Quantitative Endogenous Formate Analysis in Plasma Using Headspace Gas Chromatography Without a Headspace Analyzer

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Abstract

The objective was to develop a simple routine method for quantitative measurement of endogenous formic acid in plasma and whole blood using headspace gas chromatography—flame ionization detection. (GC-FID). Two-hundred microliters of sample was placed in a 1-mL glass vial. Fifty microliters of aqueous ethanol (10%) was added as an internal standard and a derivatizing agent. Ethylformate formation was enhanced by addition of 200 μL concentrated sulfuric acid as a catalyst. The vials were then sealed immediately and placed in a water bath for 15 min at 60°C. One milliliter of this headspace gas was siphoned using a gas-tight syringe and injected into a GC-FID fitted with a capillary column. Ethanol eluted at approximately 3.0 min, and ethylformate eluted around 4.7 min. The limit of quantitation for ethylformate was 0.026 mmol/L, and the limit of detection was 0.020 mmol/L. Imprecisions for spiked plasma samples at 0.25 and 1 mmol/L were 10% and 9%, respectively, and recoveries were at 100% and 108%, respectively. A simple, reliable, and highly specific headspace analysis method for quantifying endogenous formate without the use of a headsapce analyzer was developed. This method enables the routine clinical analysis of formate in plasma and whole blood samples.

Introduction

Formic acid and its conjugate base formate are essential endogenous one-carbon metabolites in most living organisms participating in vital one-carbon pool of intermediary metabolism (1,2). Studies have shown that formate is the metabolite of methanol (MeOH) and responsible for the toxicity observed in methanol poisoning (3-5). Other sources of formate are through the diet, environmentally through inhalation of methanol vapors, and production by intestinal microflora and certain dietary supplements (6,7). For methanol toxicity to occur, it first has to be metabolized by alcohol dehydrogenase (ADH) to formaldehyde and then to formic acid (Figure 1). The toxicity of formate includes optic nerve damage, an increased anion gap, and metabolic acidosis (8,9).

Early laboratory diagnosis of methanol poisoning as well as monitoring of formate levels, whether for occupational health or clinical research purposes, is essential. In the case of methanol poisoning, analyses by gas chromatography (GC)-based methods are the most common. Most hospitals use osmolal and anion gap to aid in their diagnosis of alcohol ingestion. Methanol increases the osmolal gap while formate increases the anion gap (3,10,11). Metabolic acidosis and increased anion and osmolal gaps might not be seen at lower concentrations of plasma methanol (< 20mM) and formate (10,12,13). Furthermore, increases in osmolal and anion gap could also be due to ethylene glycol ingestion (14). Given the ubiquitous nature of formate and its deleterious effect at higher levels, it is important to have a reliable quantitative method for the measurement of plasma formate at all levels (subclinical and clinical). Most routine clinical methods involve enzymatic analysis and/or GC with flame-ionization detection (FID) (15-18). Although these methods are reliable, their sensitivity at lower endogenous levels is poor. Mass spectrometry (MS) may provide a better sensitivity, but it is expensive and has longer turnaround time because of sample preparation. We describe here a simple, quantitative GC-FID method that is sensitive at lower formate levels and provides a faster turnaround time.
Experimental

Materials

Pure formic acid standard (88%, v/v) was obtained from Fisher Scientific (Fair Lawn, NJ). Sulfuric acid and anhydrous ethyl alcohol were obtained from J.T. Baker (Phillipsburg, NJ) and Commercial Alcohols (Brampton, ON, Canada), respectively. A gas-tight syringe (Hamilton 1.25 mL; 22/2”/2) was purchased from Hamilton Company (Reno, NV). A working stock solution of 100 mmol/L formic acid was prepared in deionized water. Aqueous serial dilutions were made from each of the stock at concentrations of 20.0 mmol/L, 10.0 mmol/L, 4.0 mmol/L, 2.0 mmol/L, 1.0 mmol/L, 0.5 mmol/L, 0.25 mmol/L, 0.125 mmol/L, and blank. A 10% (v/v) ethanol in deionized water was prepared as an internal standard. These working standards and the ethanol were prepared fresh each time.

