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Accurate assignment of ethanol origin in postmortem urine: liquid chromatographic–mass spectrometric determination of serotonin metabolites☆

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Abstract

Toxicological examination of fatal aviation accident victims routinely includes analysis of ethanol levels. However, distinguishing between antemortem ingestion and postmortem microbial formation complicates all positive ethanol results. 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 h after acute ingestion. The liquid–liquid extracts of postmortem urine samples were subjected to liquid chromatography–mass spectrometry (LC–MS) for the simultaneous quantitation of these two analytes, yielding detection limits of 0.1 ng/ml for each. Examination of the 5-HTOL/5-HIAA ratio was undertaken for 44 urine samples known to be antemortem ethanol-positive or antemortem ethanol-negative. Recent ethanol ingestion was conveniently and accurately separated using a 5-HTOL/5-HIAA ratio of 15 pmol/nmol, a value previously suggested using human volunteers. All 21 ethanol-negative postmortem samples were below this cutoff, while all 23 ethanol-positive postmortem samples were above this cutoff. Thus, we recommend the employment of this cutoff value, established using this straightforward LC–MS procedure, to confirm or deny recent antemortem ethanol ingestion in postmortem urine samples.

Keywords: Postmortem urine; Ethanol; Serotonin

1. Introduction

Ethanol analysis, most commonly accomplished by headspace gas chromatography (GC), is one of the most common and routine tests performed on forensic specimens. The presence of ethanol in fatal aircraft accident victims constitutes an important part of aircraft accident investigations and related litigation. An ethanol-positive identification of a sample invites legal scrutiny as to its validity. With the precision of today's analytical techniques, there is a high degree of certainty associated with the quantitative determination of ethanol found in a biological specimen. However, whether the ethanol found in a specimen is derived from postmortem microbial formation or antemortem ethanol consumption is another, and obviously more important, variable to consider when interpreting ethanol results.

The microbial formation of ethanol in postmortem specimens is by far the most likely complication encountered when examining ethanol results. The first report dealing with postmortem formation of ethanol in corpses appeared in 1936 [1,2]. Postmortem ethanol was found, surprisingly, to be present in a sample known to involve no prior antemortem ingestion. This result established that simple postmortem existence of ethanol could not be used as proof of antemortem ingestion. Today, it is known that many different microbes are responsible for postmortem formation of ethanol in animals [2]. Investigations have been performed to identify the particular species of bacteria, yeast and/or fungi responsi-

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ble for ethanol production and the mechanism by which it is formed [3–6]. *Candida albicans* has been the microbe most often ascribed to be responsible for postmortem production of ethanol in humans [7,8]. This species of yeast is commonly found in humans in vivo [9]. Located ubiquitously throughout the body, the highest concentrations of *C. albicans* are typically found in the mouth and on the skin [9]. Glucose is the most prevalent substrate in the human body used by these microbes to form ethanol [10]. Other endogenous compounds can also be utilized as substrates including, but not limited to, lactate, mannitol, galactose, maltose, sucrose and lactose [10–13]. However, it should be noted that approximately 100 species of bacteria, yeast and fungi have been shown capable of producing postmortem ethanol [3].

When working with forensic specimens, postmortem ethanol formation, beyond that which might have already occurred prior to specimen receipt, is generally felt to be reasonably suppressed by storage at $-20\,^{\circ}C$ and the addition of a preservative such as sodium fluoride [14]. These stabilization precautions, however, obviously do not eliminate ethanol formation prior to sample collection and preservation [15]. Furthermore, studies have demonstrated that if a specimen is not rigorously kept below freezing, the effect of the preservative sodium fluoride in blocking postmortem formation of ethanol is substantially diminished [16,17]. Although forensic laboratories routinely employ these preservation procedures quite strictly, the exact source of any postmortem ethanol may obviously still be in doubt.

Recently, possible exploitation of the metabolism of serotonin as a biological marker for ethanol consumption has begun to gain interest in the field of forensic science [18]. Serotonin (5-hydroxytryptamine, 5-HT) is an indoleamine commonly found in nature [19]. In humans, 5-HT is found throughout the body, with substantial concentrations found in the gastrointestinal tract and blood platelets [20]. The metabolism of 5-HT initially involves oxidative deamination to form the intermediate aldehyde, 5-hydroxyindole-3-acetaldehyde (5-HIAL). This intermediate can undergo either oxidation or reduction as shown in Fig. 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 [20,21]. Reduction, catalyzed by aldehyde reductase, leads to formation of 5-hydroxytryptophol (5-HTOL), a relatively minor metabolite of 5-HT [18]. However, ethanol consumption has been shown to lead to a significantly enhanced production of 5-HTOL.

