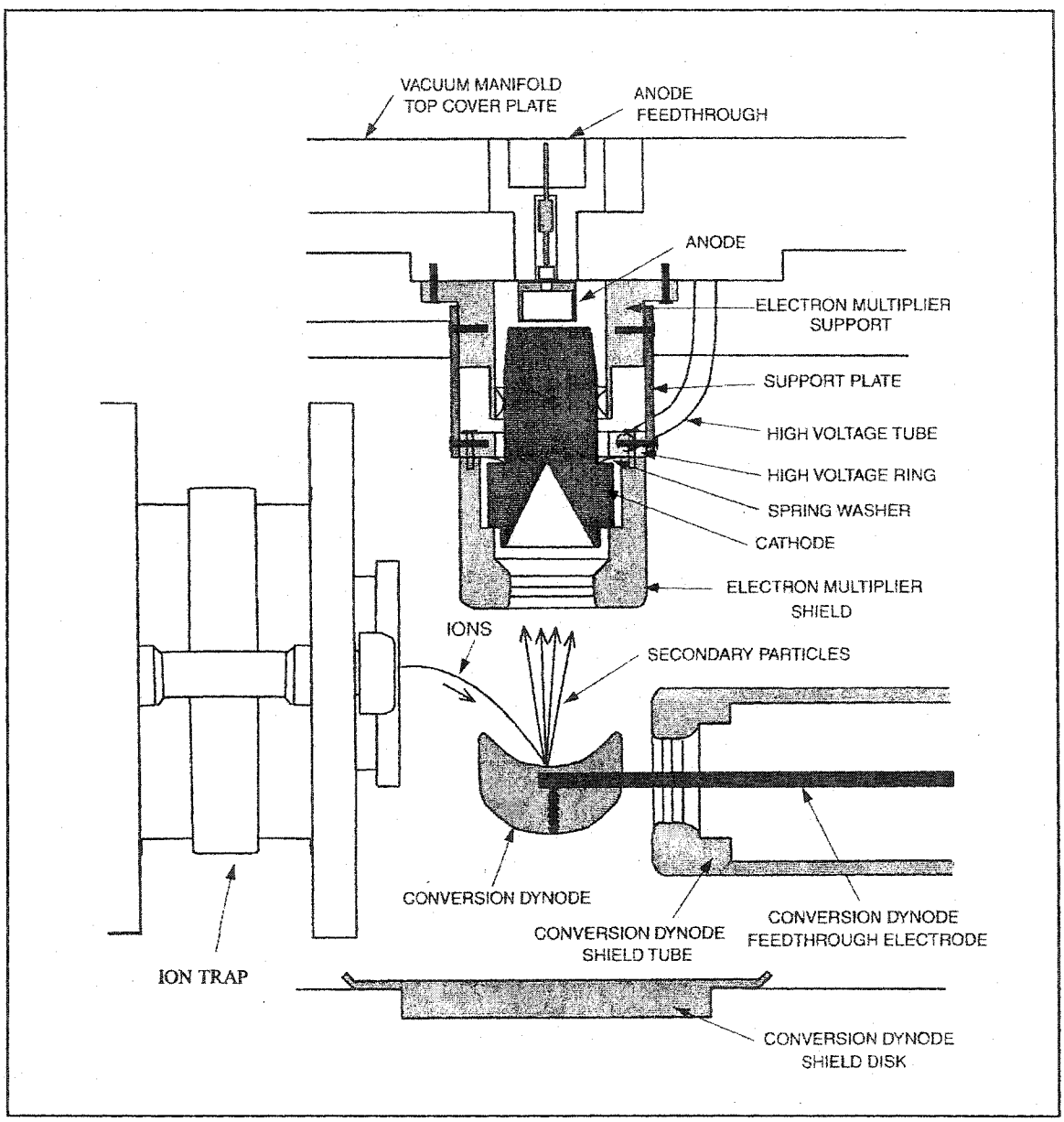


Figure 3-7. LCQ® ion detection components.[203]

IV. Conclusions

In the past twenty years the use of LC/MS as an analytical technique has grown exponentially. With improvements in technology, these instruments have become easier to use, more precise, more accurate, more versatile and more cost effective. These improvements have led to a substantially more common appearance and utilization of LC/MS in analytical laboratories. More in-depth reviews of LC can be found elsewhere[204-206] and more in-depth reviews of LC/APCI-MS can also be found elsewhere.[194, 207-211]

Establishment of an LC/MS Method for the Simultaneous Determination of 5-Hydroxyindole-3-acetic acid and 5-Hydroxytryptophol

Chapter 4

I. Introduction

Possible exploitation of the metabolism of serotonin as a biological marker for ethanol consumption began to gain considerable interest in the field of forensic science in the past decade.[133,136,136] The metabolism of 5-HT initially involves oxidative deamination to form the transitory intermediate, 5-hydroxyindole-3-acetaldehyde (5-HIAL). This intermediate can undergo either oxidation or reduction as previously shown in Figure 1-4, and discussed in chapter 1. Oxidation of the aldehyde, catalyzed by aldehyde dehydrogenase, leads to formation of 5-hydroxyindole-3-acetic acid (5-HIAA),

the predominant metabolite of 5-HT.[136, 142] Reduction, catalyzed by aldehyde reductase, leads to formation of 5-hydroxytryptophol (5-HTOL), usually a relatively minor metabolite of 5-HT.[138] However, ethanol consumption has been shown to lead to a relatively significantly enhanced production of 5-HTOL.

An increase in 5-HTOL concentration following ethanol consumption was first reported in 1967.[154] Since that time it has been clearly demonstrated that consumption of ethanol shifts 5-HT metabolism to promote formation of 5-HTOL and, some reports indicate, to reduce the formation of 5-HIAA.[20, 85, 132, 138] Thus, ingested ethanol leads to an elevation in the 5-HTOL/5-HIAA ratio. The 5-HTOL/5-HIAA ratio has been reported to remain elevated for up to six hours after ingested ethanol has been eliminated from the body.[85] As such, the 5-HTOL/5-HIAA ratio has already been applied to ethanol cessation monitoring programs as a marker of recent ethanol ingestion.[116, 130, 146] The 5-HTOL/5-HIAA ratio has also been briefly investigated for possible use in postmortem urine samples.[85] We had hoped, thus, that this ratio might serve as a more reliable, reproducible and dependable tool in analytical forensic toxicology for accurately differentiating between the postmortem microbial formation and antemortem ingestion of ethanol.

Historically, levels of 5-HTOL and 5-HIAA in individual samples have been measured using two completely different analytical techniques. 5-HIAA concentrations are typically measured at sub-nanomolar levels using liquid chromatography with electrochemical detection (LC/EC).[158, 212] 5-HTOL is also accessible by LC/EC, but the detection limits are typically insufficient to measure this compound in most pertinent biological specimens. For this reason, 5-HTOL has routinely been analyzed using gas

chromatography with mass spectrometric detection (GC/MS) following derivitization with pentafluoroproprionic acid anhydride.[156] The employment of two different analytical techniques to obtain the 5-HTOL/5-HIAA ratio in a specimen obviously decreases the precision and reliability of the final result. To date, this problem has effectively impeded the clear demonstration of this ratio as a marker for ethanol ingestion in postmortem specimens.

This chapter describes the development of a single analytical method for the simultaneous determination of both 5-HTOL and 5-HIAA using liquid chromatography with mass spectrometric detection (LC/MS).

II. Materials and Methods

A. Chemicals and Solutions

All aqueous solutions were prepared using double deionized water (DDW), which was obtained using a Milli-QT_{plus} Ultra-Pure Reagent Water System (Millipore®, Continental Water Systems, El Paso, TX).

The following chemicals were purchased from Sigma Chemical Company (St. Louis, MO) in high purity and used without any further purification.

5-hydroxytryptophol

5-hydroxyindole-3-acetic acid

5-methoxy-2-methyl-3-indoleacetic acid

ß-glucuronidase

Sodium chloride

Sodium acetate

The following chemicals were purchased from Fisher Scientific (Pittsburgh, PA) in high purity and used without any further purification.

Methanol

Acetonitrile

Ammonium hydroxide

Hydrochloric acid

Ethyl acetate

Nitric acid

Formic acid was purchased as a 97% pure solution from ICN (ICN Biomedicals, Inc., Irvine, CA) and used without any further purification.

N,O-bis[trimethylsilyl]trifluoroacetamide with 1% trimethylchlorosilane (BSTFA-1% TMCS) was purchased from Pierce (Pierce Chemicals, Rockford, IL) and used without any further purification.

B. Metabolite Stock Solutions

Two separate 10 mL stock solutions of both 5-HTOL and 5-HIAA were prepared independently at 1.00 mg/mL (5.64 mM 5-HTOL; 5.23 mM 5-HIAA) in methanol. Weighing of standards was achieved using a Sartorius semi-micro analytical balance purchased from the Aldringer Company (Aldringer Co., Dallas, TX). Each of these stock solutions was derived from a unique lot of dry chemical obtained from the manufacturer. These two stock solutions were subsequently identified as calibrators and controls. These indolic compounds are light sensitive, so care was taken to use volumetric flasks wrapped

in aluminum foil to prevent photodegradation. Once prepared, the solutions were transferred to 20 mL amber glass bottles, capped and placed in the freezer for storage at -20°C. These solutions were stable for at least 1 month (later shown stable for up to 180 days).[213] However, for maximum assurance of the quality of data, we never used any stock solutions which were over 30 days old.

C. Mobile Phase Preparation

The aqueous portion of the LC buffer was 50.0 mM formic acid adjusted to pH 5.00 with conc. ammonium hydroxide. Aqueous buffer and acetonitrile were mixed in a 98:2 ratio, respectively, to help prevent the growth of microbes, and this mixture was filtered through a vacuum filtering apparatus that incorporated a 0.45 μm GH polypro 47 mm hydrophilic, polypropylene membrane filter obtained from Pall Gelman laboratory (Pall Corp., East Hills, NY). The primary organic component of the mobile phase was LC grade methanol, which was filtered prior to use through a vacuum filter apparatus that incorporated the same type of membrane filter. The ratio of the previous aqueous mixture to methanol was 20:80 in the final LC mobile phase.

D. ß-Glucuronidase Solution

Since 5-HTOL in human-derived specimens is predominately found as the glucuronide derivative, we initially hydrolyzed the samples using the enzyme *ß*-glucuronidase. A solution of *ß*-glucuronidase was prepared by adding 2.5 mL of pH 5.00, 0.10 mM sodium acetate buffer to 250,000 units of the solid enzyme. The *ß*-glucuronidase solution was capped tightly and gently mixed for 5.00 minutes on a

Multi-Purpose Rotator, Model 151, obtained from Scientific Industries (Scientific Industries, Inc., Bohemia, NY) that was set to rotate at a slow speed. This yielded a final concentration of 100,000 units/mL. The type II lyophilized β-glucuronidase enzyme used contained 2.22 x 10^6 units/g, and was originally obtained from limpets, a type of marine gastropod. This solution was stored in the freezer at -20°C. Like the stock standards, this solution was discarded after storage for a maximum of 30 days. However, it typically was used entirely within 7 days following preparation.

E. Internal Standard Stock Solution

5-Methoxy-2-methyl-3-indoleacetic acid (5-MMIA), a compound not known to occur *in vivo*, was used as the internal standard for these experiments and was prepared at a concentration of 100 µg/mL (0.450 mM) in 100 mL of methanol by weighing 10 mg of the solid standard and quantitatively transferring it into a 100 mL volumetric flask. The volumetric flask was filled to the mark with methanol and mixed well. This solution was transferred to a clean, labeled 100 mL amber glass storage bottle, which was then placed in the freezer for storage at –20°C. This solution was stable for at least 1 month (later shown stable for up to 180 days).[213] However, for maximum assurance of data quality, we never used any stock solution which was over 30 days old.

F. LC/MS Instrumentation

Analyte separation was achieved using a Hewlett Packard 1100 LC (Hewlett Packard Co., Wilmington, DE) equipped with a Security Guard™ C-8 guard column (4.0 mm x 3.0 mm i.d., 3 µm particles) from Phenomenex® (Torrance, CA), followed

immediately by a Supelcosil™ LC-18 (150 mm x 4.6 mm i.d., 3 μm particles) analytical column from Supelco (Supelco/Sigma-Aldrich, Bellefonte, PA). Samples were injected using a Hewlett Packard G1313A autosampler. Identification and quantification were accomplished using the Finnigan model LCQ® atmospheric pressure chemical ionization (APCI) ion trap mass spectrometer (ThermoFinnigan Corp., San Jose, CA), utilizing nitrogen as the sheath gas and helium as the auxiliary gas. Control of the LC system, integration of the chromatographic peaks, and communication with the mass spectrometer were accomplished using a Gateway 2000 E-4600-SE personal computer using Xcalibur™ LC/MS software (ThermoFinnigan Corp., San Jose, CA).

G. LC/MS/MS and LC/MS/MS/MS Method Developement

Working with standard solutions prepared to contain only 5-HTOL or 5-HIAA, we began the initial investigation by examining the MS response received from these two compounds when injected directly into the LCQ®. Initial ionization evaluation of these compounds by direct injection into the LCQ® indicated that positive chemical ionization (PCI), creating the $[M+H]^+$ ions, was much more effective in signal production than negative chemical ionization (NCI), which formed the $[M-H]^-$ ions. APCI-PCI-MS conditions were optimized separately for each compound by infusing each individually into the system at a concentration of approximately 10 μg/mL and a constant flow rate of 25 μL/min. The infused solutions were prepared by dilution from the stock solutions using methanol as the diluent. Tuning the MS for the desired ions was then accomplished using the autotune feature of the Xcalibur™ software. Unfortunately, this initial investigation yielded mixed results. Figures 4-1 and 4-2 demonstrate the results of

analyzing these two compounds as pure standards when optimized, successively, for each of these two species. Optimization for the 5-HTOL MS detection required a mobile phase adjusted to a pH of 6.00, while optimization for the 5-HIAA MS detection required a mobile phase adjusted to a pH of 3.00. Figure 4-1 shows the results of the mobile phase optimized for 5-HTOL, ionization, which worked quite well for this analyte. Unfortunately, the same conditions worked very poorly, as shown in the same Figure 4-2, for 5-HIAA.