Analysis of clinical sample and recovery experiments

Pooled plasma was spiked with formic acid to required concentrations (0.125 and 1 mmol/L). Limits of detection (LOD) and quantitation (LOQ) were determined in aqueous samples because of the ubiquitous nature of formate in biological samples. Five levels (10 replicates of each) were prepared at 0.010, 0.020, 0.125, 0.250, and 1.000 mmol/L. Data obtained was analysed using EP Evaluator software version 7 (David G. Rhoads Associates, Kennett Square, PA).

Plasma samples (personal information/identification removed) were obtained from 69 pregnant women whose plasma samples were sent for prenatal panel (rubella Ig G, syphilis, hepatitis B virus antigen, and HIV antigen). Another 15 serial plasma samples were collected from a female patient admitted into the intensive care unit for methanol poisoning. An additional 10 samples were obtained from a pharmacokinetic study using pigs (31–38 kg, n = 4), which were injected intravenously with 237 mg/kg formate.

Sample preparation

Two linear calibration curves were constructed. One standard curve ranged from 0 to 4.0 mmol/L, for low formate samples, and the other from 0 to 20.0 mmol/L for high formate samples. Two-hundred-microliter plasma samples and aqueous standards were each placed in a 1-mL glass vial. Fifty microliters of aqueous ethanol (10%, v/v) was added as internal standard and as a derivatizing agent. The formation of ethylformate was enhanced by addition to the reaction mixture of 200 µL concentrated sulfuric acid as a catalyst. The vials were then sealed immediately and placed in a waterbath for 15 min at 60°C to allow the ethylformate formed to equilibrate in the headspace above the reaction mixture in the vial. One milliliter of this headspace gas was siphoned using a gas-tight syringe and injected into the GC.

Instrumentation

Analysis of ethylformate was done on a GC fitted with an FID (Hewlett-Packard series II 5890) fitted with a DB-ALC1 capillary column (30 m x 0.53-mm i.d., 3.0 µm, J&W Scientific, Folsom, CA). Other parameters were set as outlined in Table I. Integration of the internal standard peak (ethanol) and

<table>
<thead>
<tr>
<th>Table I. Parameter Values for the Gas Chromatograph</th>
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<tbody>
<tr>
<td>Parameter</td>
</tr>
<tr>
<td>Carrier gas</td>
</tr>
<tr>
<td>Oven</td>
</tr>
<tr>
<td>Injector</td>
</tr>
<tr>
<td>Detector</td>
</tr>
<tr>
<td>Detector gas</td>
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<tr>
<td>Column</td>
</tr>
</tbody>
</table>

Figure 2. Chromatographic spectrum of ethyl formate.

Figure 3. Standard curves of low (A) and high (B) aqueous standards of formic acid.
ethylformate was done using HP 3365 series II Chemstation (version A.03.21, Hewlett-Packard). Quantitation of the ethylformate was done using the ratio of the ethylformate peak area to that of ethanol using standard curves (Chemistry Software for Windows®, ChemSW™).

Results

Figure 2 shows the chromatogram with the internal standard (ethanol) eluting at 2.9 min and ethylformate at 4.5 min. A six-point linear calibration curve was constructed for the aqueous standards. The chromatography of these aqueous standards showed complete separation of the analyte and the internal standard (ethanol). Spiked samples also showed chromatography consistent with that seen in the aqueous standards. The amount of ethanol added as an internal standard resulted in very high levels (final concentration 311 mM, 14.29 g). Thus, any ethanol present in the sample would not have an effect on the quantitation.

Linearity

A six-point calibration curve was constructed for the low standards (Figure 3A) and a five-point curve for the high standards (Figure 3B). The dynamic range selected was based on physiological levels of formate (4,8,19,20). The low standard curve calibration points included a blank, 0.125, 0.250, 0.500, 1.000, 2.000, and 4.000 mmol/L. Linear regression equation was \( y = 0.0537x + 0.00075 \) (\( r = 0.9999; r^2 = 0.9985 \)). The high standard curve calibration points included a blank, 2, 4, 10, and 20 mmol/L. Linear regression equation was \( y = 0.00502x - 0.00034 \) (\( r = 0.9999; r^2 = 0.9999 \)).