An increase in 5-HTOL concentration following ethanol consumption was first reported in 1967 [22]. 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 [18,23–25]. Two primary factors contribute to an increase in the 5-HTOL/5-HIAA ratio. Both factors are associated with the oxidation of ethanol to acetaldehyde. First, as acetaldehyde is being produced from ethanol by alcohol dehydrogenase, it is substantially oxidized by aldehyde dehydrogenase to form acetic acid. This oxidation of acetaldehyde effectively occupies the substrate site of aldehyde dehydrogenase preventing the use of this site by 5-HIAL [26]. Secondly, normal metabolism of ethanol to acetaldehyde and acetic acid leads, in both of these enzymatic steps, to relatively excessive consumption of the available oxidized nicotinamide adenine dinucleotide (NAD^+) cofactor and relatively excessive production of the corresponding reduced NADH. This enhanced level of NADH

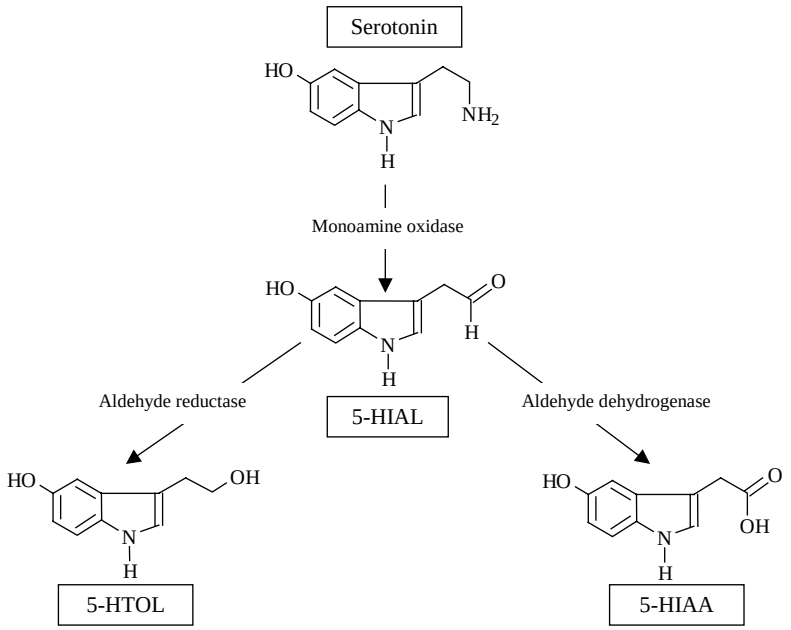

Fig. 1. The metabolism of serotonin.

may then add impetus for promoting conversion of 5-HIAL to 5-HTOL rather than to 5-HIAA [27]. These two factors act independently and together to cause an increase in the 5-HTOL/5-HIAA ratio following ethanol ingestion [26]. The 5-HTOL/5-HIAA ratio remains elevated for many hours after ingested ethanol has been eliminated from the body [24]. As such, the 5-HTOL/5-HIAA ratio has already been applied to ethanol cessation monitoring programs as a marker of recent ethanol ingestion in urine specimens [27–29]. The 5-HTOL/5-HIAA ratio has also been briefly investigated for possible use in postmortem urine samples [24]. We had hoped, thus, that this ratio might serve as a reliable, reproducible tool in analytical forensic toxicology for accurately differentiating between the postmortem 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) [30,31]. 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 typically been analyzed using gas chromatography with mass spectrometric detection (GC–MS) [32]. 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. In this study, we describe a remarkably precise method with very low detection limits for the rapid and simultaneous determination of 5-HTOL and 5-HIAA using liquid chromatography with mass spectrometric detection (LC–MS). Following validation of the method, we investigated the postmortem 5-HTOL/5-HIAA ratios in both antemortem ethanol-positive and antemortem ethanol-negative urine samples.

2. Materials and methods

2.1. 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). All chemicals were purchased in the highest possible purity and used without any further purification. Sodium chloride, sodium acetate, acetic acid, β-glucuronidase, 5-hydroxytryptophol, 5-hydroxyindole-3-acetic acid, and 5-methoxy-2-methyl-3-indoleacetic acid were purchased from Sigma (St. Louis, MO). Methanol, acetonitrile, ammonium hydroxide, hydrochloric acid, ethyl acetate, and nitric acid were purchased from Fisher Scientific (Pittsburgh, PA). Formic acid (97%) was purchased from ICN Biomedicals Inc. (Irvine, CA). *N*,*O*-bis[Trimethylsilyl]trifluoroacetamide with 1% trimethylchlorosilane (BSTFA/1% TMCS) was purchased from Pierce (Pierce Chemicals, Rockford, IL). All acetate buffers described further were prepared by first making stock solutions of the desired final concentration of both sodium acetate and acetic acid. One of these was then titrated with the other until the desired pH was achieved. The pH of all solutions was measured using a Corning model 430 pH meter (Corning Life Sciences, Acton, MA) connected to a Corning 3-in-1 model pH electrode.

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. 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. 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 10 ml of methanol. 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) [33]. However, for maximum assurance of the quality of data, we never used any stock solutions which were over 30 days old.

Since 5-HTOL in human-derived specimens is predominately found as the glucuronide derivative, we initially hydrolyzed the samples using the enzyme β-glucuronidase. A solution of β-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 and mixing to dissolve. This yielded a final concentration of 100,000 units/ml. 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.