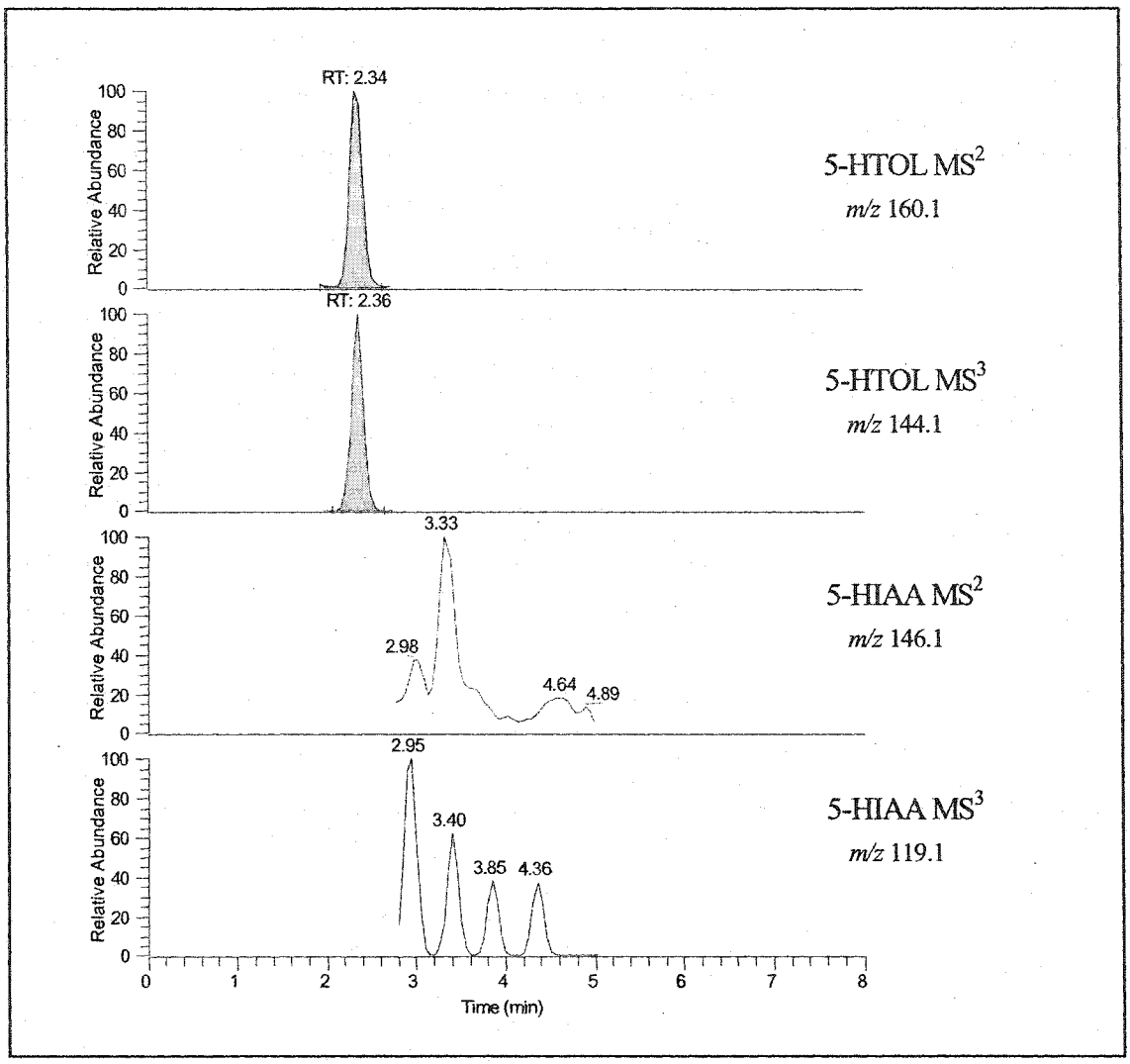


Figure 4-1. Initial analysis of 5-HTOL and 5-HIAA pure standards incorporating a pH 6.00 mobile phase optimized for 5-HTOL ionization.

Figure 4-2 demonstrates the results of our initial investigation utilizing a mobile phase that enhanced the ionization of 5-HIAA. This clearly provided very good results for 5-HIAA, but, unfortunately, very poor results for 5-HTOL.

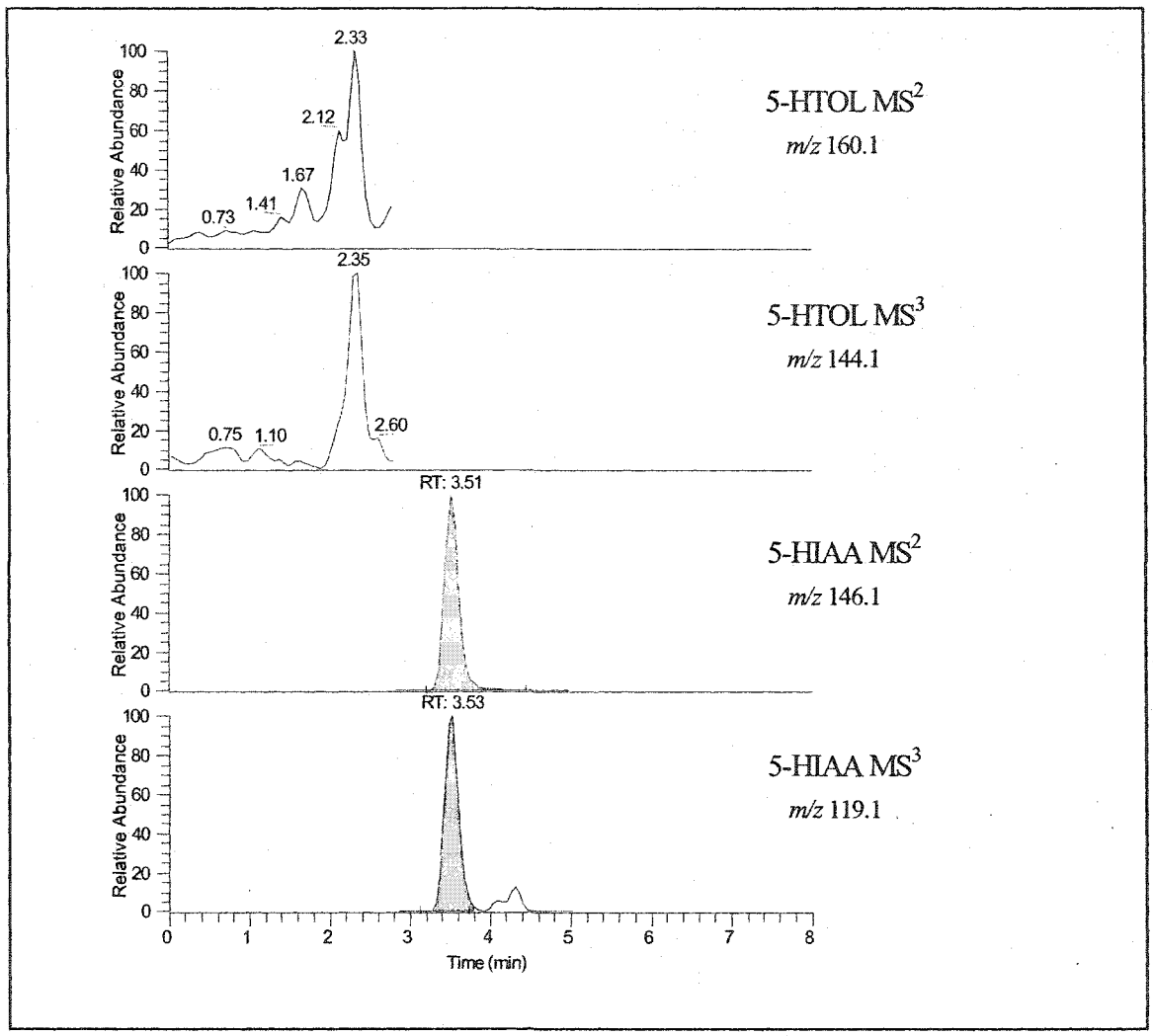


Figure 4-2. Initial analysis of 5-HTOL and 5-HIAA pure standards incorporating a pH 3.00 mobile phase optimized for 5-HIAA ionization.

Similar investigations were undertaken for all pH values between 2.00 and 8.00 at 0.50 pH increments. Substitution of acetonitrile for methanol and substitution of acetate

buffer components for the formate buffer components were attempted. Buffer concentration was also investigated, with concentrations ranging from 10 to 100 mM. Trifluoroacetic acid (TFA) is a commonly used ion-pairing agent for reversed-phase chromatography. Addition of varying amounts of this agent (0.01% to 0.1%) were also investigated. All of these modifications of the LC eluting solvent, unfortunately, yielded ionization conditions favorable to, at most, only one of the two analytes of concern. It became clear from the results of this initial investigation that no one mobile phase could be readily developed that would allow for the efficient ionization of both compounds simultaneously. Therefore, as will be described in detail below, 5-HTOL and 5-HIAA were routinely derivatized with TMS to form 5-HTOL-TMS and 5-HIAA-TMS derivatives. The internal standard, 5-MMIA, did not react with TMS to form a derivative, but provided adequate response levels in its underivatized form. The LC solvents selected for these derivatives, based on observations for the underivatized species discussed above and limited further pH investigations, contained 80% methanol, and 50.0 mM formic acid pH 5.00 as the buffering agent. Initial ionization evaluation of these derivatives again indicated that positive chemical ionization (PCI), creating the $[M+H]^+$ ions, was more effective than negative chemical ionization (NCI). APCI-PCI-MS conditions were optimized by separately infusing each of the desired compounds, at approximately 10 μg/mL following derivitization, directly into the LCQ® at 25 μL/min. Tuning the MS for the desired ion was accomplished using the autotune feature of the Xcalibur™ software. Figure 4-3 represents the results of an injection of the pure 5-HTOL and 5-HIAA standards after TMS derivatization.

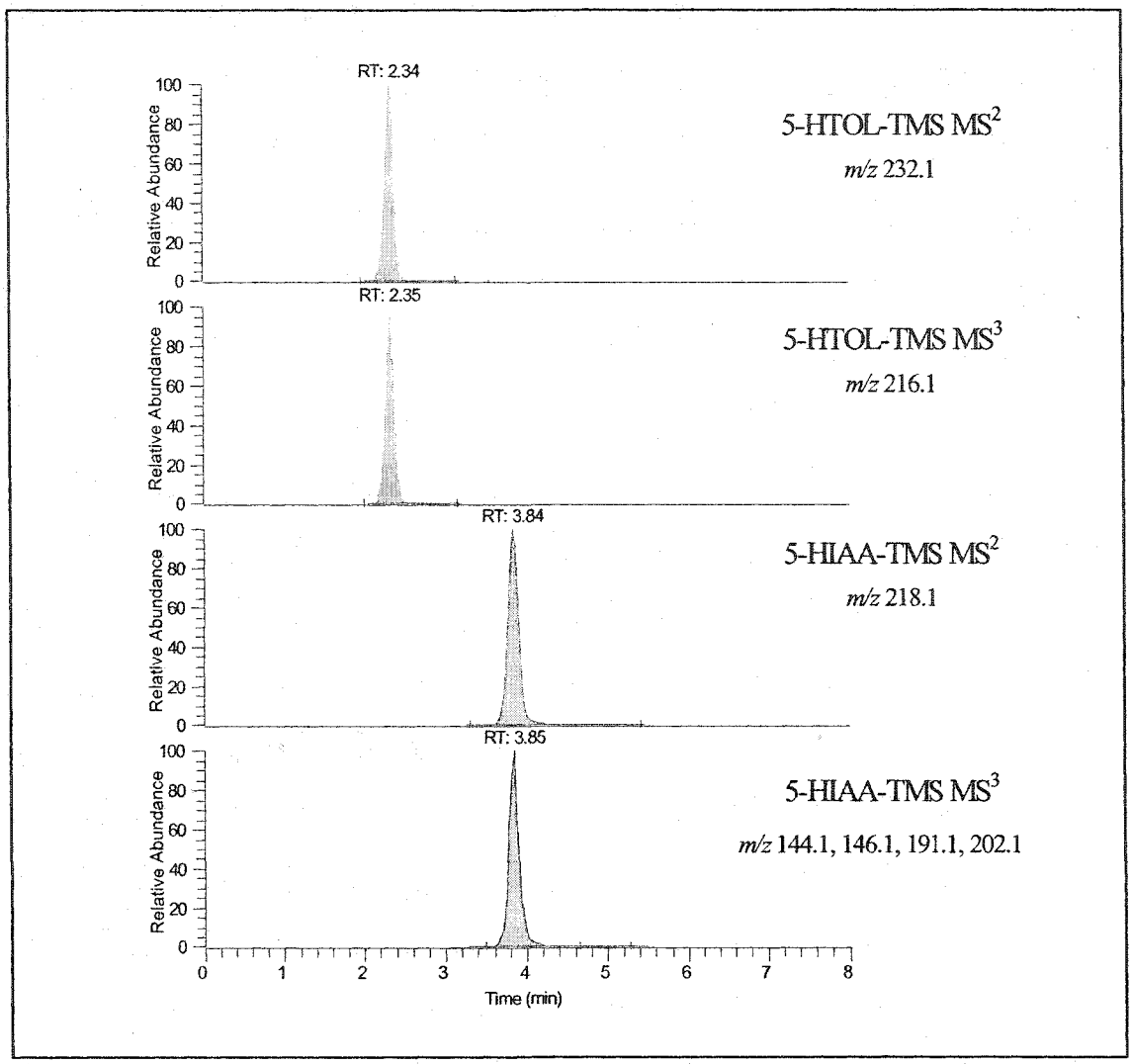


Figure 4-3. Initial analysis of 5-HTOL and 5-HIAA derivatized pure standards.

After optimizing the separation of the two derivatized target compounds, 5-MMIA was included as an internal standard. As mentioned above 5-MMIA does not form a TMS derivative under the conditions of this experiment. During an investigation of possible internal standards for this experiment, we had established a retention time for 5-MMIA of 1.7 minutes, which resulted in complete resolution from the TMS derivatives of both 5-HTOL and 5-HIAA, so the separation of all three components could easily be

achieved under these conditions. As a result of these preliminary APCI-PCI-MS investigations, each individual sample analysis was subsequently split into 3 unique data collection segments.

The operating conditions for data collection segment 1, used for analysis of 5-MMIA, were as follows: APCI capillary temperature, 150°C; APCI vaporizer temperature, 450°C; source voltage, 10.0 kV; source current, 5.0 μA; capillary voltage, 8.0 V; tube lens offset, 25.0 V; octapole 1 offset, -1.75 V; octapole 2 offset, -6.5 V; interoctapole lens voltage, -16.0 V; ion trap DC offset, -10 V; multiplier voltage, 0.0 V; micro-scan injection time, 200 msec. Segment 1 was further split into 2 separate scan events. Scan event 1 involved collection of the $[M+H]^+$ parent ion at *m/z* 220.1, and scan event 2 collected the daughter ion at *m/z* 174.1 following collision induced dissociation (CID) of the parent ion using a collision energy of 42%.

The operating conditions for data collection segment 2, used for the TMS derivative of 5-HTOL, were as follows: APCI capillary temperature, 150°C; APCI vaporizer temperature, 450°C; source voltage, 10.0 kV; source current, 5.0 μA; capillary voltage, 17.0 V; tube lens offset, 25.0 V; octapole 1 offset, -3.25 V; octapole 2 offset, -7.0 V; interoctapole lens voltage, -16.0 V; ion trap DC offset, -10 V; multiplier voltage, 0.0 V; micro-scan injection time, 200 msec. Segment 2 was further split into 3 separate scan events. Scan event 1 involved collection of the $[M+H]^+$ parent ion at *m/z* 250.1. Scan event 2 collected the daughter ion at *m/z* 232.1 following CID of the parent ion using a collision energy of 38%. Scan event 2 collected the granddaughter ion at *m/z* 216.1 following CID of the daughter ion using a collision energy of 48%.

