Simultaneous measurement of ethanol and ethyl-d₅ alcohol by stable isotope gas chromatography–mass spectrometry

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Efforts to define gender- and ethnic-dependent differences in ethanol first-pass metabolism by gastric mucosa and liver have been limited by a lack of analytical tools that distinguish ethanol concurrently administered by oral and intravenous routes. A stable isotope gas chromatography–mass spectrometry method for simultaneous measurement of ethanol and ethyl-d₅ alcohol in serum was developed to meet this need. The assay was linear from 1 to 30 mmol/L. The limit of quantification was 1 mmol/L. Analytical imprecision (CV) was <10%. Analytical recovery was >90%. Specificity was based on retention time and reproducibility of ion ratios. The assay was free from interference by other volatile alcohols. Simultaneous oral administration of ethanol and ethyl-d₅ alcohol produced nearly identical pharmacokinetic profiles. Simultaneous oral ingestion of ethanol and intravenous infusion of ethyl-d₅ alcohol, adjusted for gastric emptying time, revealed decreased bioavailability of ethanol by the oral route. The method described is sufficient to study the first-pass metabolism of ethanol.

INDEXING TERMS: bioavailability • metabolism, first-pass • pharmacokinetics

Excessive exposure to ethanol (ethyl alcohol) may increase an individual’s risk for alcoholic hepatitis and cirrhosis. However, total dose-related exposure does not reliably predict whether a given individual will progress to organ injury [1, 2]. For example, alcoholic cirrhosis develops in only a subgroup of alcoholics [3, 4]. The reasons for varied susceptibility to this complication are poorly understood. Previous studies suggest that genetic factors are important [5]. In general, women are more vulnerable to alcoholic cirrhosis than are men [6]; in women, progression of hepatic injury occurs in response to lower amounts of alcohol intake over a shorter time [7–11]. This gender-dependent difference has been attributed to an apparently greater bioavailability of orally administered ethanol in women compared with men [12, 13]. Lower gastric mucosal alcohol dehydrogenase (ADH) activity in women compared with men and, thus, less extensive first-pass oxidative metabolism by the stomach, purportedly accounts for the difference in alcohol bioavailability [14, 15]. Increased concentrations of ethanol, particularly in the portal circulation, may account for the greater susceptibility of women to alcoholic cirrhosis. Women also have a lower percent total body water relative to body weight when compared with men. Since alcohol distributes into body water, a dose of alcohol based on body weight would produce higher blood alcohol concentrations in women than in men [11]. This, too, may contribute to the greater vulnerability of women to alcohol-induced liver injury.

Ethnic differences in alcohol first-pass metabolism also may exist. Seventy percent of Japanese men and women lack gastric α-ADH, typically found in Caucasians and African-Americans [16, 17]. The absence of this form of gastric ADH might account for diminished gastric ADH activity among Japanese individuals.

Studies designed to measure the oral bioavailability of ethanol are potentially confounded by an inability to distinguish and measure the alcohol simultaneously administered by intravenous

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This work was reported in part in the following abstracts:


Received September 27, 1995; accepted November 22, 1995.

¹ Nonstandard abbreviations: ADH, alcohol dehydrogenase; AUC, area under the concentration vs time curve; and GC-MS, gas chromatography–mass spectrometry.
and oral routes [18]. In particular, measuring intravenously and orally administered alcohol in separate experiments introduced variability into previous measurements of ethanol bioavailability [19]. The present study was conducted to develop and validate a stable isotope gas chromatography–mass spectrometry (GC-MS) method for simultaneous measurement of ethanol and ethyl-d₅ alcohol in serum. This analytical tool was designed to permit measurement of alcohol simultaneously administered by intravenous and oral routes and to provide better assessment of ethanol bioavailability.