The aqueous portion of the HPLC buffer was 50.0 mM formic acid adjusted to pH 5.00 with concentrated 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 HPLC 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 HPLC mobile phase.

2.2. Instrumentation

Analyte separation was achieved using a Hewlett-Packard 1100 HPLC (Hewlett-Packard Co., Wilmington, DE) equipped with a Security Guard™ C-8 guard column (4.0 mm × 3.0 mm i.d., 3 μm particles) from Phenomenex® (Torrance, CA), followed immediately by a Supelcosil™ LC-18 (150 mm × 4.6 mm i.d., 3 μm particles) analytical column from Supelco/Sigma–Aldrich (Bellefonte, PA). Samples were injected using a Hewlett-Packard G1313A autosampler. Identification and quantitation were accomplished using a Finnigan model LCQ atmospheric pressure chemical ionization (APCI) ion trap mass spectrometer (ThermoFinnigan Corp., San Jose, CA), which utilized nitrogen as the sheath gas and helium as the reagent gas. Control of the HPLC 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.).

2.3. LC–MS/MS and LC–MS/MS/MS methods

For all determinations, the HPLC was operated in an isocratic mode with a flow rate of 1.0 ml/min. The sample injection volume was 10 μl. The HPLC 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. As described in detail further, 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. Initial ionization evaluation of the derivatized compounds and the underivatized 5-MMIA 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 of the three compounds by infusing the desired compound at a concentration of approximately 10 μg/ml, prepared by dilution from the stock solutions using methanol, into the LCQ at a constant rate of 1.0 ml/min. Tuning the MS for the desired ions was then accomplished using the autotune feature of the Xcalibur™ software. As a result of these preliminary APCI–PCI–MS investigations, each sample analysis was subsequently split into three unique data collection segments.

The operating conditions for segment 1, which was 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 ms. Segment 1 was further split into two 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 segment 2, which analyzed 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 ms. Segment 2 was further split into three 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 segment 3, which analyzed 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 ms. Segment 3 was further split into three 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%.

2.4. Specimen preparation and extraction

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 further, were prepared and extracted in the following manner.

Three milliliters aliquots of individual samples were transferred to 16 mm × 150 mm screw-topped culture tubes. 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. β-Glucuronidase solution (7500 units; 75 μl) followed by 1.00 ml of 0.10 mM sodium acetate buffer (pH 6.00) was added to each sample. The samples were vortexed briefly and incubated at 70 °C for 45 min to facilitate hydrolysis of the glucuronide conjugate. In our initial investigations complete hydrolysis of the 5-HTOL–glucuronide conjugate was achieved after incubation with β-glucuronidase for 30 min at 70 °C. However, an incubation time of 45 min was chosen to ensure that specimens with extremely elevated 5-HTOL concentrations were also completely hydrolyzed. Following hydrolysis, samples were allowed to cool to room temperature. Then, 2.00 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 rotary mixing wheel and gently mixed for 20 min by simple rotation of the wheel at 6 rpm. Following mixing, the samples were centrifuged at 820 × *g* for 5 min. The organic (upper) layer of each sample was transferred using a disposable pipette to a clean 10 ml conical tube and dried in a water bath 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 heating block at 80 °C for 20 min. Following derivatization, the tubes were removed from the heating block, allowed to cool to room temperature, and subsequently evaporated to dryness in a water bath 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.

2.5. Extraction efficiency

The recovery of each analyte was determined using the following procedure [34]. Two groups, X and Y, of controls prepared using negative urine diluent were extracted in the same manner as described earlier. 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 between 1.0 and 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) = percent 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 further.

2.6. Postmortem urine specimens

Postmortem urine specimens received by our laboratory are always stored at −20 °C. However, no specimens are retained in our storage facility for more than 5 years. Classification of postmortem urine specimens in our laboratory as being positive or negative for antemortem ethanol ingestion has routinely been done using criteria that mirrors those 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 cutoff value. In our laboratory, fluid or tissue samples containing ethanol at or above this limit are declared positive for ethanol, while those below this limit are declared negative for ethanol.

3. Results and discussion

3.1. Method validation

Derivatization of 5-HTOL and 5-HIAA with TMS, as described earlier, was found to be essential for this method. Derivatization of these compounds achieved two necessary objectives. First, the underivatized compounds provided poor chromatographic resolution under mobile phase conditions amenable to LC–MS. 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 latter observation was not totally unexpected since one compound is an acid and the other a primary alcohol. By producing a TMS derivative of each compound, excellent chromatographic resolution was achieved, and the undesirable competing effects in the APCI–PCI ionization were eliminated. Additionally, derivatization increased the fundamental mass of each of the compounds, practically allowing use of MS/MS and MS/MS/MS and enhancing the associated detection limits by ca. 1000 times compared with the underivatized forms.