The operating conditions for data collection segment 3, used for the TMS derivative of 5-HIAA, were as follows: APCI capillary temperature, 150°C; APCI vaporizer temperature, 450°C; source voltage, 10.0 kV; source current, 5.0 μA; capillary voltage, 3.0 V; tube lens offset, 10.0 V; octapole 1 offset, -4.25 V; octapole 2 offset, -7.0 V; interoctapole lens voltage, -22.0 V; ion trap DC offset, -10 V; multiplier voltage, 0.0 V; micro-scan injection time, 200 msec. Segment 3 was further split into 3 separate scan events. Scan event 1 involved collection of the $[M+H]^+$ parent ion at *m/z* 264.1. Scan event 2 collected the daughter ion at *m/z* 218.1 following CID of the parent ion using a collision energy of 36%. Scan event 2 collected the granddaughter ions at *m/z* 144.1, 146.1, 191.1 and 202.1 following CID of the daughter ion using a collision energy of 48%.

For all determinations, the LC was operated in an isocratic mode with a flow rate of 1.0 mL/min. The sample injection volume was 10 μL. The LC column was routinely equilibrated overnight prior to use. Following use, the column was washed and stored in a 50:50 mixture of methanol:H_2O. After the completion of an analysis sequence, the LC column was washed for 1 hour with 50:50, methanol:H_2O at a flow rate of 1 mL/min. Following this wash, the LCQ® vaporizer temperature was set to 500°C and the capillary temperature was set to 200°C. After temperature equilibration a mixture of 50:50, methanol:H_2O was introduced into the LCQ® at 1.00 mL/min for 15 minutes with the LCQ® running. This procedure was routinely used to remove any remaining mobile phase salts from the capillary tubing inlet in the LCQ® and ensure optimal performance the following day.

H. Calibration Curves and Controls

Calibration curves were prepared by dilution utilizing human certified negative urine as the diluent. Human certified negative urine, as obtained from the manufacturer (UTAK Laboratories Inc., Valencia, CA) is guaranteed to be free of any artificial pharmaceutical compounds and abnormal organic volatiles. Through the course of our initial investigations this processed urine was also found to be negative for both 5-HTOL and 5-HIAA. The calibrators were prepared from one set of the original stock standard solutions of 5-HTOL and 5-HIAA. Controls were prepared in a similar manner to calibrators, using the same human certified negative urine as diluent, but employing the second set of original stock solutions. Calibrators, controls and postmortem urine specimens, all referred to as simply "samples" below, were prepared and extracted in the following manner.

I. Specimen Extraction

It should be noted that 5-HTOL is extensively conjugated *in vivo* in humans as a glucuronide adduct. Therefore, to facilitate excretion of this compound from the body, an initial hydrolysis step was performed on all samples. Three mL aliquots of individual samples were transferred to 15 mL screw-topped culture tubes obtained from Fisher Scientific (Pittsburgh, PA). To each sample, 1.00 mL of a 1000 ng/mL (4.50 μM) internal standard solution, prepared by dilution of its stock solution with water, was added. Then, *ß*-glucuronidase solution (7500 units; 75 μL) followed by 1.00 mL of 0.10 mM pH 5.00 sodium acetate buffer was added to each sample. The samples were vortexed briefly on a Maxi Mix II type 37600 mixer obtained from Thermolyne

(Barnstead/Thermolyne Inc., Dubuque, IA) and incubated at 70°C for 45 minutes to facilitate hydrolysis of any glucuronide conjugates. Following hydrolysis, samples were allowed to cool to room temperature. Then 3.00 mL of a 0.10 M sodium acetate buffer, pH 6.00 and 0.50 mL of a saturated sodium chloride solution were added to each sample, and the tubes were briefly vortexed. Ethyl acetate (9.00 mL) was added to each tube, and the tube was tightly capped. The mixture was then placed on a Multi-Purpose Rotator, Model 151 obtained from Scientific Industries (Scientific Industries, Inc., Bohemia, NY) and gently mixed for 20 minutes by simple rotation at 6 rpm. Following mixing, the samples were centrifuged at 820xg for 5 minutes in a Jouan C-422 bench-top centrifuge, obtained from Jouan, Inc. (Winchester, VA). Following centrifugation, the organic (upper) layer of each sample was collected and transferred using a disposable pipette to a clean 10 mL conical tube obtained from Kimble Glass (Kimble Glass, Inc., Vineland, NJ). The organic layer was evaporated in a TurboVap® LV evaporator obtained from Zymark (Zymark, Inc., Hopkinton, MA) set at 40°C under a constant stream of nitrogen. Once dryness was achieved, the samples were removed from the evaporator. Both ethyl acetate (50 µL) and BSTFA/1%-TMCS (50 µL) were added to each sample. The tubes were capped, vortexed briefly and placed in a Multi-Blok™ heating block obtained from Lab-Line Instruments (Lab-Line Instruments, Inc., Melrose Park, IL) and set at 80°C for 20 minutes. Following derivitization, the tubes were removed from the heating block, allowed to cool to room temperature, and subsequently evaporated to dryness in a TurboVap® LV evaporator at 40°C under a constant stream of nitrogen. The samples were reconstituted in 50 µL of methanol, vortexed briefly, and transferred using a 50 µL pipette to micro-vials for LC/MS analysis.

J. Extraction Efficiency

The recovery of each analyte was determined using the following procedure.[214] Two groups, X and Y, of controls prepared using negative urine diluent were extracted in the same manner as described immediately above. Group X was spiked with a precisely known amount of both 5-HTOL and 5-HIAA prior to extraction, and group Y was spiked with the same precisely known amount of 5-HTOL and 5-HIAA following the liquid/liquid extraction step. The amounts of 5-HTOL and 5-HIAA varied from 1.0 ng/mL to 800 ng/mL. Upon analysis, the average response factor obtained from group X was divided by the average response factor obtained from group Y to yield the percent recovery value (100 * X/Y = % recovery) for each of the compounds. The response factor employed for each compound was the ratio of its peak area to that of the internal standard, as described below.

K. Extraction Accuracy, Precision and Metabolite Stability

Intra-day (within day) as well as the inter-day (between days) accuracy and precision was examined for this extraction. This procedure also served to elucidate the stability of these two compounds when stored at refrigerator temperatures (4°C) for a period of 8 days. A calibration curve and 5 replicates each of two different control concentrations were extracted and analyzed on day 1 of the experiment. The urine control lot was then stored in the refrigerator at 4°C. On days 2, 4 and 8 five replicates of each control concentration were extracted and compared against the calibration curve ran on day 1. Five replications of each control concentration extracted on one day were used

to determine the intra-day accuracy and precision of this extraction. Comparing controls extracted from various days to the original calibration curve was undertaken to examine the inter-day accuracy and precision and also to examine any possible degradation of the metabolites over time. The two metabolite concentrations used for this experiment were 1 and 10 ng/mL.

L. Long Term Metabolite Stability

Since actual postmortem urine samples are typically stored in our laboratory for extended periods of time at -20°C, and not 4°C, the long-term stability of 5-HTOL and 5-HIAA at -20°C was also investigated. Controls were separated into 4 mL aliquots and stored at -20°C. Five control replicates were then analyzed on days 1, 14, 30, 60, 90, 180 and 365. Fresh calibration curves were prepared and analyzed on each day of analysis. There was no statistical decrease in concentration for any of the controls for all times investigated. Therefore, the degradation of 5-HTOL and 5-HIAA over time in specimens stored at -20°C should not be of significant concern.

III. Results and Discussion

A. Method Validation

Derivitization of 5-HTOL and 5-HIAA with TMS, as described above, was found to be essential for this method. Derivatization of these compounds achieved two desirable objectives. First, derivatization increased the fundamental mass of each of the

compounds, practically allowing the use of MS/MS and MS/MS/MS and enhancing the associated detection limits by ca. 1000 times compared with the underivitized forms. Second, optimizing the mobile phase to increase APCI-PCI ionization for one of the underivatized compounds dramatically decreased the ionization efficiency for the other. This observation was not totally unexpected since one compound is an acid and the other a primary alcohol. But, by producing a TMS derivative of each compound it became possible to employ a mobile phase that enhanced the ionization of both derivatives while simultaneously retaining the capability for the low level detection of each.

An ion trap mass spectrometer is a collection device that allows for the "trapping" or isolation of ions from a target compound followed by subsequent elucidation of a hopefully unique spectrum from these individual ions. APCI is a soft ionization technique and, when used in the PCI mode, becomes an excellent source of $[M+H]^+$ parent ions. This ionization technique, in combination with an ion trap, enabled us to perform MS/MS/MS on the 5-HTOL-TMS and 5-HIAA-TMS derivatives, and MS/MS on 5-MMIA. 5-HTOL-TMS had a $[M+H]^+$ parent ion at *m/z* 250.1. The parent ion was collected by the ion trap and subjected to CID, resulting in a daughter ion at *m/z* 232.1. Collecting the *m/z* 232.1 ion and performing CID on it resulted predominantly in a granddaughter ion at *m/z* 216.1. The same process was used on 5-HIAA-TMS, which had a parent $[M+H]^+$ ion at *m/z* 264.1, a daughter ion at *m/z* 218.1 and several granddaughter ions with high-abundance, including three predominant ones at *m/z* 146.1, 191.1 and 202.1. The internal standard had a $[M+H]^+$ ion at *m/z* 220.1, which, when subjected to CID, resulted in a daughter ion at *m/z* 174.1. The full scan MS/MS spectra for 5-HIAA-TMS and 5-MMIA and the MS/MS/MS spectra for 5-HTOL-TMS provided

the "fingerprints" used for analyte identification and confirmation. The full scan spectra are shown in Figures 4-4 through 4-8. Probable fragmentation mechanisms, as projected by the Mass Frontier™ 3.0 computer program provided by Finnigan with the LCQ® MS software package, and viable routes for the achievement of both the 5-HIAA-TMS MS/MS and 5-HTOL-TMS MS/MS/MS product ions are shown in Figures 4-9 and 4-10.

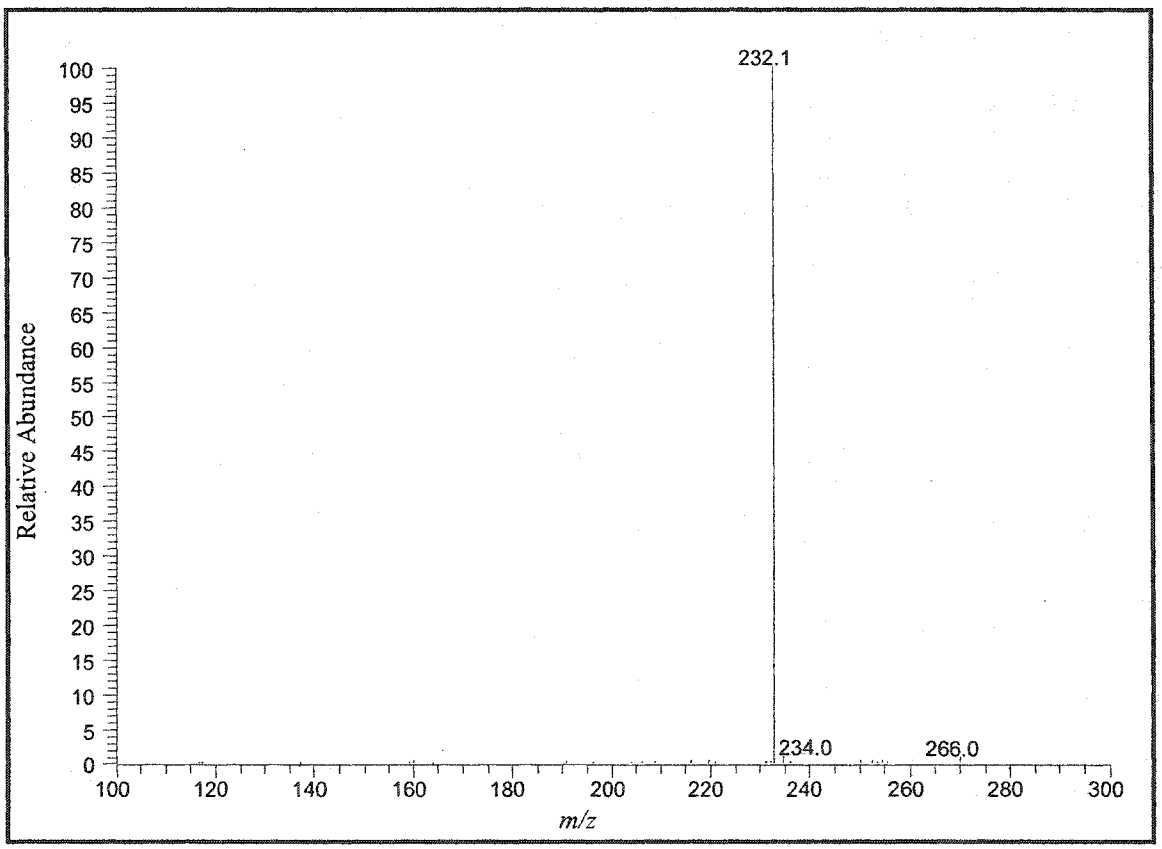


Figure 4-4. MS/MS spectrum of 5-HTOL (*m/z* 250.1 → spectrum).

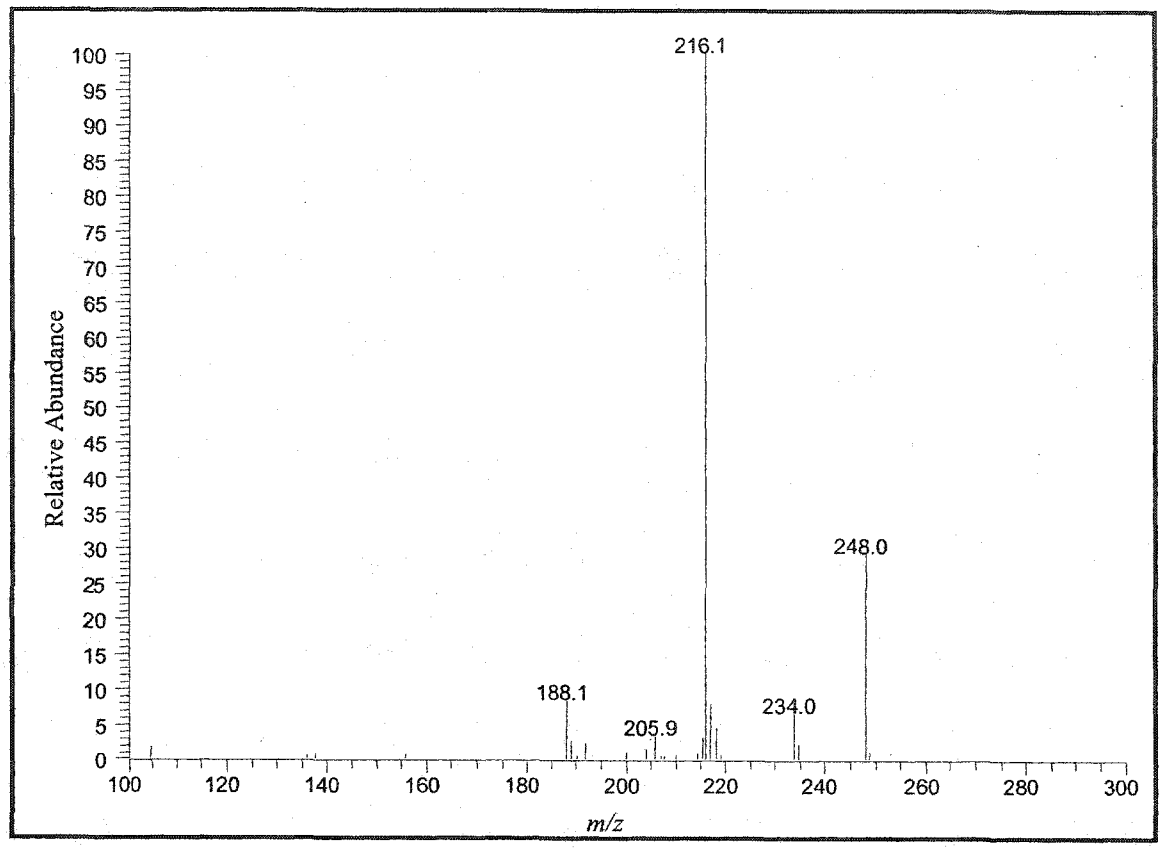


Figure 4-5. MS/MS/MS spectrum of 5-HTOL (*m/z* 250.1 → *m/z* 232.1 → spectrum).