**Materials and Methods**

**REAGENTS**

Absolute ethanol and anhydrous ethyl-d₅ alcohol for preparation of analytical calibrators were from Aaper Alcohol and Chemical Co. (Shelbyville, KY) and Aldrich Chemical Co. (Milwaukee, WI), respectively. Anhydrous ethyl-d₅ alcohol also was used for oral and intravenous administration. Eighty-proof vodka (Smirnoff, Menlo Park, CA) was used for oral administration. n-Butanol and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ) and Mallinckrodt (Paris, KY), respectively. Fresh frozen plasma was obtained from Transfusion Medicine, Indiana University Medical Center (Indianapolis, IN) and was analyzed by the Sports Medicine and Drug Identification Laboratory, Indiana University Medical Center, to ensure that it was drug-free. Crimp-top vials (11-mm clear), rubber caps, and 100-μL flat-bottom insert tubes were purchased from Sun International (Wilmington, NC).

**PREPARATION OF CALIBRATORS**

Stock calibrators were prepared by weighing 46 mg of ethanol and 51 mg of ethyl-d₅ alcohol into separate 10-mL aliquots of drug-free fresh frozen plasma. Working calibrators of ethanol and ethyl-d₅ alcohol were prepared in plasma to yield final concentrations of 1, 2.5, 5, 10, 20, and 30 mmol/L.

**SAMPLE EXTRACTION**

Subject sera and calibrators (100 μL of each) were combined with 200 μL of 500 mg/L n-butanol (internal standard) in acetonitrile. Samples were sealed, vortex-mixed, and allowed to stand at room temperature for 10 min; they were then centrifuged at 15,000g for 10 min. The resulting supernates were transferred to 100-μL flat-bottom insert tubes and sealed in 11-mm clear crimp-top vials. One microliter was injected onto the analytical column.

**CHROMATOGRAPHIC APPARATUS AND CONDITIONS**

Analyses were performed with a 5890 Series 11 gas chromatograph equipped with a 5971 Mass Selective Detector from Hewlett-Packard (Palo Alto, CA). The analytical column, Supelcowax™ 10 (30 m × 0.25 mm (i.d.) × 0.25 μm film), was from Supelco (Bellefonte, PA). The chromatographic conditions were as follows: injection port temperature 150 °C, split ratio 70:1, purge vent 1-3 mL/min, head pressure 34.5 kPa, linear velocity 30 cm/s, and column flow 0.90 mL/min. Helium was the carrier gas. The oven temperature was held at 60 °C for 2 min, and then ramped to 175 °C at 5 °C/min. The column oven temperature was held at 175 °C for 1 min, and then programmed to 200 °C at 20 °C/min. The mass selective detector was operated in the selected ion monitoring mode, to measure the abundance of the molecular ions and two additional ions for each alcohol. The monitored ions were as follows: ethanol (m/z 31, 45, 46), ethyl-d₅ alcohol (m/z 33, 49, 51), and n-butanol (m/z 55, 56, 57).

**ASSAY VALIDATION**

Calibration curves were constructed by least-squares regression of the ratios of abundances of the m/z 45 ion (ethanol) and the m/z 49 ion (ethyl-d₅ alcohol) to the abundance of the m/z 55 ion (n-butanol) vs the concentrations of ethanol and ethyl-d₅ alcohol in calibrator samples. Limits of detection were defined as concentrations that produced at least three times the background signal produced by alcohol-free plasma. The limits of quantification were defined as the lowest alcohol concentrations tested for which the lower 99% confidence interval from the calibration curves did not intersect the x-axis. Specificity was based on retention time and stability of ion ratios compared with pure calibrators. Potential interference in measurement of ethanol by ethyl-d₅ alcohol and vice versa was assessed by comparing the calibration curves for each alcohol in the absence and presence of a large excess (30 mmol/L) of the other alcohol. Potential interference by an excess (30 mmol/L) of other volatile alcohols also was examined. Within-run precision was determined by repeated (n = 10) analysis of high-, intermediate-, and low-concentration controls in single and multiple runs.

