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Simultaneous determination of formate and acetate in whole blood and urine from humans using gas chromatography–mass spectrometry

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Abstract

We devised a sensitive and simple method for simultaneous determination of formate and acetate in whole blood and urine from humans using gas chromatography–mass spectrometry. Formate and acetate were alkylated with pentafluorobenzyl bromide in the mixture of acetone and phosphate buffer (pH 6.8). The derivatives obtained were analyzed using gas chromatography–mass spectrometry in positive-ion electron ionization (EI) mode. The lower limit of detection for both compounds was 0.02 mM. The calibration curves for formate and acetate were linear over the concentration range from 0.05 to 5.0 mM. Accuracy and precision of the method were evaluated and the coefficients of variation were within 10%. With use of this method, levels of formate and acetate in whole blood can be determined in forensic cases.

Keywords: Formate; Acetate

1. Introduction

Formate and acetate in whole blood and urine are sometimes analyzed in forensic cases. Formate is considered to be an indicator for formaldehyde [1–3], methanol [4–10], and formic acid poisoning [11,12] since methanol and formaldehyde are metabolized to formic acid. As ethanol is metabolized to acetic acid, acetate is considered to be an indicator for oral ingestion of ethanol [13,14].

Short-chain fatty acids such as formate and acetate are also produced from blood or urine degraded during postmortem changes. When both formate and acetate were found to be over the control level in blood and urine of healthy persons, it is assumed that these acids had been produced by degradation. Therefore, it is useful that formate and acetate are simultaneously determined in forensic cases in order to determine the origin of these acids.

There are reports on the simultaneous determination of formate and acetate as the phenacyl esters [15], the *p*-bromophenacyl esters [16] or the pentafluorobenzyl esters [17,18]. However, these methods do not determine formate and acetate in biological samples. The pentafluorobenzyl ester of formate in biological samples could not be determined because of the instability of the ester [19].

We earlier developed a sensitive and simple method to determine sulfide [20], polysulfides [21], thiosulfate [22], cyanide [23], thiocyanate [23], azide [24], nitrite and nitrate [25,26] in whole blood using pentafluorobenzyl bromide as the alkylating agent. In this report, we derivatized formate and acetate in biological samples simultaneously for possible use in forensic cases.

2. Experimental

2.1. Reagents

A solution of internal standard (IS) was prepared by dissolving 1,3,5-tribromobenzene (TBB) in n-hexane to yield a concentration of 0.1 mM. TBB was purchased from Wako Pure Chemical Industries (Osaka, Japan). An alkylating agent, pentafluorobenzyl bromide (PFBBr; Aldrich, Milwaukee, WI, USA), was dissolved in acetone at a concentration of 100 mM. A stock standard solution (100 mM)

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of formate and acetate were prepared by dissolving sodium formate and sodium acetate in distilled and deionized water. This solution was further diluted with this water to prepare 0.1–50 mM solutions. The other reagents used were of analytical grade.

2.2. Preparation of whole blood and urine samples

Samples to be tested were prepared by adding the standard solution to whole blood and urine, both of which were collected from a healthy volunteer. The whole blood collected was added to an anticoagulant (heparin) and stored at 4 °C. For analysis of formate and acetate, 0.2 ml samples of whole blood and urine were directly used in the alkylation procedure without deproteinization.

When formate or acetate concentration in whole blood and urine is over the quantitation limit of 5.0 mM, the sample was diluted.

2.3. Alkylation procedure

A 1.0-ml volume of 100 mM PFBBr solution in acetone was put into a 10-ml volume glass-stoppered test tube with 0.2-ml of 0.5 M phosphate buffer (pH 6.8). 0.2 ml of the sample solution was added to the mixture and the preparation was vortexed for 1 min at room temperature, maintained at 60 °C in a water bath for 60 min. 2.0 ml of 0.1 mM TBB solution in *n*-hexane was added to the preparation. The preparation was vortexed for 1 min at room temperature then centrifuged at 1400 × *g* for 15 min. The organic phase was placed in another test tube and 1.0-μl aliquot of the solution was injected onto the gas chromatography–mass spectrometry (GC–MS) apparatus.