An ion trap mass spectrometer is a collection device which allows for the “trapping” or isolation of ions from a target compound followed by subsequent formation of unique spectra 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 and 5-HIAA derivatives, and MS/MS on 5-MMIA. 5-HTOL 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 the granddaughter ion at *m/z* 216.1. The same process was used on 5-HIAA, 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

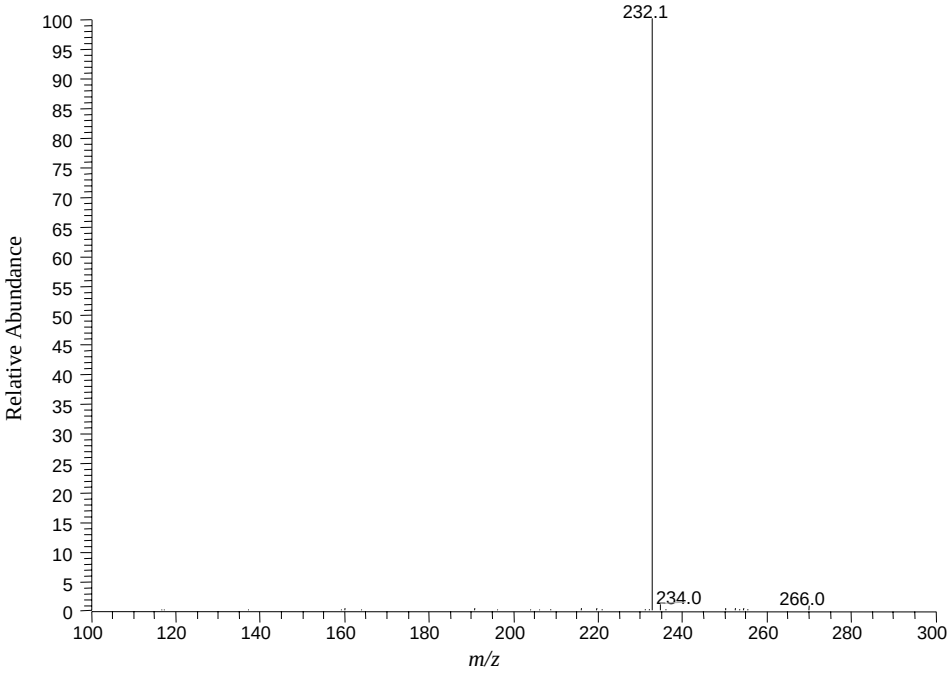

Fig. 2. MS/MS spectrum of 5-HTOL (*m*/*z* 250.1 → spectrum).

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-MMIA and the MS/MS/MS spectra for 5-HTOL and 5-HIAA provided the "fingerprints" used for analyte identification and confirmation. The full scan spectra are shown in Figs. 2–6.

Quantitation 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 calculated by dividing the area of the analyte peak by the area of the internal standard peak. The MS/MS/MS ion at *m*/*z* 216.1 was

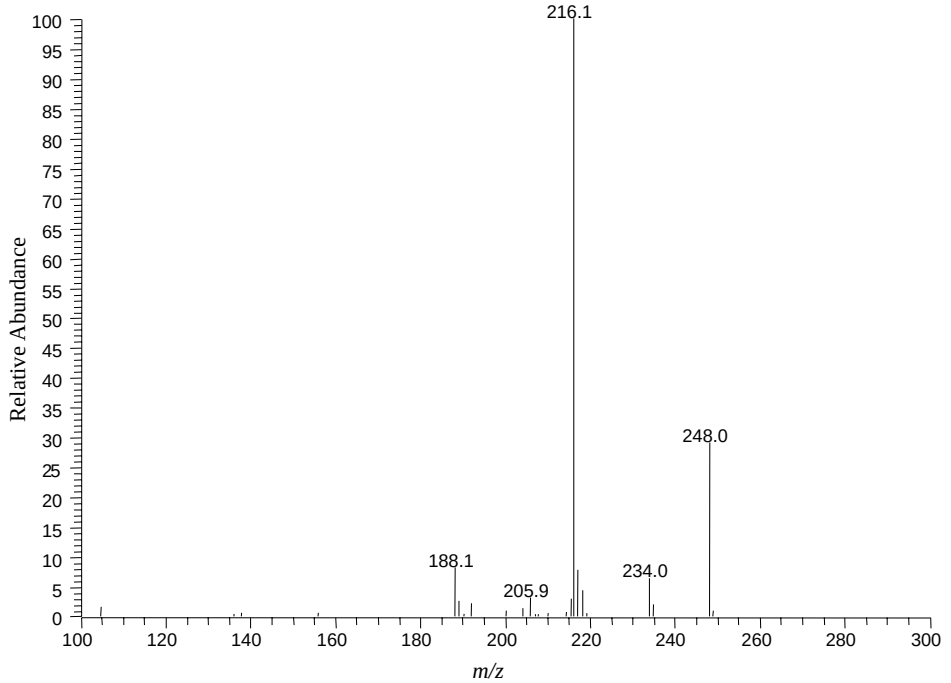

Fig. 3. MS/MS/MS spectrum of 5-HTOL (*m*/*z* 250.1 → *m*/*z* 232.1 → spectrum).