**HUMAN SUBJECT**

The participation of a human subject in the experimental protocol was reviewed and approved by the Institutional Review Board, Indiana University–Purdue University at Indianapolis, and was consistent with the Helsinki Declaration of 1975 as revised in 1983. After review of the purpose and risks of participation in the study, the subject completed an informed consent statement. Medical history revealed that the subject, a 27-year-old Caucasian man, was a nonsmoker on no medications. He had some experience with social drinking but no personal or family history of alcohol abuse or alcoholism. The latter was excluded by using the Michigan Alcoholism Screening Test [20]. The subject was deemed healthy by physical examination.

**EXPERIMENTAL PROTOCOL**

In each of three sessions, the subject was asked to abstain from beverage alcohol for 48 h and to fast for 8 h before alcohol administration. A breath-alcohol content was measured with an Alcosensor IV Intoximeter (St. Louis, MO) before the initiation of each session. Vital signs and body weight were recorded. A 20-gauge angiocatheter for blood sampling was placed in an antecubital vein. Patency of the vein was maintained by infusion of isotonic saline at a rate of 20 mL/h. At 0800, the subject was given 15 min to eat a meal consisting of two scrambled eggs, two strips of bacon, two pieces of toast with two pats of butter and jam, and 224 mL of orange juice. Oral administration of ethanol
began at 0900 and was completed within 10 min, while the subject was seated at a 50° angle on a cart.

In the first session, the subject ingested 3.7 MBq (100 μCi) of 99Tc-labeled technicium sulfate mixed with water and vodka (80 mL/L) to yield 0.3 g of ethanol per kg of body weight. Gamma emission over the stomach was recorded each minute for 1 h with a gamma camera. Counts per minute were corrected for the decay rate of 99Tc, and the rate of gastric emptying was calculated.

In a second session, the subject was given the same oral dose of ethanol as vodka in water but without 99Tc. An equimolar dose of ethyl-d₅ alcohol, diluted to 80 mL/L with sterile isotonic saline, was infused intravenously via a second angiocatheter placed in an antecubital vein of the arm opposite that used for blood collection. The intravenous infusion was adjusted to deliver alcohol at a rate similar to that defined by gastric emptying in the first session. For this subject, intravenous alcohol was infused at 3.33 mL/min for 11 min and at 5.14 mL/min for 75 min. Separate 1-mL whole-blood samples were obtained via the angiocatheter at 0, 7, 15, and every 15 min thereafter until 210 min after initiation of alcohol ingestion. Serum, recovered by centrifugation of these whole-blood specimens at 1400g for 5 min, was transferred to a clean polypropylene tube, frozen in solid CO₂, and stored at −70°C until assayed for ethanol and ethyl-d₅ alcohol concentration by GC-MS.

In a third session, the subject simultaneously ingested 0.15 g of ethanol per kilogram of body weight and an equimolar amount of ethyl-d₅ alcohol. The combined dose was diluted in water to ~80 mL/L. Separate 1-mL whole-blood samples were collected and processed and the resulting serum specimens were stored and analyzed for ethanol and ethyl-d₅ alcohol by GC-MS as in the second session.

**Results and Discussion**

The stable isotope GC-MS assay described in the present study allowed simultaneous detection and measurement of ethanol and ethyl-d₅ alcohol in serum. The limits of detection (sensitivity) for both ethanol and ethyl-d₅ alcohol were 0.5 mmol/L. The limits of quantification for both compounds were 1 mmol/L with at least 99% confidence (Fig. 1). These limits of detection and quantification are comparable with those routinely achieved in our clinical laboratory for analysis of whole-blood alcohol by gas chromatography with flame ionization detection. The calibration curves for ethanol and ethyl-d₅ alcohol were linear from 1 to 30 mmol/L (Fig. 1). The slope ± SE and y-intercept ± SE for the abundance of m/z 45 ion derived from ethanol relative to the m/z 55 ion derived from n-butanol (internal standard) vs the ethanol concentration were 0.082 ± 0.001 and 0.022 ± 0.018, respectively. The coefficient of determination (r²) was 0.992. For ethyl-d₅ alcohol, the slope ± SE and y-intercept ± SE for the abundance of the m/z 49 ion relative to the abundance for the m/z 55 ion vs concentration were 0.068 ± 0.001 and 0.003 ± 0.018, respectively. The coefficient of determination (r²) was 0.995. Linearity of the assay for higher alcohol concentrations was not assessed.