2.4. GC–MS conditions

Gas chromatography–mass spectrometry (GC–MS) was done using on a Hewlett-Packard HP 5790 A gas chromatograph (Palo Alto, CA, USA) interfaced to a JEOL AX505 A mass spectrometer (Tokyo, Japan). The column was a J&W fused-silica capillary tube of DB-225 (30 m × 0.32 mm i.d., 0.25 μm film thickness). A splitless injection mode was selected with a valve off-time of 1.5 min. The initial temperature of the column was held at 50 °C for 3 min, and then programmed to rise to 220 °C at 10 °C/min. The injection port, separator and ion source were kept at 220, 200, and 220 °C, respectively. Helium was used as the carrier gas at a flow-rate of 2 ml/min. Mass spectra were obtained by positive-ion electron ionization (EI) mode and negative-ion chemical ionization (CI) mode, scanning every 0.5 s from 45 to 400 *m/z* and from 25 to 400 *m/z*, respectively. The ionization energy of the EI condition was 70 eV. The ionization energy and reagent gas of the CI conditions were 200 eV and isobutane, respectively.

2.5. Preparation of calibration graphs

Whole blood and urine samples were prepared to contain formate and acetate at concentrations of 0.02–5.0 mM. These samples were extracted and derivatized in the same manner as described above. Calibration graphs were obtained by plotting the peak-area ratio of the molecular peak ion, *m/z* $[226]^+$ or *m/z* $[240]^+$, of the derivative of formate or acetate to the base peak ion, *m/z* $[314]^+$, of TBB (IS) against the concentration of formate or acetate, using mass chromatography in positive-ion EI mode.

3. Results and discussion

3.1. Analysis by GC–MS

Mass spectra of the derivative of formate and acetate using positive-ion EI and negative-ion CI modes, are shown in Figs. 1 and 2, respectively. As shown in Fig. 1, the molecular ion of the derivative of formate was observed at *m/z* 226 when using positive-ion EI mode, and the base peak ion was observed at *m/z* 181 $[M–HCOO]^+$. Using negative-ion CI mode, the base peak ion of the derivative of formate was $[HCOO]^-$ at *m/z* 45. The mass spectral pattern indicated that the derivative obtained was PFB-OCOH. As shown in Fig. 2, the molecular ion of the derivative of acetate was observed at *m/z* 240 when using the positive-ion EI mode, and the base peak ion was observed at *m/z* 181 $[M–CH_3COO]^+$. Using negative-ion CI mode, the base peak ion of the derivative of acetate was $[CH_3COO]^-$ at *m/z* 59. The mass spectral pattern indicated that the derivative obtained was PFB-$OCOCH_3$. The most abundant ion of TBB was observed at *m/z* 314 $[M + 2]^+$ using positive-ion EI mode. Using negative-ion CI mode, the base peak ion of TBB was observed at *m/z* 79 $[Br]^-$.

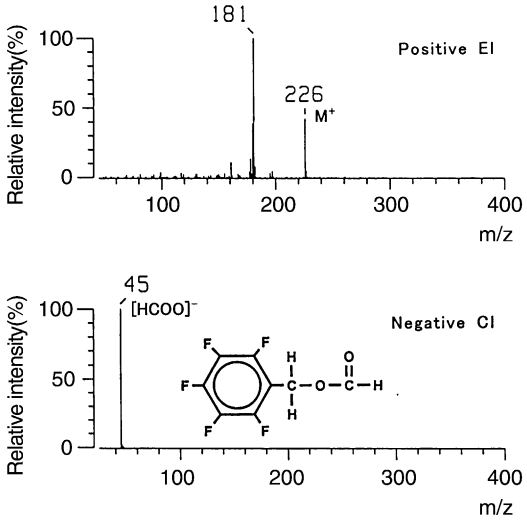

Fig. 1. Mass spectra of the derivative of formate, using positive-ion EI and negative-ion CI modes of GC–MS.