Precision and recovery

The limit of detection (LOD), determined in aqueous standards, was 0.02 mmol/L, corresponding to imprecision (coefficient of variation, CV) of 24%. Limit of quantification (LOQ) was 0.026 mmol/L (CV = 20%). The LOQ was obtained by fitting a curve using the equation CV = A + B x (1/mean) to estimate the relationship between mean and CV. Based on this model the concentration at whose upper 95% confidence interval for the curve had a CV of 20% was determined as the LOQ (Figure 4 and Table II).

Recovery and intra-assay precision of formate in plasma were determined at two levels (0.125 mmol/L and 1.000 mmol/L). Recovery and precision at both spiked levels were acceptable (101-108%). Imprecision at 0.125 mmol/L was 10.5%, and at 1 mmol/L, it was 8.6% (Table III).

Clinical samples

The results of formate analysis on 69 pregnant women are summarized in Table IV. The mean ± SD was 0.202 ± 0.198 mmol/L. This was consistent with that found by other workers (4,6,8,19,21). The formate profile of a female patient admitted into the intensive care unit for suspected methanol poisoning is shown in Figure 5. This patient was on treatment for methanol poisoning that included hemodialysis. Plasma formate profile with time in pigs (n = 4) administered a bolus intravenous injection of formate (237 mg/kg, as a sodium formate) is shown in Figure 6.

Discussion

Formate is an important compound that is present in humans, animals and plants. Most methods available in hospitals for diagnosis of methanol poisoning do not quantitate endogenous formate levels and rely instead on methanol levels. GC–MS methods are able to quantitate such levels but require lengthy sample preparation. Such instruments are also expensive. Therefore, such a method is not suited for routine clinical settings. Our method combines both high sensitivity and simplicity accompanied by faster turnaround time. The instrument, GC–FID, is far less expensive than GC–MS, making it a more accessible instrument for both hospitals and research labs. Unlike the method developed by Abolin et al. (15), this method cannot be used to monitor ethanol levels encountered in ethanol intoxi-
Table III. Recovery and Precision of Aqueous Spiked Plasma Samples

<table>
<thead>
<tr>
<th>Sample Concentration Type</th>
<th>Number of Samples</th>
<th>Mean (mmol/L)</th>
<th>SD (mmol/L)</th>
<th>CV (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>1</td>
<td>1.075</td>
<td>0.092</td>
<td>8.60</td>
<td>108</td>
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<tr>
<td></td>
<td>0.125</td>
<td>0.126</td>
<td>0.013</td>
<td>10.49</td>
<td>101</td>
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<tr>
<td>Aqueous</td>
<td>0.125</td>
<td>0.125</td>
<td>0.010</td>
<td>8.16</td>
<td>100</td>
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<tr>
<td></td>
<td>0.020</td>
<td>0.020</td>
<td>0.004</td>
<td>20.45</td>
<td>101</td>
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<tr>
<td></td>
<td>0.010</td>
<td>0.004</td>
<td>0.004</td>
<td>90.44</td>
<td>43</td>
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</table>

Table IV. Summary Statistics of Plasma Formate in 69 Pregnant Women

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Value (mmol/L)</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.202</td>
<td>0.154-0.249</td>
</tr>
<tr>
<td>Standard deviation (SD)</td>
<td>0.198</td>
<td>0.170-0.238</td>
</tr>
<tr>
<td>Range</td>
<td>0.011-0.957</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>0.108</td>
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</tbody>
</table>

Figure 5. Plasma formate, methanol, and ethanol profile of a female patient intoxicated with approximately 5 cups of methyl hydrate. Treatment included a standard ethanol drip (10 h post-ingestion); hemodialysis (from 12 h to 20 h post-ingestion); folate (50 mg), vitamin B6 (500 mg), and thiamine (200 mg/9 h post-ingestion and repeated at 20 h post-ingestion). At admission (9 h post-ingestion), pH was 7.4.

Figure 6. Semilogrithmic plasma formate versus time plot in young pigs (n = 4) administered 237 mg/kg formate by i.v. bolus injection. Each time point shows mean formate level.

Conclusions

We have demonstrated the quantitative assay of plasma formate at various levels as seen in the pig and pregnant population experiments described. The method can also be used in research settings as demonstrated by the data on formate disappearance in pigs administered formate as well as in the methanol-poisoned patient. The turnaround time from sample receipt to results was approximately 25 min.

References