The within- and between-run analytical imprecision, expressed as CV, for low- (1 mmol/L), intermediate- (15 mmol/L), and high- (30 mmol/L) concentration ethanol and ethyl-d₅ alcohol controls were stable (Table 1). As expected, between-run imprecision was greater than within-run imprecision, but only marginally so. Analytical recoveries for these same controls were consistently ≥90% with some dependence on concentration (Table 1). Analytical recoveries at concentrations >2.5 mmol/L were in excess of 95% (data not shown). Use of the internal standard, n-butanol, served to stabilize assay imprecision and analytical recovery.

The analytical specificity of this method was based, in part, upon the reproducibility of analyte retention time on capillary column gas chromatography. Repetitive analysis of the low-, intermediate-, and high-concentration controls containing ethanol and ethyl-d₅ alcohol plus n-butanol revealed imprecision <0.5% for within- and between-run retention times. Representative chromatograms show complete gas chromatographic resolution of unidentified matrix-related peaks and n-butanol from ethanol and ethyl-d₅ alcohol, but only minimal resolution of the latter two compounds (Fig. 2). Highly reproducible electron
impact ionization with selective ion monitoring mode mass spectrometry provided additional analytical specificity. Selected ions derived from ethanol (m/z 31, 45, and 46) and ethyl-d₅ alcohol (m/z 33, 49, and 51) were used to qualitatively identify each compound. Ion abundance ratios for ethanol (m/z 31:m/z 45 and m/z 46:m/z 45) and ethyl-d₅ alcohol (m/z 33:m/z 49 and m/z 51:m/z 49) were deemed characteristic of each alcohol in experimental specimens when they fell within ranges equivalent to those for the 30 mmol/L calibrators ± 20% (Fig. 3). Ion ratios typically fall outside this range at concentrations below the limit of detection or when a coeluting peak produces significant quantities of m/z ions characteristic of the analyte to be measured.

The low molecular masses of both alcohols precluded identification of highly specific ions. Although the abundances of m/z 45 and m/z 49 were used to quantify the amounts of ethanol and ethyl-d₅ alcohol, respectively, in experimental samples, the m/z 45 ion was not entirely specific to ethanol. Ionization of ethyl-d₅ alcohol produces low abundance of m/z 45 ion, the ion selected for quantitative measurement of ethanol. Because ethanol and ethyl-d₅ alcohol were not resolved by gas chromatography, the m/z 45 ion derived from the deuterated compound interfered with the quantification of the nondeuterated alcohol. The impact of this interference was illustrated as a consistent positive bias in an ethanol calibration curve prepared from calibrators containing excess (30 mmol/L) ethyl-d₅ alcohol (Fig. 4).

The abundance of m/z 45 ions derived from ionization of ethyl-d₅ alcohol was directly proportional to the abundance of the m/z 49 ion. A consistent abundance ratio for the m/z 45 ion to the m/z 49 ion, both derived from ethyl-d₅ alcohol, allows for

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Target, mmol/L</th>
<th>Mean ± SD, mmol/L</th>
<th>CV, %</th>
<th>Recovery, %</th>
<th>Mean ± SD, mmol/L</th>
<th>CV, %</th>
<th>Recovery, %</th>
</tr>
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<tbody>
<tr>
<td>Ethanol</td>
<td>1.0</td>
<td>0.90 ± 0.03</td>
<td>3.3</td>
<td>90</td>
<td>0.90 ± 0.06</td>
<td>6.7</td>
<td>90</td>
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<tr>
<td></td>
<td>15.0</td>
<td>16.4 ± 1.1</td>
<td>6.7</td>
<td>109</td>
<td>15.5 ± 1.3</td>
<td>8.4</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>30.0</td>
<td>28.4 ± 0.6</td>
<td>2.1</td>
<td>95</td>
<td>29.0 ± 1.2</td>
<td>4.1</td>
<td>97</td>
</tr>
<tr>
<td>Ethyl-d₅ alcohol</td>
<td>1.0</td>
<td>1.04 ± 0.06</td>
<td>5.8</td>
<td>104</td>
<td>1.03 ± 0.10</td>
<td>9.7</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>15.6 ± 0.06</td>
<td>0.1</td>
<td>104</td>
<td>15.1 ± 0.8</td>
<td>5.3</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>30.0</td>
<td>30.9 ± 1.17</td>
<td>3.8</td>
<td>103</td>
<td>29.8 ± 1.79</td>
<td>6.0</td>
<td>99</td>
</tr>
</tbody>
</table>