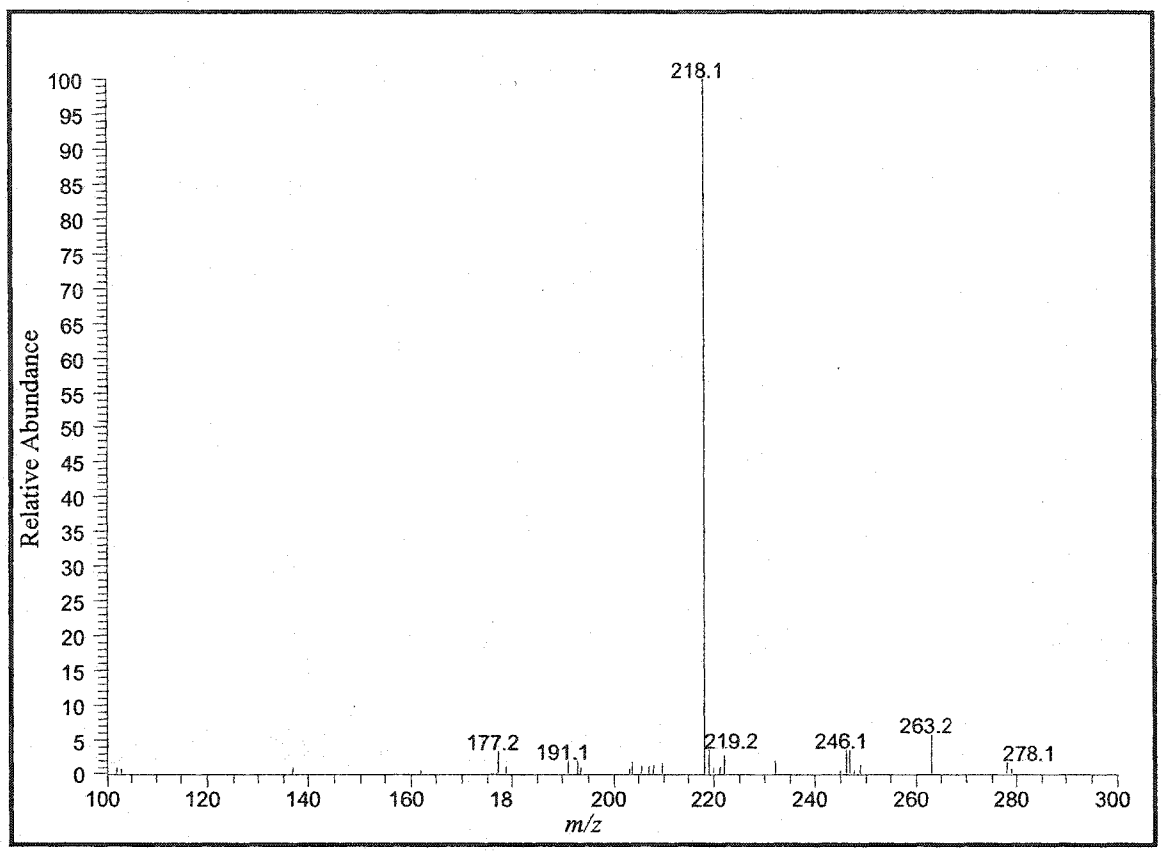


Figure 4-6. MS/MS spectrum of 5-HIAA (*m/z* 264.1 → spectrum).

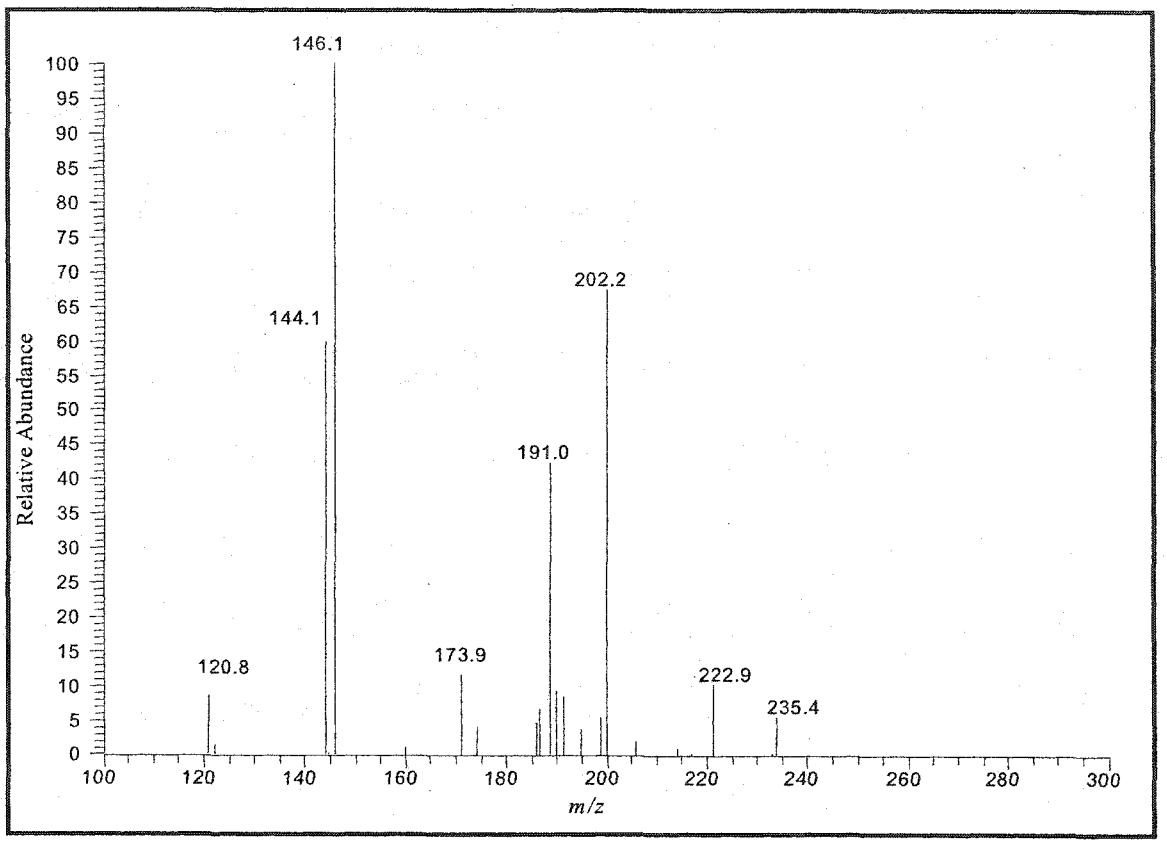


Figure 4-7. MS/MS/MS spectrum of 5-HIAA (*m/z* 264.1 → *m/z* 218.1 → spectrum).

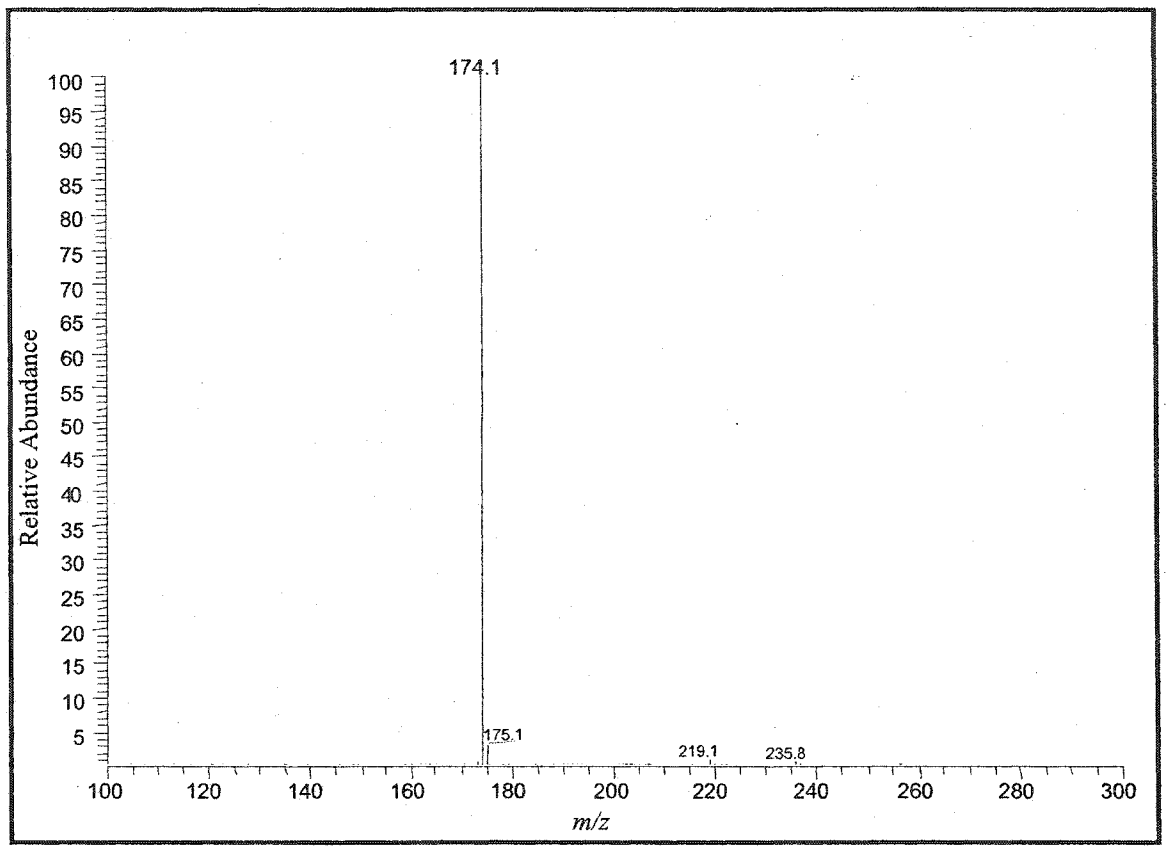


Figure 4-8. MS/MS spectrum of 5-MMIA (*m/z* 220.1 → spectrum).

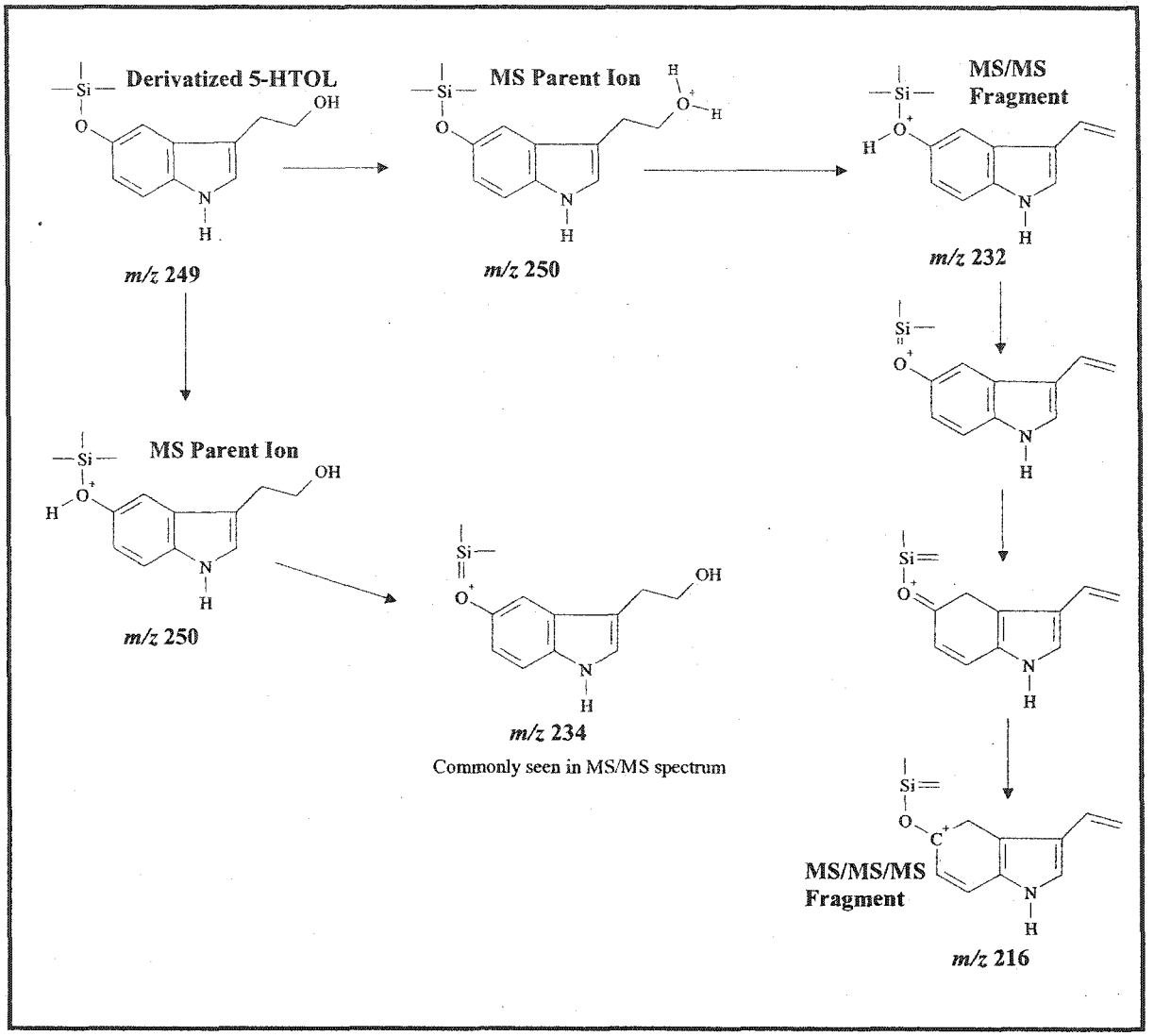


Figure 4-9. Probable APCI-MS fragmentation mechanism yielding the 5-HTOL-TMS MS/MS/MS quantification ion.