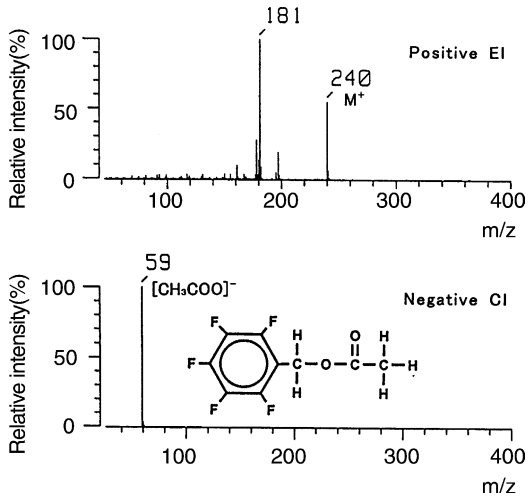

Fig. 2. Mass spectra of the derivative of acetate, using positive-ion EI and negative-ion CI modes of GC–MS.

3.2. Determination of formate and acetate in whole blood and urine

Mass chromatograms of the derivatized extracts from whole blood with added 1.0 mM each of formate and acetate, using positive-ion EI mode, are shown in Fig. 3. Sharp and symmetrical peaks of the derivative from formate, acetate, and TBB (IS) were observed, with retention times of 9.1, 9.7, and 13.9 min, respectively. The calibration curves were linear within the concentration range from 0.05 to 5.0 mM for formate and acetate in whole blood and urine. The equations and r values for the curves were: $y = 0.133x + 0.011$ ($r = 0.998$) for formate in whole blood; $y = 0.173x + 0.019$ ($r = 0.998$) for formate in urine; $y = 0.150x + 0.013$ ($r = 0.998$) for acetate in whole blood; $y = 0.151x + 0.014$ ($r = 0.999$) for acetate in urine. Recoveries of formate and acetate in the whole blood and urine were determined by comparing the peak area ratios of

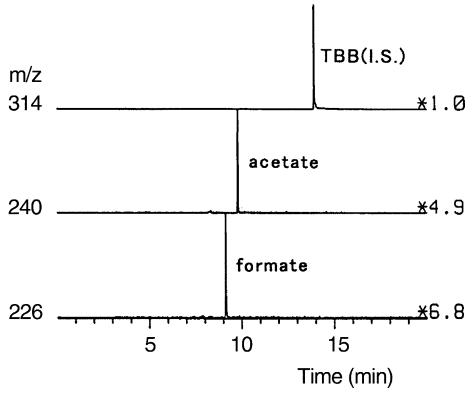

Fig. 3. Mass chromatograms of the derivatized extract obtained from blood with added 1 mM each of formate and acetate, using positive-ion EI mode of GC–MS. The numbers to the right of the chromatograms indicate the degree of enlargement used to obtain similar-sized peaks in the chromatograms.

Table 1
Accuracy and precision of formate and acetate determination in human whole blood[a]

Compound	Added (mM)	n	Detected (mM)		R.S.D. (%)
			Mean	S.D.	
HCOOH	0	5	0.07	0.007	9.4
	1.0	5	1.02	0.089	8.7
	2.0	5	2.06	0.087	4.3
CH_3COOH	0	5	0.08	0.007	9.6
	1.0	5	1.06	0.066	6.3
	2.0	5	2.11	0.086	4.1

[a] S.D.: standard deviation; R.S.D.: relative standard deviation.

m/z 226 of formate derivative, or m/z 240 of acetate derivative, to m/z 314 of TBB in samples with those in water samples, using mass chromatography. The gross recoveries of formate in the whole blood and urine were 65 and 85%, respectively, while those of acetate in both samples were 70%.

The lower limit of detection for formate and acetate in whole blood and urine, based on a concentration giving a signal three times stronger than the average noise intensity, was approximately 0.02 mM, the same level as in reported methods. The lower limits of detection of formate in reported GC methods were 0.05 mM [27], 0.02 mM [28,29], and 0.03 mM [30]. The lower limits of detection of acetate in reported GC and GC–MS methods were 0.05 mM [31] and 0.01 mM [32], respectively. Within-day precisions were obtained using two different concentrations (1.0 and 2.0 mM) by adding formate and acetate to blank whole blood and urine. The coefficients of variation (CV) ranged from 3.4 to 9.6% (Tables 1 and 2).