Fig. 2. Representative total ion chromatogram.
GC-MS analysis of serum collected from the subject 120 min after combined oral administration of ethanol and ethyl-d₅ alcohol showed complete resolution of unidentified matrix-related peaks (1) and the internal standard, n-butanol (4), from ethanol (2) and ethyl-d₅ alcohol (3). Resolution of the latter two peaks was only marginal. Chromatography of serum from this subject obtained before alcohol administration revealed no peaks at retention times corresponding to peaks 2 and 3 (not shown).

Fig. 3. Concentration-dependent stability of ion abundance ratios and analytical sensitivity.

(A) ion abundance ratios for m/z 31:m/z 45 (●) and m/z 46:m/z 45 (○) vs ethanol concentration. (B) ion abundance ratios for m/z 33:m/z 49 (●) and m/z 51:m/z 49 (○) vs ethyl-d₅ alcohol concentration. The dotted lines represent the ion abundance ratios ± 20% for either the 30 mmol/L ethanol (A) or ethyl-d₅ alcohol (B) calibrator. The resulting ranges provide qualitative specificity for each alcohol. If abundance ratios for either alcohol in experimental samples exceeded the ranges indicated by the dotted lines, that alcohol was considered undetected.
the following calculation of m/z 45 ion abundance derived from ethanol:

\[ A_{45}^{Eth} = A_{45}^{Total} - A_{45}^{Eth-d_5} \]

where \( A_{45}^{Eth} \) = abundance of the m/z 45 ion derived from ethanol, \( A_{45}^{Total} \) = the total abundance of the m/z 45 ion, and \( A_{45}^{Eth-d_5} \) = abundance of the m/z 45 ion derived from ethyl-d_5 alcohol calculated by multiplying the abundance of the m/z 49 ion by the abundance ratio of the m/z 45 ion to the m/z 49 ion for ethyl-d_5 calibrators.

The calculated abundance of the m/z 45 ion derived from ethanol was used to determine the concentration of ethanol in the presence of ethyl-d_5 alcohol. Effectiveness of this correction is demonstrated by elimination of positive bias observed in a calibration curve based on ethanol calibrators supplemented with excess (30 mmol/L) ethyl-d_5 alcohol (Fig. 4). The magnitude of the correction was typically <10% when the concentrations of the two alcohols were equimolar and provided accurate measurement of ethanol in the presence of ethyl-d_5 alcohol. Ethyl-1,1-d_2 alcohol from Merck & Co. (Rahway, NJ) and ethyl-d_5 alcohol from Aldrich were evaluated as alternatives to ethyl-d_5 alcohol for use in this assay. However, ionization of these compounds produced mass spectra that were even less distinct than the mass spectra of ethyl-d_5 alcohol when compared with mass spectrum of ethanol. By contrast, ethanol produced no background abundance for the m/z 49 ion. Thus, ethanol did not interfere with quantification of ethyl-d_5 alcohol. Analysis of ethanol and ethyl-d_5 alcohol by direct GC-MS analysis of whole blood, serum, or plasma was not successful because of matrix-related interferences. Treating plasma or serum samples with acetonitrile eliminated the matrix-associated interferences, presumably by deproteinization. The assay was free from interference by acetone and other volatile alcohols (Table 2).