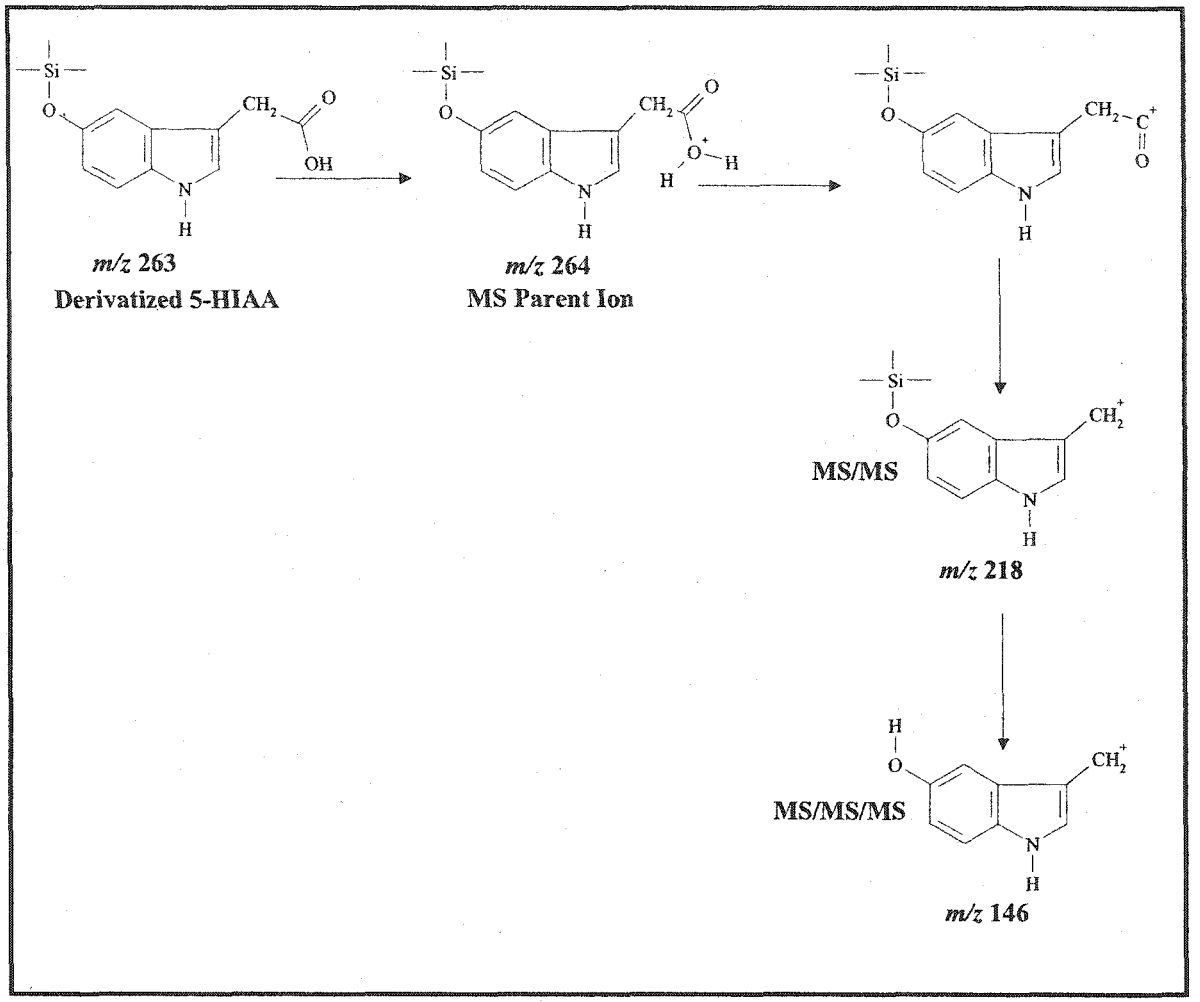


Figure 4-10. Probable APCI-MS fragmentation mechanism yielding the 5-HIAA-TMS MS/MS quantification ion.

Quantification of 5-HTOL and 5-HIAA in samples was achieved via an internal standard calibration procedure. Response factors for both compounds were determined for each sample. The response factor for each analyte was then calculated to be the area of the analyte peak divided by the area of the internal standard peak. The MS/MS/MS ion at *m/z* 216.1 was used for 5-HTOL quantification, while the MS/MS ion at *m/z* 218.1 was used for 5-HIAA quantification. The MS/MS ion at *m/z* 174.1 was used for the internal standard, 5-MMIA. Calibration curves were prepared by plotting a linear regression of the analyte/internal standard response factor versus the analyte concentration for the calibrators, and these curves were used to determine the concentrations of 5-HTOL and 5-HIAA in controls and specimens.

As can be seen in Figure 4-11, 5-MMIA, 5-HTOL and 5-HIAA were completely resolved chromatographically. The peak shape was excellent for all three compounds, and the noise was notably found to be negligible for all specimens examined. These compounds experienced no interference from endogenous sample matrix components. Typical retention times were 1.59, 2.34 and 3.30 minutes for 5-MMIA, 5-HTOL and 5-HIAA, respectively. The number of theoretical plates calculated for each compound ranged from 2000 to 5000.

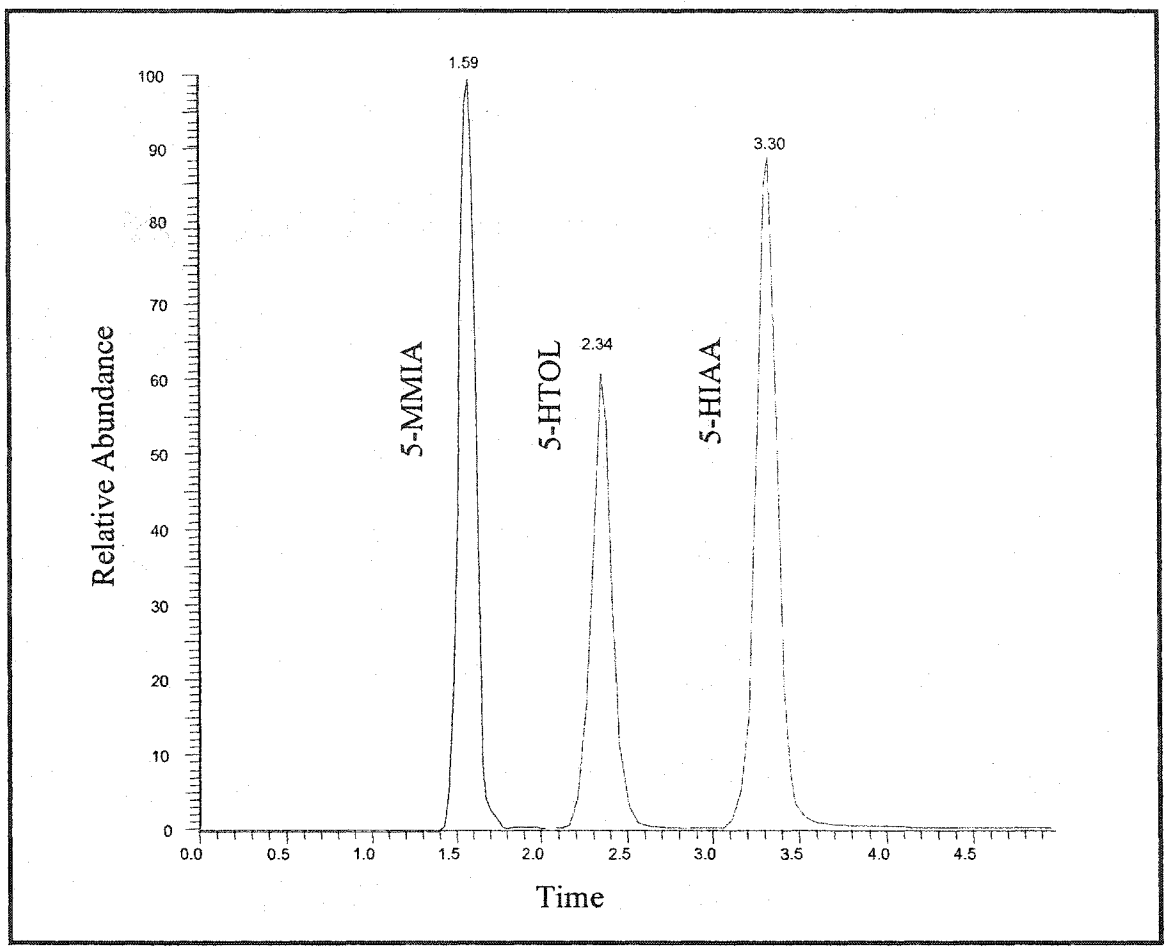


Figure 4-11. Representative concatenated chromatogram of 5-MMIA, 5-HTOL and 5-HIAA. Chromatographic peaks represent ions monitored in SIM mode for each compound as follows: 5-MMIA MS/MS ion at *m/z* 174.1; 5-HTOL MS/MS/MS ion at *m/z* 216.1; 5-HIAA MS/MS ion at *m/z* 218.1. Peaks obtained from a 10 µL injection of a standard with an original concentration of each analyte of 10 µg/mL. The chromatogram is constructed by monitoring for 5-MMIA from 0-2.0 min, for 5-HTOL from 2.0-2.8 min and for 5-HIAA from 2.8-5.0 min. The 100% relative abundance corresponds to the peak current observed for the 5-MMIA peak.

The extraction efficiency of 5-HTOL and 5-HIAA from postmortem urine samples, as described in the experimental section, was determined at 1, 10, 50 and 800 ng/mL. The individual recovery values are presented in Table 4-1. The recovery of 5-HTOL and 5-HIAA across this broad concentration range averaged 82% and 80%, respectively. These values exceeded our initial expectations, considering the relative simplicity of the extraction procedure.

Table 4-1. Percent recovery ± s.d. for 5-HTOL & 5-HIAA.*

Compound	1 ng/mL	10 ng/mL	50 ng/mL	800 ng/mL
5-HTOL	77 ± 10	81 ± 7	82 ± 4	89 ± 8
5-HIAA	83 ± 6	89 ± 2	87 ± 3	59 ± 2

* n=5 for all determinations.

The limit of detection (LOD), limit of quantification (LOQ) and linear dynamic range (LDR) for each analyte are listed in Table 4-2. The LOD was defined as the lowest concentration of analyte having a minimum signal-to-noise (S/N) ratio of five, in addition to meeting a MS/MS and MS/MS/MS spectral "fingerprint" confirmation and ±2% retention time criteria. The "fingerprint" criterion was fundamentally qualitative in nature. However, it can be stated that, for an MS spectra with all the usual other peaks being below 10% of the base peak, we would never accept as valid a sample which produced any peak to be greater than 25% of the same base peak. For the unusual case of the MS/MS/MS spectra of 5-HIAA, there were four peaks observed to routinely be ≥ 40% of the base peak at *m/z* 146.1. In this case, no specimens were accepted unless they demonstrated all four major ions in approximately the same ratios as the standard

and simultaneously had no additional extraneous ions that were ≥ 25% of the base peak. The LOQ was defined as the lowest concentration meeting all LOD criteria plus having a S/N ratio of 10 and having a measured value for a corresponding standard within ±20% of its target concentration. The LOD was found to be 0.10 ng/mL for both 5-HTOL and 5-HIAA. The LOQ was found to be 0.39 ng/mL and 0.78 ng/mL for 5-HTOL and 5-HIAA, respectively. The LDR for these two compounds was 0.39 - 800 ng/mL for 5-HTOL and at least 0.78 - 12800 ng/mL for 5-HIAA. The correlation coefficients for both of the LDR curves exceeded 0.99. Non-linearity was observed with 5-HTOL at concentrations greater than 800 ng/mL, while concentrations above 12800 ng/mL for 5-HIAA were not evaluated.

Table 4-2. LOD, LOQ and LDR values determined for 5-HTOL & 5-HIAA.

Compound	LOD (ng/mL)	LOQ (ng/mL)	LDR (ng/mL)
5-HTOL	0.10	0.39	0.39-800
5-HIAA	0.10	0.78	0.78-12800

Carryover from one sample to the next was not found to be a problem. It was, however, initially investigated and subsequently monitored by the use of blanks with each group of samples investigated. A methanol blank initially injected following the highest calibrator showed no carryover contamination. Subsequently, blanks were used after every third specimen throughout the sample sequence to verify that no sample-to-sample contamination had occurred.

Intra-day (within day) and inter-day (between days) accuracy and precision were examined for this extraction. The accuracy was measured as the percent relative error between the experimentally determined and target concentrations of a sample. The precision was measured as the percent relative standard deviation (RSD) for repeated sample types. Urine controls at 1 and 10 ng/mL were prepared in pools on day 1 and stored in the refrigerator at 4°C until extracted.

For intra-day analyses, a calibration curve was extracted along with 5 replicates of each control concentration on day 1 of the experiment. The intra-day relative error and RSD for 5-HTOL were +9% and 2% at 1 ng/mL and -4% and 8% at 10 ng/mL, respectively. The intra-day relative error and RSD for 5-HIAA were 0% and 8% at 1 ng/mL and -8% and 2% at 10 ng/mL, respectively. These data are summarized in the beginning of Table 4-3 below.

Inter-day accuracy and precision were evaluated by extracting 5 replicates of each of two control concentrations on days 2, 4 and 8, and basing the quantification on the calibration curve originally prepared on day one. The results obtained after storage of each control lot at 4°C for 2, 4 and 8 days can be seen in Table 4-3 below. At 2 days of storage the relative error and RSD for 5-HTOL were 0% and 6% at 1 ng/mL and -14% and 5% at 10 ng/mL, respectively. The relative error and RSD for 5-HIAA were -1% and 5% at 1 ng/mL and -12% and 4% at 10 ng/mL, respectively. At 4 days of storage, the relative error and RSD for 5-HTOL were -4% and 9% at 1 ng/mL and -5% and 4% at 10 ng/mL, respectively. The relative error and RSD for 5-HIAA were -3% and 7% at 1 ng/mL and -14% and 2% at 10 ng/mL, respectively. At 8 days of storage, the relative error and RSD for 5-HTOL were -1% and 4% at 1 ng/mL and -13% and 1% at 10 ng/mL,

respectively. The relative error and RSD for 5-HIAA were -9% and 8% at 1 ng/mL and -17% and 1% at 10 ng/mL, respectively.

After 8 days of storage at 4°C, the 1.00 ng/mL control was found to have a 5-HTOL concentration of 0.99 ± 0.04 ng/mL and a 5-HIAA concentration of 0.91 ± 0.07 ng/mL. The 10.0 ng/mL control was found to have a 5-HTOL concentration of 8.7 ± 0.1 ng/mL and a 5-HIAA concentration of 8.3 ± 0.1 ng/mL. The decrease in concentrations at 10.0 ng/mL was not unexpected, however, due to the relative ease of autoxidation of these compounds. These relatively minor decreases were found to be acceptable for general use and agree well with previously reported short-term stability studies for these compounds under similar conditions.[158] Nonetheless, as a good laboratory practice and in an effort to maintain a high degree of accuracy, we would recommend preparing new calibration curves at the beginning of each new analysis or at least once per day.

Table 4-3. Intra-day accuracy and precision for repeated determinations* Over 8 Days.