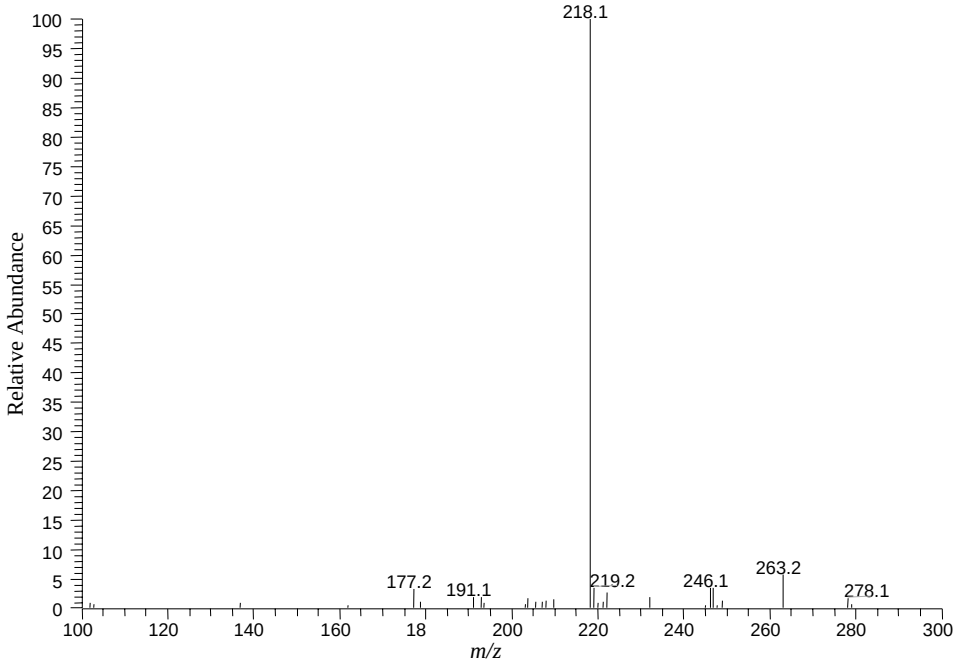

Fig. 4. MS/MS spectrum of 5-HIAA (*m*/*z* 264.1 → spectrum).

used for 5-HTOL quantitation, while the MS/MS ion at *m*/*z* 218.1 was used for 5-HIAA quantitation. 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 used to determine the concentrations of 5-HTOL and 5-HIAA in controls and specimens.

As can be seen in Fig. 7, 5-MMIA, 5-HTOL and 5-HIAA are well shaped, completely resolved, and readily distinguished from noise when analyte levels are at/near the limit of quantitations (LOQs) in real samples. The 5-HTOL and 5-HIAA portions of this chromatogram were multiplied by 250 to make the peaks visible on the scale shown; however, this amplification also clearly demonstrates the relatively innocuous noise levels seen in the neighborhood

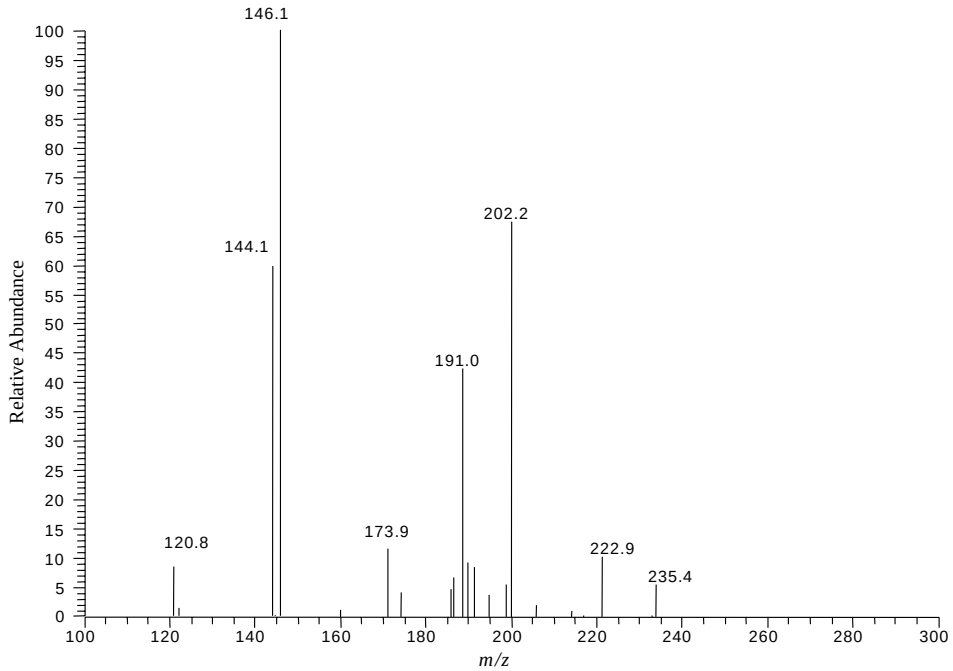

Fig. 5. MS/MS/MS spectrum of 5-HIAA (*m*/*z* 264.1 → *m*/*z* 218.1 → spectrum).