The utility of this assay was demonstrated by measurement of serum ethanol and ethyl-d_5 alcohol after simultaneous oral administration of both compounds to a human subject (Fig. 5). This route of coadministration produced nearly identical concentration vs time profiles. The area under the concentration vs time curve (AUC) from 0 to 210 min for ethanol (1406 mmol/L·min) and ethyl-d_5 alcohol (1480 mmol/L·min) were similar. Despite the similar profiles and areas under these curves, the concentration of ethyl-d_5 alcohol was consistently equal to or slightly greater than that of ethanol. Over the duration of this experiment, the concentration of ethyl-d_5 alcohol was on average 0.3 mmol/L greater than that for ethanol. This difference is small relative to the analytical imprecision of the method and may be due to the latter. Alternatively, this difference may be due to slight overcorrection of ethanol concentrations. The possibility of a minimal isotope effect cannot be excluded.

When equimolar doses of ethanol and ethyl-d_5 alcohol were

![Fig. 4. Correction of potential interference from ethyl-d_5 alcohol in the quantification of ethanol.](image)

A calibration curve for ethanol in the presence of excess (30 mmol/L) ethyl-d_5 alcohol (∗) illustrates a consistent positive bias relative to calibration in the absence of ethyl-d_5 alcohol (●). Mathematical correction for this interference, as described in Results and Discussion, produces a corrected calibration curve (dashed line) that is not significantly different from that for ethanol in the absence of ethyl-d_5 alcohol.

![Fig. 5. Simultaneous oral administration of ethanol and ethyl-d_5 alcohol.](image)

Serum ethanol (●) and ethyl-d_5 alcohol (○) concentrations after simultaneous oral administration of both compounds to a human subject show nearly identical profiles vs time. The sum of both alcohol concentrations also is shown (∗).

### Table 2. Chromatographic specificity.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention time, min</th>
<th>k'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>2.01</td>
<td>0.44</td>
</tr>
<tr>
<td>Acetone</td>
<td>2.42</td>
<td>0.73</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>2.47</td>
<td>0.76</td>
</tr>
<tr>
<td>Ethanol</td>
<td>3.00</td>
<td>1.14</td>
</tr>
<tr>
<td>Ethyl-d_5 alcohol</td>
<td>3.02</td>
<td>1.16</td>
</tr>
<tr>
<td>n-Propanol</td>
<td>4.10</td>
<td>2.00</td>
</tr>
<tr>
<td>n-Butanol</td>
<td>5.83</td>
<td>3.16</td>
</tr>
<tr>
<td>Isoamyl alcohol</td>
<td>6.81</td>
<td>3.86</td>
</tr>
<tr>
<td>Amyl alcohol</td>
<td>7.57</td>
<td>4.40</td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td>15.12</td>
<td>9.80</td>
</tr>
</tbody>
</table>

Acetone and volatile alcohols listed above, prepared at a concentration of 30 mmol/L, were well resolved from and did not interfere with GC-MS analysis of ethanol and ethyl-d_5 alcohol.
Ethanol

Dean

References

This work was supported by a Public Health Service grant AA07611.

Fig. 6. Simultaneous oral administration of ethanol and intravenous administration of ethyl-d₅ alcohol.

The oral route of administration for ethanol (●) produced serum concentrations consistently lower than that observed with ethyl-d₅ alcohol (○). The sum of both alcohol concentrations is shown (▲).

simultaneously administered by oral and intravenous routes, respectively, the serum concentration vs time profiles were dissimilar (Fig. 6). With different routes of administration, the AUC for ethanol (1336 mmol/L · min) was substantially less than the AUC for ethyl-d₅ alcohol (1594 mmol/L · min). This difference supports the notion that the bioavailability of ethanol is diminished by oral ingestion and the resulting first-pass metabolism. Failure of the ethanol and ethyl-d₅ alcohol curves to rise at the same rate and peak at the same time suggests that intravenous infusion rates need to be optimized for future studies. Nevertheless, the analytical method described proved sufficient for study of ethanol first-pass metabolism and is currently being used to assess the role of gender and ethnicity.

This work was supported by a Public Health Service grant AA07611.

References