	5-HTOL		5-HIAA	
	Day 1		Day 1	
Target Conc. (ng/mL)	1	10	1	10
Mean ± SD (ng/mL)	1.09 ± 0.02	9.6 ± 0.8	1.00 ± 0.08	9.2 ± 0.2
Relative Error	+9%	-4%	0%	-8%
R.S.D.	2%	8%	8%	2%
	Day 2		Day 2	
Target Conc. (ng/mL)	1	10	1	10
Mean ± SD (ng/mL)	1.00 ± 0.06	8.6 ± 0.4	0.99 ± 0.05	8.8 ± 0.3
Relative Error	0%	-14%	-1%	-12%
R.S.D.	6%	5%	5%	4%
	Day 4		Day 4	
Target Conc. (ng/mL)	1	10	1	10
Mean ± SD (ng/mL)	0.96 ± 0.09	9.5 ± 0.4	0.97 ± 0.07	8.6 ± 0.2
Relative Error	-4%	-5%	-3%	-14%
R.S.D.	9%	4%	7%	2%
	Day 8		Day 8	
Target Conc. (ng/mL)	1	10	1	10
Mean ± SD (ng/mL)	0.99 ± 0.04	8.7 ± 0.1	0.91 ± 0.07	8.3 ± 0.1
Relative Error	-1%	-13%	-9%	-17%
R.S.D.	4%	1%	8%	1%

*n=5 at each concentration for each day, controls were run on day 1, 2, 4 and 8.

Since actual postmortem urine samples are stored for extended periods of time at -20°C, and not 4°C, the long-term stability of 5-HTOL and 5-HIAA at -20°C was also investigated. Controls were separated into 4 mL aliquots and stored at -20°C. Five control replicates were then analyzed on days 1, 14, 30, 60, 90, 180 and 365. Fresh calibration curves were prepared and analyzed on each day of analysis. As can be seen in Table 4-4 below, there was no statistical decrease in concentration for any of the controls for all times investigated. Therefore, the degradation of 5-HTOL and 5-HIAA over time in properly stored specimens should not be of significant concern for at least 365 days.

Table 4-4. Long-term stability of 5-HTOL and 5-HIAA.*

	Day	1	14	30	60	90	180	365
5-HTOL	Mean	405	406	402	398	397	394	383
	s.d.	3	3	5	5	3	4	12
	RSD	1	1	1	1	1	1	3
5-HIAA	Mean	397	396	397	400	386	395	377
	s.d.	10	4	8	5	15	16	13
	RSD	2	1	2	1	4	4	3

*n=5 for all determinations.

IV. Conclusions

The procedure described in this chapter provides a rapid, accurate and reproducible method for the simultaneous extraction and quantification of 5-HTOL and 5-HIAA in postmortem human urine specimens. A liquid-liquid extraction procedure in combination with LC/APCI-PCI-MS provides both superior separation of these two compounds and detection limits that are well below concentrations expected in the usual postmortem specimens. Through the course of these experiments we demonstrated the effectiveness of this novel LC/MS method for the simultaneous analysis of two serotonin metabolites. We validated this method by demonstrating the LOD, LOQ, LDR, intra/inter-day reproducibility and stability of the compounds over extended storage times. One of the most important aspects of this novel method was the relative simplicity by which the simultaneous analysis of both compounds was achieved. The relative simplicity of this method made it more readily applicable to reliable and routine use in the field of toxicology. We were then prepared to apply this method to postmortem urine specimens obtained from the victims of aviation accidents.

LC/MS Determination of the 5-HTOL/5-HIAA Ratio in Postmortem Urine Specimens

Chapter 5

I. Introduction

The ratio of two serotonin metabolites, 5-hydroxytryptophol (5-HTOL) and 5-hydroxyindole-3-acetic acid (5-HIAA), is reasonably accepted to be altered upon the ingestion of ethanol. It is also known, as discussed in chapter 1 of this dissertation, that there are numerous isozymes of both ADH and ALDH available for the oxidation/reduction of both alcohols and aldehydes. Thus, one might reasonably expect to see variations in the shift of this ratio associated with genetic subsets. However, genetic variations in ethanol metabolism in various ethnic groups has surprisingly been shown to have no discernible effect on the basal 5-HTOL/5-HIAA ratio or the ratio

following ethanol consumption.[37, 39, 116, 147, 167]. Several studies have been conducted on living humans examining the shift in the urinary 5-HTOL/5-HIAA ratio following consumption of ethanol.[85, 132, 138, 158, 165] These reports have clearly demonstrated 5-HTOL/5-HIAA ratios significantly above those of urine baseline levels following ethanol ingestion. They also found that, in general, the more ethanol an individual consumed the higher the 5-HTOL/5-HIAA ratio became.[147] Furthermore, 5-HTOL/5-HIAA ratios remained elevated above baseline levels for hours after ethanol could no longer be detected in the body. In one study, both men and women were dosed with 0.80 g/kg ethanol, resulting in an average peak urinary alcohol concentration of 87 mg/dL.[138] They found that while ethanol could no longer be detected in the body 10 hours after dosing, the 5-HTOL/5-HIAA ratio remained significantly elevated for up to 16 hours after dosing. In a separate study, Hagan *et al.* dosed subjects at 600 mg/kg.[132] They too found that the 5-HTOL/5-HIAA ratio remained significantly elevated for up to 16 hours after dosing, a time which was significantly longer than ethanol could be detected in the body.[132] Helander *et al.* have proposed a cutoff value for the 5-HTOL/5-HIAA ratio of 15 pmol/nmol, below which it would be assumed that substantial amounts of ethanol had not been consumed for at least 14 hours prior to sampling.[37, 116] It is, however, noteworthy, that those prior investigations of the 5-HTOL/5-HIAA ratio in urine were conducted on living volunteers.

The stability of the 5-HTOL/5-HIAA ratio in urine as a function of time and temperature after sampling has also been investigated. In one such experiment, Helander *et al.* divided each of three individual urine specimens with varying 5-HTOL/5-HIAA ratios into two separate samples. One of the samples was stored at 4°C and the other

sample was stored at 22°C.[85] After 4 weeks of storage, the specimens stored at 4°C demonstrated no change in the 5-HTOL/5-HIAA ratio. The three specimens stored at 22°C, on the other hand, exhibited contrasting alterations in the 5-HTOL/5-HIAA ratio. Two of the specimens demonstrated a decrease in the 5-HTOL/5-HIAA ratio, with the largest decrease being approximately 15%. The other specimen stored at 22°C demonstrated an increase of approximately 20% in the 5-HTOL/5-HIAA ratio over the 4 week storage time.[85] These same authors also included a cursory examination of postmortem urine samples with respect to their 5-HTOL/5-HIAA ratios. While finding substantial individual postmortem variations, a seemingly reasonable correlation between ethanol levels and 5-HTOL/5-HIAA ratios was demonstrated.[85] However, no work has been performed to date on postmortem samples, as opposed to the living volunteers discussed above, to establish and validate an appropriate ratio of 5–HTOL/5-HIAA which could be employed independent of any additional (including even ethanol analyses) data to confirm or deny antemortem ethanol consumption. In this portion of our current study, we investigated the 5-HTOL/5-HIAA ratio in postmortem urine specimens with the intent to establish a cutoff value or, perhaps, range of values, for 5-HTOL/5-HIAA which would allow reasonably definitive discrimination between none and some antemortem alcohol consumption.

The FAA receives biological samples from approximately 450 fatal aviation accidents per year. Each of these cases is routinely screened for ethanol upon arrival. The FAA further has a toxicological database that allows researchers to query all fatal aviation cases that are currently in storage. This program allows the user to select the biological specimen, the compound of interest and the range of accident dates to be

queried. This tool allowed us to choose urine specimens from the last 5 years that had been assigned to be either positive or negative for antemortem ethanol ingestion upon initial screening for use in the current study.

Urine specimens from 44 aviation accidents that occurred between the years 1998 and 2003 were obtained for examination. The distribution of these cases by year is shown in Table 5-1.

Table 5-1. Distribution of cases obtained by year.

Year	1998	1999	2000	2001	2002	2003
# Of Cases	3	6	10	12	10	3

Of the 44 specimens analyzed, the FAA had previously reported 21 of these specimens to be negative with respect to antemortem ethanol consumption. The remaining 23 specimens were previously reported to be positive with respect to antemortem ethanol consumption. The urine specimens which had ethanol values exceeding the FAA's in-house cutoff of 10 mg/dL were initially further verified as ethanol-positive by showing the postmortem urinary ethanol concentration was self-consistent with the postmortem ethanol values found in to at least two other postmortem fluid and/or tissue samples from the same subject. Then the number and identity of other volatile organic compounds (VOCs) was also investigated in all of the original urine specimens. Finally, a subject was only assigned permanently to be ethanol-positive if both the distribution of ethanol between the three postmortem specimens was appropriately correlated as expected and

the presence of other volatile organic compounds was considered sufficiently low to be in the "normal" range, indicating no postmortem microbial ethanol formation.

The initial focus of this study was to establish a cutoff 5-HTOL/5-HIAA ratio in postmortem urine, so that we could claim, with reasonable statistical certainty, that the victim of a fatal aviation accident had or had not consumed ethanol prior to death. Following the establishment of an appropriate 5-HTOL/5-HIAA ratio for the determination of antemortem or no antemortem consumption of ethanol using the 44 samples, we applied this novel method to additional cases where antemortem consumption had not previously been assigned and/or could not reasonably have been assigned with certainty.

II. Materials and Methods

A. Specimen Selection

Postmortem urine specimens received by the FAA laboratory are always stored at -20°C. However, no specimens are retained in the storage facility for more than five years. Classification of postmortem urine specimens as being positive or negative for antemortem ethanol ingestion has routinely been done using criteria established by the College of American Pathologists (CAP). This agency has established a rigid 10 mg/dL blood ethanol level, as determined by a headspace GC procedure using flame ionization detection, as the standard cutoff value to distinguish between none and some antemortem ethanol consumption. At the FAA, both fluid or tissue samples containing ethanol at or above this limit are also declared positive for antemortem ethanol ingestion, while those

below this limit are declared negative. This is a quite restrictive and/or demanding cutoff value in that the corresponding legally impaired limit for pilots is only 40 mg/dL and that for motor vehicle operators is typically only 80-100 mg/dL

The CAMI toxicological database developed by DiscoverSoft Development, LLC, was used for specimen selection in this study. Version 2.21i of this database was used in the archive mode to create a QA report that queried all cases received by CAMI between 1998 and 2003. Limiting it to cases that had urine remaining for further analysis initially narrowed the database search. Selecting urine specimens from cases that were initially found to contain ethanol provided further limitation to this search. Once ethanol-positive urine specimens had been isolated, these cases were searched for both other fluid and/or tissue specimens that also contained ethanol and other volatile organic compounds so that an evaluation of ethanol distribution between fluids and tissues and an evaluation of extraneous VOCs could be accomplished. If a selected case met these criteria for an assessment of antemortem ethanol ingestion, the urine from that case was retrieved from storage for further analysis. Ethanol-negative urine specimens were selected at random from cases that (1) had urine remaining for analysis and (2) contained no ethanol upon initial screening (3) had VOCs which, if detectable at all, were present at concentrations considered to be entirely normal and consistent with no antemortem alcohol consumption.

B. Chemicals and Solutions

Please refer to Chapter 4, section II A.

C. Metabolite Stock Solutions

Please refer to Chapter 4, section II B.

D. Mobile Phase Preparation

Please refer to Chapter 4, section II C.

E. ß-glucuronidase Solution

Please refer to Chapter 4, section II D.

F. Internal Standard Stock Solution

Please refer to Chapter 4, section II E.

G. LC/MS Instrumentation

Please refer to Chapter 4, section II F.

H. Calibration Curves and Controls

Please refer to Chapter 4, section II H.

I. Postmortem Specimen Preparation and Extraction

Please refer to Chapter 4, section II I.

III. Results and Discussion

A. Forensic Urine Analysis

To ascertain the utility of our LC/MS procedure to simultaneously determine 5-HTOL and 5-HIAA in routine toxicological investigations, we examined a total of forty-four urine specimens obtained from fatal aviation accident victims. Of the 44 specimens analyzed, 21 specimens had been assigned to be negative for antemortem ethanol consumption, while 23 specimens had been assigned to be positive for antemortem ethanol consumption. The prior establishment of positive with respect to antemortem ethanol ingestion was not only based on the urine ethanol level, but also included corroboration by (1) appropriate ethanol levels in at least two other tissue and/or fluids and (2) appropriately low or moderate levels for other analyzed volatile organic compounds (VOCs). Such corroborating values for all positives are shown in Table 5-2.

Table 5-2. Corroborating evidence accumulated for the 23 ethanol-positive specimens.

Specimen Number	Ethanol Concentration (mg/dL or mg/hg)			VOCs Present (mg/dL)
	Urine	Alternate 1	Alternate 2	
22	81	43 (blood)	75 (VH*)	acetaldehyde (1)
23	220	289 (VH)	246 (blood)	none
24	344	255 (VH)	235 (blood)	none
25	21	16 (blood)	20 (VH)	none
26	261	178 (VH)	159 (blood)	none
27	520	290 (VH)	274 (blood)	acetone (1)
28	89	63 (blood)	40 (muscle)	none
29	58	40 (blood)	35 (brain)	none
30	141	97 (brain)	103 (muscle)	none
31	130	83 (VH)	61 (blood)	acetaldehyde (1)
32	111	127 (VH)	120 (blood)	acetaldehyde (4)
33	277	216 (blood)	159 (muscle)	acetaldehyde (2)
34	13	12 (VH)	9 (blood)	sec-butanol (2)
35	68	35 (brain)	42 (kidney)	isobutanol (3), methanol (1)
36	188	126 (VH)	90 (blood)	none
37	181	91 (VH)	86 (blood)	none
38	18	20 (blood)	14 (muscle	none
39	25	24 (VH)	15 (blood)	n-propanaol (1)
40	44	17 (liver)	15 (muscle)	n-propanol (1), acetaldehyde (5)
41	205	155 (blood)	187 (VH)	acetone (1), acetaldehyde (2)
42	20	17 (blood)	19 (VH)	n-propanol (2)
43	36	24 (muscle)	22 (brain)	none
44	11	9 (VH)	5 (brain)	none

*VH = vitreous humor

The treatment of victim specimens upon initial receipt by the FAA is pertinent to the current samples employed. If many different tissues and fluids for a given victim are received, the FAA would initially examine one such specimen for ethanol and other VOCs. The order of specimen types that would be initially selected would generally be urine, vitreous humor, blood and tissue specimen(s). If the first specimen examined proves to be below the ethanol cutoff of 10 mg/dL and all other VOCs were undetectable and/or present at levels indicative of no antemortem alcohol consumption, the victim is declared ethanol-negative. Even if more specimens exist for this victim, none are further examined for ethanol content. This procedure is a safety feature, which guarantees that the majority of any misidentified samples would turn out to be false negatives instead of false positives. The FAA subscribes to this general philosophy, considering the many undesirable legal and other ramifications of a false positive. Thus, in short, all the ethanol-negative samples in the current study have no ethanol data for other tissues and/or fluids; however, they do have corresponding data for other VOCs in urine as seen in Table 5-3.