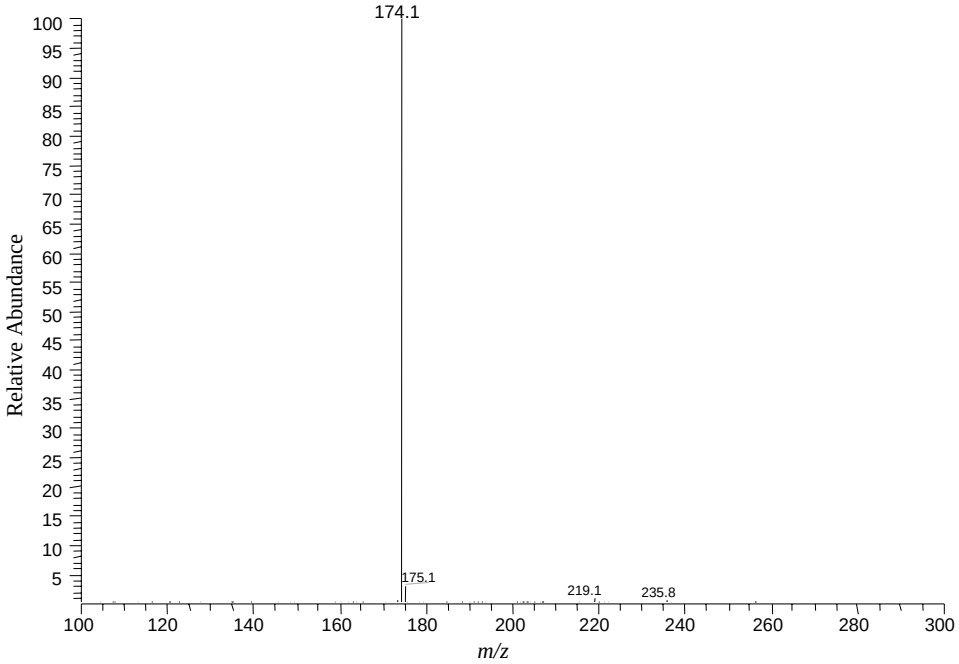

Fig. 6. MS/MS spectrum of 5-MMIA (*m*/*z* 220.1 → spectrum).

of these peaks at these low levels of injected analytes. Construction of a similar chromatogram for an extracted, processed-urine-negative control exhibited detector response throughout the analyte regions which was always less than 0.1% of the internal standard peak height. The compounds determined experienced no interference from endogenous sample matrix components. Typical retention times were 1.73, 2.44 and 4.11 min for 5-MMIA, 5-HTOL and 5-HIAA, respectively. The average number of theoretical plates calculated for each compound ranged from 2000 to 5000.

The extraction efficiency of 5-HTOL and 5-HIAA from postmortem urine samples, as described in Section 2.5, was determined at 1, 10, 50 and 800 ng/ml. The individual recovery values are presented in Table 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 simplicity of the extraction procedure.

The limit of detection (LOD), limit of quantitation and linear dynamic range (LDR) were determined for each analyte. The LOD was defined as the lowest concentration of analyte having a minimum signal-to-noise (S/N) ratio of 5, in addition to meeting a MS/MS and MS/MS/MS spectral "fingerprint" confirmation and ±5% 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

Table 1
Percent recovery ± S.D.for 5-HTOL and 5-HIAA[a]

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

[a] $n = 5$ for all determinations.

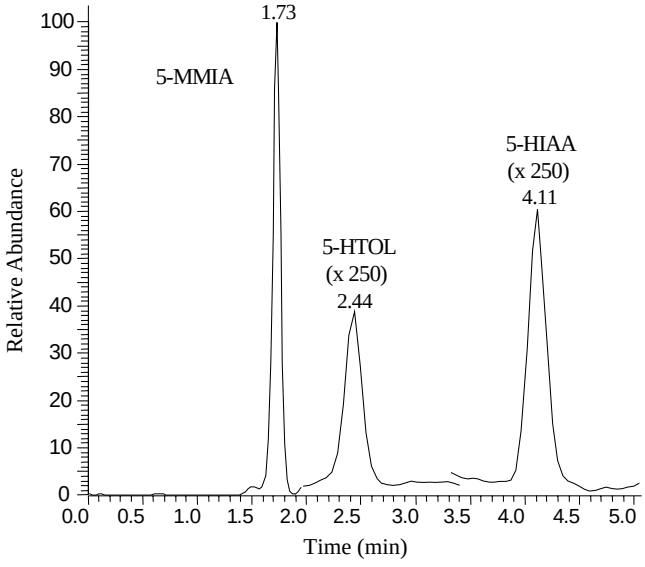

Fig. 7. Representative concatenated chromatogram of 5-MMIA, 5-HTOL and 5-HIAA in an extracted urine calibrator. Chromatographic peaks represent ions monitored in SIM mode for each compound as follows: 5-MMIA MS/MS ion at *m*/*z* 174.1; 5-HTOL MS/MS/MS ion at *m*/*z* 216.1; 5-HIAA MS/MS ion at *m*/*z* 218.1. Peaks obtained from a 10 μl injection of a 780 pg/ml calibrator. The chromatogram is constructed by monitoring for 5-MMIA from 0 to 2.0 min, for 5-HTOL from 2.0 to 3.4 min and for 5-HIAA from 3.4 to 5.0 min. The 100% relative abundance corresponds to the peak current observed for the 5-MMIA peak.

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 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 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.

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 solvent injections. A methanol blank initially injected following the highest calibrator showed no carryover contamination. Subsequently, blanks were used randomly throughout the sample sequence to verify that no sample-to-sample contamination occurred.

Intra-day (within-day) and inter-day (between-day) 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 (R.S.D.) for the experimentally determined concentrations. 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 five replicates of each control concentration on day 1 of the experiment. The intra-day relative error and R.S.D. 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 R.S.D. 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 2.

Inter-day accuracy and precision were evaluated by extracting five replicates of each of two control concentrations on days 2, 4 and 8, and basing the quantitation on the calibration curve originally prepared on day 1. The results obtained after storage of each control lot at 4 °C for 2, 4 and 8 days can be seen in Table 2. At 2 days of storage, the relative error and R.S.D. for 5-HTOL were 0 and 6% at 1 ng/ml and −14 and 5% at 10 ng/ml, respectively. The relative error and R.S.D. 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 R.S.D. for 5-HTOL were −4 and 9% at 1 ng/ml and −5 and 4% at 10 ng/ml, respectively. The relative error and R.S.D. 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 R.S.D. for 5-HTOL were −1 and 4% at 1 ng/ml and −13 and 1% at 10 ng/ml, respectively. The relative error and R.S.D. 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

Table 2
Intra-day accuracy and precision for repeated determinations over 8 days[a]

	5-HTOL		5-HIAA	
Day 1				
Target concentration (ng/ml)	1	10	1	10
Mean ± S.D. (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				
Target concentration (ng/ml)	1	10	1	10
Mean ± S.D. (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				
Target concentration (ng/ml)	1	10	1	10
Mean ± S.D. (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				
Target concentration (ng/ml)	1	10	1	10
Mean ± S.D. (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

[a] $n = 5$ at each concentration for each day, controls were run on days 1, 2, 4 and 8.

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 [30]. 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.

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 and 180. 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 properly stored specimens should not be of significant concern for at least 180 days.

3.2. Forensic urine analysis

Several studies have been conducted on living humans examining the shift in the urinary 5-HTOL/5-HIAA ratio following consumption of ethanol [18,24,25,30,35]. 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 ratios were. 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 [18]. They found that while ethanol could no longer be detected in the body 10 h after dosing, the 5-HTOL/5-HIAA ratio remained significantly elevated for up to 16 h after dosing. In a separate study, Hagan and Helander dosed subjects at 0.60 g/kg [25]. They too found that the 5-HTOL/5-HIAA ratio remained significantly elevated for up to 16 h after dosing, a time which was significantly longer than ethanol could be detected in the body [25]. Helander et al. have established a cutoff value for the 5-HTOL/5-HIAA ratio of 15 pmol/nmol, below which ethanol had not been consumed for at least 14 h prior to sampling [28,36,37]. These same authors also included a cursory examination of postmortem urine samples with respect to their 5-HTOL/5-HIAA ratios, and, while finding substantial individual variations, demonstrated a reasonable correlation between ethanol levels and 5-HTOL/5-HIAA ratios [24].

To ascertain the utility of our procedure for routine toxicological investigations, we examined 44 urine specimens obtained from fatal aviation accident victims. Of the 44 specimens analyzed, 21 specimens were known to be negative for antemortem ethanol consumption, while 23 specimens were known to be positive for antemortem ethanol consumption. Urinary ethanol concentrations obtained from the cases examined were corroborated by investigation of a minimum of one other tissue or fluid from the same case. All ethanol-negative urine specimens had a separate corresponding ethanol-negative tissue or fluid, while all ethanol-positive urine specimen had a separate corresponding ethanol-positive tissue or fluid. The data collected from all 44 specimens are illustrated in Fig. 8a. The results show a general trend of increasing 5-HTOL/5-HIAA ratio with increasing urine ethanol concentration. There is, however, substantial inter-individual variation in 5-HTOL/5-HIAA ratios at ethanol concentrations below approximately 150 mg/dl. This area has been expanded and can be seen in more detail in Fig. 8b. These variations are not unexpected since the specimens utilized in this study are from postmortem sources with unknown dietary, medical and other potentially pertinent information.