Table 5-3. Corroborating evidence accumulated for the 21 ethanol-negative specimens.

Specimen Number	Ethanol Concentration (mg/dL)	VOCs Present (mg/dL)
1	<10	sec-butanol (1), acetone (1)
2	<10	none
3	<10	none
4	<10	acetaldehyde (2)
5	<10	none
6	<10	none
7	<10	n-propanol (3), acetaldehyde (1)
8	<10	acetone (2)
9	<10	none
10	<10	none
11	<10	none
12	<10	sec-butanol (2)
13	<10	none
14	<10	isobutanol (3), methanol (1)
15	<10	none
16	<10	acetaldehyde (3)
17	<10	none
18	<10	n-propanol (4)
19	<10	none
20	<10	none
21	<10	none

LC/MS results for 5-HTOL, 5-HIAA, and the 5-HTOL/5-HIAA ratio for 44 specimens analyzed is presented in Tables 5-4 and 5-5 below. Data obtained from the analysis of the 21 assigned antemortem ethanol negative specimens are shown in Table 5-4. The data obtained from the analysis of the 23, assigned antemortem ethanol-positive specimens are shown in Table 5-5.

Table 5-4. 5-HTOL and 5-HIAA data collected for the 21 ethanol-negative specimens.

Specimen Number	5-HTOL (ng/mL)	5-HIAA (μg/mL)	Ethanol Conc. (mg/dL)	5-HTOL* (pmol)	5-HIAA** (nmol)	5-HTOL/5-HIAA Ratio (pmol/nmol)
1	5.1	26	<10	86	$4.1x10^2$	0.21
2	1.2	13	<10	20	$2.0x10^2$	0.10
3	21	16	<10	$3.6x10^2$	$2.5x10^2$	1.4
4	3.8	25	<10	64	$3.9 x10^2$	0.16
5	160	68	<10	$2.7x10^3$	$1.1 x10^3$	2.5
6	32	3.8	<10	$5.4 x10^2$	60	9.1
7	22	15	<10	$3.7x10^2$	$3.4 x10^2$	1.6
8	9.2	12	<10	$1.6 x10^2$	$1.9 x10^2$	0.83
9	290	70	<10	$4.9 x10^3$	$1.1 x10^3$	4.5
10	1.6	36	<10	27	$5.6 x10^2$	0.05
11	2.0	8.3	<10	34	130	0.26
12	88	10	<10	$1.5 x10^3$	$1.6 x10^2$	9.5
13	40	11	<10	$6.6 x10^2$	$1.7 x10^2$	3.8
14	0.93	90	<10	16	$1.4 x10^3$	0.01
15	4.4	1.1	<10	74	17	4.3
16	3.0	49	<10	51	$7.7 x10^2$	0.07
17	68	120	<10	$1.2 x10^3$	$1.9 x10^3$	0.61
18	4.9	1.4	<10	83	22	3.8
19	20	3.2	<10	$3.4 x10^2$	50	6.7
20	3.8	12	<10	64	$1.9 x10^2$	0.34
21	5.5	1.9	<10	93	30	3.1

* total pmol contained in 3.0 mL urine sample

** total nmol contained in 3.0 mL urine sample

Table 5-5. 5-HTOL and 5-HIAA data collected for the 23 ethanol-positive specimens.

Specimen Number	5-HTOL (ng/mL)	5-HIAA (μg/mL)	Ethanol Conc. (mg/dL)	5-HTOL* (pmol)	5-HIAA** (nmol)	5-HTOL/5-HIAA Ratio (pmol/nmol)
22	220	5.9	81	$3.7x10^3$	94	40
23	$1.2 x10^3$	6.9	220	$2.4x10^4$	$1.1x10^2$	190
24	1.8	0.0063	344	30	0.1	$3.0x10^2$
25	260	13	21	$4.4x10^3$	$2.0x10^2$	22
26	14	0.060	261	240	940	250
27	460	9.1	520	$7.8x10^4$	140	550
28	45	2.5	89	760	39	19
29	18	.24	58	$3.0x10^2$	3.8	81
30	35	440	141	590	6.9	86
31	86	1.1	130	$1.5x10^3$	17	84
32	250	4.9	111	$4.2x10^3$	77	55
33	28	0.12	277	470	1.9	250
34	190	5.8	13	$3.2x10^3$	91	35
35	29	0.53	68	490	8.3	59
36	260	2.1	188	$4.4x10^3$	33	130
37	4.1	0.033	181	69	0.52	130
38	23	0.57	18	390	8.9	44
39	350	5.9	25	$6.0x10^3$	93	65
40	670	20	44	$1.1x10^4$	310	36
41	870	10	205	$1.5x10^4$	160	94
42	230	5.6	20	$3.9x10^3$	88	44
43	2.9	0.15	36	49	2.4	21
44	13	0.73	11	220	11	19

* total pmol contained in 3.0 mL urine sample
** total nmol contained in 3.0 mL urine sample

The data collected from all 44 specimens are illustrated in Figure 5-1a as a plot of the 5-HTOL/5-HIAA ratio vs. the ethanol content in the urine samples. The results show a general trend of increasing postmortem 5-HTOL/5-HIAA ratio with increasing

postmortem urine ethanol concentration. There is, however, substantial inter-individual variation in 5-HTOL/5-HIAA ratios at ethanol concentrations below approximately 150 mg/dL. Thus, the area below 150 mg/dL has been expanded and is shown in more detail in Figure 5-1b. These kinds of variations, which upon preliminary examination appear to be considerable, are, in fact, not unexpected since the specimens utilized in this study are from human postmortem sources with unknown dietary, medical and other relevant information.

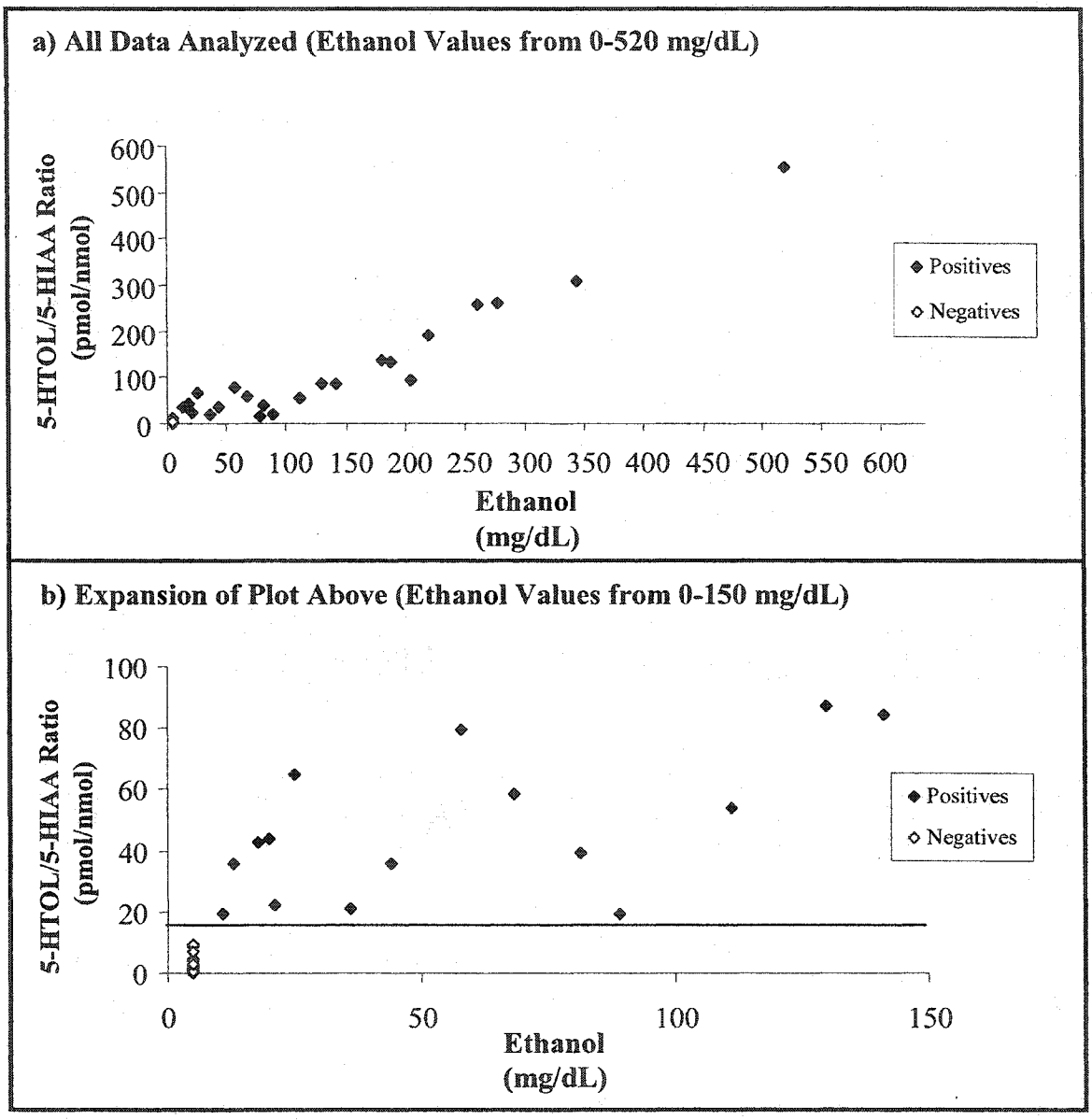


Figure 5-1. 5-HTOL/5-HIAA ratio as a function of postmortem urine ethanol levels.

The 21 ethanol-negative urine specimens investigated had corresponding 5-HTOL/5-HIAA ratios that ranged from 0.01 - 9.5 pmol/nmol, with an "average" of 2.52 ± 2.88 pmol/nmol (mean ± s.d.). 5-HTOL concentrations in these specimens ranged from 0.930 - 301 ng/mL with an "average" of 40.1 ng/mL. 5-HIAA concentrations ranged from 1.1 – 49 μg/mL with an "average" of 27 μg/mL. The 23 ethanol-positive specimens had ethanol concentrations ranging from 11 to 520 mg/dL. The corresponding 5-HTOL/5-HIAA ratios ranged from 19 to 550 pmol/nmol. Careful examination of Figure 5-1 shows that the lowest ethanol-positive ratio is 19, with only three other ethanol-positives near this ratio (19, 21 and 22). The highest ratio for the ethanol negatives, on the other hand was 9.5 with only one other ethanol-negative (9.1) near this upper value. The gap between 9.5 and 19 and clearly separates all negatives from all positives. However, we wanted to establish a cutoff that would be applicable to all future such investigations. In selecting such a cutoff, we decided that improper assignment of a sample as being ethanol-positive was of much greater concern than mis-assignment as an ethanol-negative due to all the legal ramifications that are associated with positive assignment. Thus, we wanted to select a cutoff, or dividing line, closer to the lowest observed positives and farther away from the highest observed negatives. While many values in this range could be appropriately selected with these criteria, the value finally chosen was 15 pmol/nmol for the 5-HTOL/5-HIAA ratio. This cutoff is a conservative one leading to a minimization of false positives. And, as stated, we feel this cutoff is completely appropriate in consideration of the substantial personal, professional and legal consequences of a positive declaration of antemortem ethanol consumption in a toxicological result. This cutoff value is notably more than 4 standard deviations above

the average result obtained for all 21 ethanol-negative specimens. By a simple statistical consideration this would lead to, at most, a false positive rate of no more than 1 in 10,000. Figure 5-2 demonstrates graphically the differences seen between the 5-HTOL/5-HIAA ratios of ethanol-positive specimens when compared to ethanol-negative specimens. This figure also demonstrates the significance of the average ethanol-negative 5-HTOL/5-HIAA ratio, which was found to be more than 4 standard deviations lower than the closest ethanol-positive result.

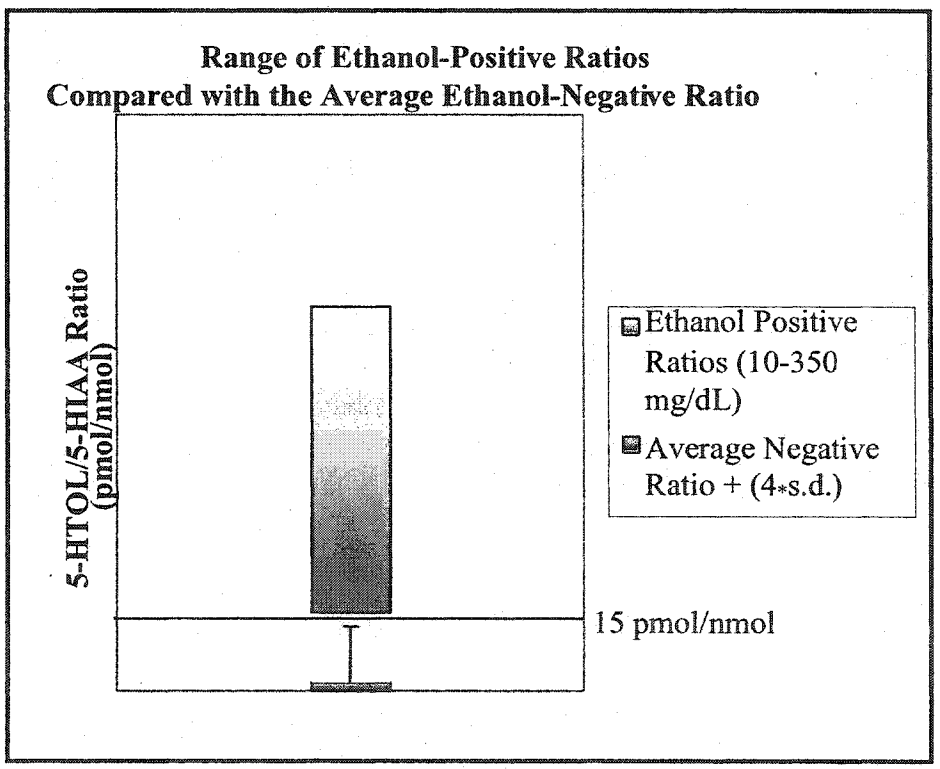


Figure 5-2. Average ethanol negative 5-HTOL/5-HIAA ratio (+ 4 s.d.) vs. range of ethanol positive ratios.

With the 15 pmol/nmol cutoff ratio established above we decided to examine our data in another way. Figure 5-3 shows the results obtained by grouping the cases according to the ethanol concentrations, expressed as increasing ranges of values, and plotting the 5-HTOL/5-HIAA ratio for each of these somewhat arbitrary groups.