The 21 ethanol-negative postmortem urine specimens investigated had corresponding 5-HTOL/5-HIAA ratios that ranged from 0.01 to 9.38 pmol/nmol, with an average of 2.52 ± 2.94 pmol/nmol (mean ± S.D.). 5-HTOL concentrations in these specimens ranged from 0.930 to 301 ng/ml with an average of 40.1 ng/ml. 5-HIAA concentrations

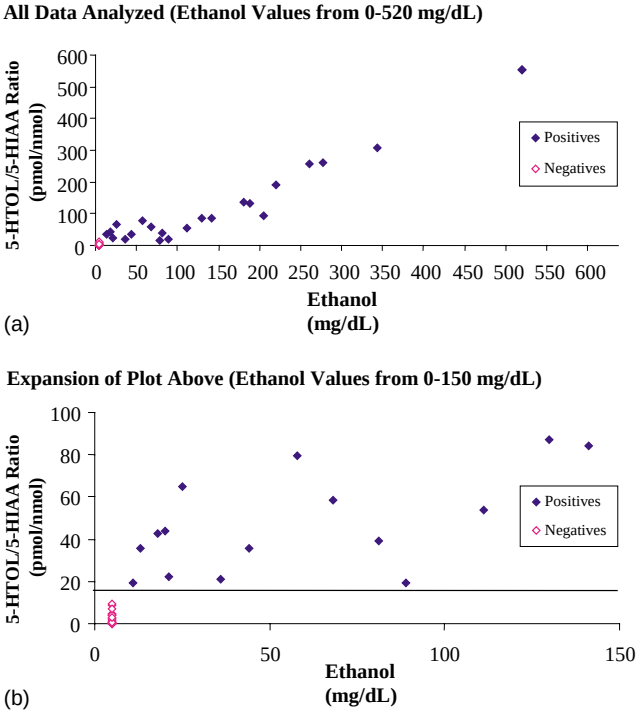

Fig. 8. (a and b) 5-HTOL/5-HIAA ratio as a function of postmortem urine ethanol.

ranged from 1.1 to 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 551 pmol/nmol. As seen in Fig. 8, we initially examined a 15 pmol/nmol ratio for 5-HTOL/5-HIAA as the cutoff between positive and negative values for antemortem ethanol consumption. We were actually a bit surprised to see that this cutoff, originally established for living human beings, was seemingly appropriate for postmortem samples as well. However, it should be noted that selection of the 15 pmol/nmol cutoff appears to be a conservative one leading to a minimization of false positives. We feel this is completely appropriate due to the substantial personal, professional and legal consequences of a positive postmortem declaration in toxicological examinations. This cutoff value is more than 4 standard deviations above the average result obtained for all 21 ethanol-negative specimens. By simple statistical consideration this would lead to, at most, a false positive rate of no more than 1 in 10,000.

We applied this novel LC–MS method to urine samples from two unusual cases of postmortem ethanol formation, which could have easily lead to a false ethanol-positive. Case 1 was obtained from a fatal aviation accident that occurred in remote, mountainous terrain. The victim was not recovered from the accident scene for more than 24 h. This case had a blood ethanol concentration of 92 mg/dl, well above the antemortem ethanol cutoff of 10 mg/dl. The case also had a positive urinary ethanol concentration of 21 mg/dl. But, the vitreous humor ethanol concentration, which is normally similar to the urine value, was 0 mg/dl, a clearly negative value. These specimens were each also 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 [3,8,17,38–42]. However, the use of other volatiles as a marker for postmortem ethanol formation can be misleading and, therefore, caution must be exercised [3]. 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, which was substantially below the established 15 pmol/nmol cutoff. This result clearly indicates an absence of recent ethanol ingestion and indicates that the ethanol present in this case was due to postmortem microbial formation.

Case 2 was an aviation fatality which was recovered from water approximately 2.5 months after the accident occurred. The urine and blood ethanol concentrations were 31 and 16 mg/dl, respectively. Vitreous humor, heart and skeletal muscle were also analyzed and were found to have ethanol concentrations of 17 mg/dl, 8 and 12 mg/hg, respectively. The distribution of ethanol in these fluids and tissues supports a conclusion of antemortem ethanol consumption. Various volatiles, including large amounts of acetaldehyde, *n*-propanol and *n*-butanol were also present in these samples supporting the opposing mechanism of microbial formation of ethanol. The blood and tissue samples were noted as putrefied by simple inspection, and the urine was bloody. While the distribution of ethanol between fluid and tissues as well as the levels of ethanol were quite consistent with antemortem ethanol consumption, the visual confirmation of sample putrefaction and the presence of the various volatiles suggests that the ethanol found in this case is due to postmortem microbial formation. We found the 5-HTOL/5-HIAA ratio to be 1.9 pmol/nmol. This value is well below the 15 pmol/nmol cutoff, thus strongly indicating the absence of recent antemortem ethanol ingestion. The presence of ethanol in this case was reported as being due to postmortem microbial formation.

4. Conclusion

The procedure described in this paper provides a rapid, accurate and reproducible method for the simultaneous extraction and quantitation of 5-HTOL and 5-HIAA in postmortem human urine specimens. A liquid–liquid extraction procedure in combination with HPLC–APCI–PCI–MS provides both superior separation of these two compounds and detection limits that are well below concentrations expected in the usual postmortem specimen.

The application of this procedure shows the effectiveness of LC–MS in the analysis of 5-HTOL and 5-HIAA. It also demonstrates the utility of the 5-HTOL/5-HIAA ratio in determining ethanol origin in postmortem urine 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. The relative simplicity of this procedure should make the 5-HTOL/5-HIAA ratio methodology readily applicable to the assessment of antemortem versus postmortem origination of ethanol found in postmortem samples in toxicological fluid and tissue analyses.

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