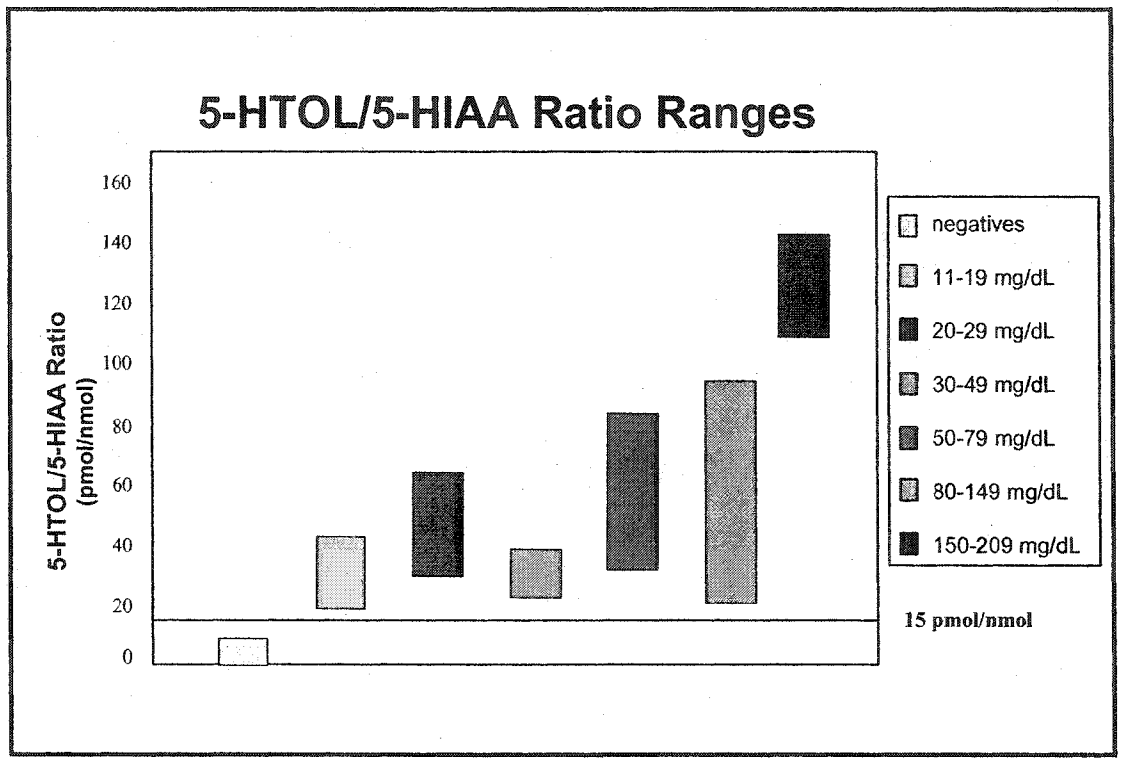


Figure 5-3. 5-HTOL/5-HIAA ratios for both ethanol-negative and ethanol-positive specimens and their relationship to the established 15 pmol/nmol cutoff.

Figure 5-3 demonstrates the range of the 5-HTOL/5-HIAA ratios in these sets of samples that were grouped according to their ethanol concentrations. As can be seen, the group of negative specimens displayed a range of 5-HTOL/5-HIAA ratios that fell distinctly below the 15 pmol/nmol 5-HTOL/5-HIAA cutoff, while all of the ethanol positive groups are above the cutoff. Also, it can be generally seen that, as the ethanol concentration of the

group increases, so does the 5-HTOL/5-HIAA ratio range generally increase for that group. It should be noted that one datum point was removed from this figure. That datum point had an ethanol concentration of 520 mg/dL and a corresponding 5-HTOL/5-HIAA ratio of 550 pmol/nmol. The results for this sample, if plotted in the same figure, caused the information currently shown to become completely indiscernible. Therefore, this one datum point was excluded from the figure.

All the above results can be taken to reasonably justify the selection of 15 pmol/nmol of the postmortem 5-HTOL/5-HIAA ratio in urine to discriminate between antemortem ethanol consumption and no antemortem ethanol consumption.

B. Forensic Cases Containing Postmortem Ethanol of Unknown Origin

Following establishment of this 15 pmol/nmol cutoff, we felt it appropriate to apply this methodology to a variety of aviation cases in which the postmortem production of ethanol was considered to be a possibility upon initial ethanol screening, but could not be firmly established.

1. Case #1

Case #1 was obtained from a fatal aviation accident that occurred in a remote, mountainous terrain. The victim was not recovered from the accident scene for more than 24 hours. This case had a blood ethanol concentration of 92 mg/dL, well above the cutoff of 10 mg/dL employed by the FAA to indicate antemortem ethanol consumption. The case also had a positive urinary ethanol concentration of 21 mg/dL. But, the vitreous humor ethanol concentration, which is normally 90 -100% of the urine value, was 0

mg/dL, clearly negative. These specimens were each notably missing other volatiles such as acetaldehyde, acetone, sec-butanol, isopropanol, n-propanol and n-butanol. The lack of other commonly analyzed volatiles suggests the absence of microbial postmortem ethanol production.[8, 47, 56, 77, 101, 105, 106, 181] Conversely, the abnormal nature of the ethanol distribution in these three biological matrices suggests postmortem microbial ethanol formation. To conclusively determine if recent ethanol ingestion occurred, we investigated the 5-HTOL/5-HIAA ratio. We found the 5-HTOL/5-HIAA ratio to be 1.6 pmol/nmol in urine, which was substantially below the established 15 pmol/nmol cutoff. This result clearly supports an absence of recent ethanol ingestion and indicates that the ethanol present in this case was due to postmortem microbial formation.

2. Case #2

Case #2 originated from an aviation accident that occurred in a dry creek bed in upstate New York. The body of the pilot was recovered and autopsied within 24 hours of the accident. Specimens examined from the victim for volatile organic compounds included: blood, urine, vitreous humor, skeletal muscle and heart muscle. The urine and blood ethanol concentrations were determined to be 31 mg/dL and 16 mg/dL, respectively following headspace GC analysis. Vitreous humor, heart and skeletal muscle were also analyzed and found to contain ethanol at concentrations of 17 mg/dL, 8 mg/hg and 12 mg/hg, respectively. The distribution of ethanol in these fluids and tissues reasonably supports a conclusion of antemortem ethanol consumption. Various volatiles, however, including large amounts of acetaldehyde, n-propanol and n-butanol were also present in these samples supporting the opposing possibility of postmortem

microbial formation of ethanol. While the distribution of ethanol between fluid and tissues as well as the levels of ethanol were quite consistent with antemortem ethanol consumption, a visual confirmation of sample putrefaction and the presence of substantial amounts of the various volatiles could be argued to more likely indicate that the ethanol found in this case was due to postmortem microbial formation. We found the 5-HTOL/5-HIAA ratio to be 1.9 pmol/nmol in urine. This value is well below the 15 pmol/nmol cutoff, thus strongly indicating the absence of recent antemortem ethanol ingestion. As such, the presence of ethanol in this case was verified to be due to postmortem microbial formation.

3. Case #3

The third case examined that contained ethanol from an unknown origin originated from an aviation fatality due to an accident that occurred over a stretch of the Atlantic Ocean. The wreckage from the accident was located approximately 9 hours after last contact with the victim. The body of the victim, however, was not discovered until it washed up shore approximately 1.5 months after the accident occurred. Specimens examined from the victim for volatile organic compounds included blood, urine, skeletal muscle and brain. Ethanol was present in every tissue and fluid examined from this case. The concentrations of ethanol found in these specimens were 38 mg/dL, 25 mg/dL, 12 mg/hg and 39 mg/hg, respectively for the four samples mentioned above. The blood and tissue samples were noted as putrefied by visual inspection, and the urine was bloody. Also present in this case were numerous other volatile organic compounds, including n-propanol, n-butanol and acetaldehyde at concentrations substantially higher than normal.

These other volatile organic compounds, like ethanol, were present in each of the specimens analyzed. The distribution of ethanol in this case was questionable due to the fact that: 1) the highest concentration of ethanol detected was in the brain, which has a lower water content than the other specimens examined, and 2) the blood ethanol concentration considerably exceeded the urine ethanol concentration, which is possible, but not common. The presence of numerous other volatile organic compounds also seemed to support the possibility that the ethanol present in this case was due to postmortem microbial formation and not antemortem consumption. After LC/MS analysis, the 5-HTOL/5-HIAA ratio for this case was determined to be 2.1 pmol/nmol in urine. This value is well below the cutoff established for antemortem ethanol consumption, supporting the conclusion that recent ethanol consumption had not occurred prior to the accident.

4. Case #4

The fourth case examined which contained ethanol of an unknown origin also resulted from an aviation accident that occurred in the Pacific Ocean. This accident occurred in the Pacific Ocean near Alaska, and the body of the victim was not recovered for 17 days following the accident. The specimen types initially examined for ethanol and other volatile organic compounds in this case were blood, urine and brain. These specimens contained, respectively, ethanol at concentrations of 54 mg/dL, 9 mg/dL and 0 mg/hg. This case was questioned immediately after reviewing these ethanol results. The urinary ethanol concentration is below the 10 mg/dL cutoff for a report of antemortem ethanol. The blood, however, is present at a level clearly above the previously mentioned

limit of 10 mg/dL. The ethanol distribution in this case, thus, raises questions as to the ethanol origin. Analysis of other volatile organic compounds present in the victim was limited to only acetaldehyde, which was present at 12 mg/dL in the blood. This acetaldehyde result could reasonably be argued to support either antemortem consumption or postmortem microbial formation. To provide a more definitive answer to the ethanol origin question we examined the 5-HTOL/5-HIAA ratio for this case. The ratio was determined to be 3.2 pmol/nmol in urine. This value is well below the cutoff established for recent antemortem ethanol consumption, leading to the conclusion that microbes were responsible for the formation of the ethanol present in the blood of this victim.

Case #5

The next case examined, which contained ethanol of an unknown origin, resulted from the crash of a helicopter in southern California. The body of the pilot was recovered from the Pacific Ocean at a depth of approximately 70 ft. The body was not recovered until 16 days after the accident had occurred. Specimens initially analyzed for volatile organic compounds in this case were blood, urine, brain and skeletal muscle. The blood and urine contained ethanol at concentrations of 22 and 18 mg/dL, respectively. Brain and skeletal muscle were found to contain ethanol at concentrations of 27 and 9 mg/hg, respectively. The distribution of ethanol in these fluids and tissues, although not completely self-consistent, does not exclude a conclusion of antemortem ethanol consumption. Various volatiles including acetaldehyde, n-propanol, n-butanol and acetone were also present in these specimens, which suggested postmortem microbial

formation of ethanol. This case represents another classic example of inconclusive ethanol origin. Without further investigation it would not be possible to determine whether the pilot had recently consumed ethanol. Utilizing our novel method we found the 5-HTOL/5-HIAA ratio to be 0.41 pmol/nmol in urine. This value is considerably below the 15 pmol/nmol cutoff, thus strongly indicating the absence of recent antemortem ethanol ingestion. The presence of ethanol in this case was thus reported as resulting from postmortem microbial formation.

Case #6

The sixth case examined, which contained ethanol of an unknown origin, resulted from the crash of a single engine airplane in a rural region of a northern state. The accident was witnessed by numerous people, which resulted in a quick response by emergency personnel and prompt recovery of the victim. Specimens initially analyzed for volatile organic compounds in this case were blood, urine, vitreous humor, brain and skeletal muscle. The blood, urine and vitreous humor contained ethanol at concentrations of 17, 25 and 25 mg/dL, respectively. Brain and skeletal muscle were found to contain ethanol at concentrations of 146 and 170 mg/hg, respectively. The distribution of ethanol in the fluids initially examined from this case is completely consistent with antemortem ethanol consumption. The tissues examined, however, contained ethanol completely inconsistent with the fluid results. Three other volatile organic compounds were detected in these specimens including n-propanol, acetaldehyde and n-butanol. Upon closer inspection, the concentrations of the three volatile organic compounds were significantly higher in brain and muscle when compared to the fluids examined. For example, n-

propanol was detected at 14 mg/hg in the brain, but only 1 mg/dL in the blood and urine, and n-butanol was detected at 19 mg/hg in the muscle, but only 1 mg/dL in the blood and the urine. Upon specimen retrieval by the medical examiner blood is collected via containers that contain heparin, while urine and vitreous humor are simply sealed in tubes. Tissue specimens are only placed in plastic zip-top bags. When the FAA received this case it was noted that the temperature of the container carrying the specimens in questions was elevated to 10°C. It was also noted by visual inspection that the tissue specimens were putrefied and, additionally, had a foul odor. It is probable that the tissue specimens in this case were contaminated with microbes before or during collection, and that these microbes had sufficient time to produce substantial quantities of ethanol.

This case represents one of the most difficult ethanol origin scenarios possible. It appears upon initial examination that ethanol was consumed antemortem. Then as a result of trauma experienced during the accident microbes contaminated both brain and muscle specimens. Ethanol formation subsequently occurred while these tissue specimens were in transit. We applied our LC/MS method to the urine available from this case and found the 5-HTOL/5-HIAA ratio to be 67 pmol/nmol. This value is well above the 15 pmol/nmol cutoff, thus confirming the recent consumption of ethanol prior to death as suggested by the fluid ethanol distributions.

Case #7

The final case examined, which contained ethanol of an unknown origin, resulted from the crash of a single engine airplane in Florida. The accident was witnessed by an individual who immediately notified authorities. The accident site was discovered the

following day approximately 19 hours after the crash. Specimens initially analyzed for volatile organic compounds in this case included blood, urine, vitreous humor and brain. The blood, urine and vitreous humor contained ethanol at concentrations of 4, 12 and 0 mg/dL, respectively, while the brain specimen was found to contain no ethanol. The distribution of the small amounts of ethanol in the fluids and tissue examined from this case is not self-consistent, and indicated microbial ethanol formation. The urine ethanol concentration raised the biggest concern in this case since it exceeded the 10 mg/dL value. Additionally, urine was once considered immune to microbial ethanol formation because it is sealed in the bladder and not normally exposed to microbes and/or glucose. However, this view of urine has been recently questioned.[82, 91, 92, 95-97] It is now known that postmortem microbial production of ethanol in urine is quite feasible and, indeed, that appears to have occurred in this case. Upon further investigation into the toxicological findings from the deceased pilot it was noted that the urine contained glucose at an abnormally high concentration of 169 mg/dL. With this substantially elevated concentration of glucose in the urine, even modest amounts of microbes could readily produce reasonably elevated levels of ethanol.

This case represents another difficult question of ethanol origin. It appeared upon initial examination that the ethanol present in the urine must have been consumed antemortem due to the long standing belief that urine was not susceptible to microbial ethanol formation. We applied our LC/MS method to the urine available from this case and found the 5-HTOL/5-HIAA ratio to be 0.32 pmol/nmol. This value is well below the 15 pmol/nmol cutoff, thus confirming the postmortem microbial formation of ethanol.

IV. Conclusions

The application of this LC/MS procedure for the analysis of 5-HTOL and 5-HIAA and the associated 5-HTOL/5-HIAA ratio has demonstrated the utility of this approach in assessing ethanol origin in postmortem specimens. One of the most important aspects of this novel method is the simultaneous analysis of both compounds using a single extraction method and a single analytical technique. This greatly increases the precision in the methodology and, thus, the certainty of the subsequent conclusions.

We would propose that the relative simplicity of this procedure should make the 5-HTOL/5-HIAA ratio methodology readily applicable to the routine assessment of antemortem versus postmortem origination of ethanol found in postmortem samples in toxicological fluid and tissue analyses. The application of this easy, rapid and precise method is also notably not limited to postmortem samples. Indeed contested ethanol results from routine screenings in various drug programs and criminal cases could readily be evaluated through the use of this procedure.

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