Derivatization of formate and/or acetate followed by GC–MS analysis is a superior technique which can be used to identify these compounds. The pentafluorobenzylation of formate and acetate has been reported [17,18]. Ohmori et al. [19] attempted to determine formate and acetate in biological samples as the pentafluorobenzyl ester using the method reported by Chauhan and Darbre [17]. Though acetate could be determined, they could not identify formate as the ester was unstable. As acidity of formic acid is approximately as potent as that of hydrofluoric acid, the ester

Table 2
Accuracy and precision of formate and acetate determination in urine[a]

Compound	Added (mM)	n	Detected (mM)		R.S.D. (%)
			Mean	S.D.	
HCOOH	0	5	0.09	0.007	8.1
	1.0	5	1.06	0.060	5.7
	2.0	5	2.07	0.077	3.7
CH_3COOH	0	5	0.10	0.009	9.4
	1.0	5	1.02	0.048	4.7
	2.0	5	2.10	0.071	3.4

[a] S.D.: standard deviation; R.S.D.: relative standard deviation.

of formate appear to be easily hydrolyzed [19]. Jacobsson et al. [18] reported the study of extractive pentafluorobenzylation of formate and acetate with tetrahexylammmonium as the counter ion. As the reagent for extractive alkylation must be in excess to proceed the reaction at a sufficient rate, the concentration of the counter ion has to be high. They found it impossible to determine formate and acetate in the low range concentration without further purification of the counter ion of tetrahexylammonium hydrogen sulfate.

To overcome such problems, further investigation was performed based on conditions used for our derivatization [22]. We could derivatize formate and acetate simultaneously without using the counter ion. Chauhan and Darbre [17] used $NaHCO_3$ as a base, whereas in the present method alkylation was performed at pH 6.8 with use of a phosphate buffer and this explains why no apparent degradation of the ester of formate was observed. The satisfactory derivatization yield of formate was obtained under weak acid to neutral conditions. On the other hand, derivatization yield of acetate was obtained under neutral to weak base conditions. Therefore, we used phosphate buffer of pH 6.8 for the simultaneous derivatization of formate and acetate.

3.3. Practical applications

We examined two blood samples collected from a 68-year-old man who had died after drinking a formalin solution (case 1), and a 60-year-old man who died after drinking a thinner solution containing methanol (case 2). Formalin is a solution containing about 37% of formaldehyde, to which 10% of methanol was added to prevent formaldehyde polymerization. Toxicological examinations were carried out using our method described in this report. The blood levels of formate and acetate were 7.60 and 0.17 mM in case 1, and 1.35 and 0.08 mM in case 2, respectively. The blood levels of methanol were 0.21 and 0.34 mg/ml, respectively. Formate concentrations in cases 1 and 2 were 109 and 19 times higher than the control levels of 0.07 mM [33]. As the level of formate in case 1 was six times higher than that in case 2, measuring formate in addition to methanol is considered to be useful in suspected methanol poisoning in order to elucidate the solution ingested. Acetate concentrations in both cases were within a control range (0.06 mM [34], 0.03 mM [35], 0.181 mM [32]). Therefore, formate poisoning was verified.

We examined 20 blood samples from which ethanol was detected. The correlation of acetate and ethanol concentrations is shown Fig. 4. The samples were collected from persons involved traffic accidents or who died of unknown causes. The blood levels of ethanol were 1.32 ± 1.17 mg/ml. The blood levels of formate and acetate were 0.07 ± 0.03 and 1.45 ± 0.82 mM, respectively. When ethanol was detected in whole blood, the acetate levels seemed to remain elevated. So long as ethanol is present, the acetate concentrations were almost constant, 1–2 mM, but ethanol concentrations seemed to be no effect on blood acetate levels. This increased acetate level in the blood might be used as an indicator of ethanol consumption [13,14].

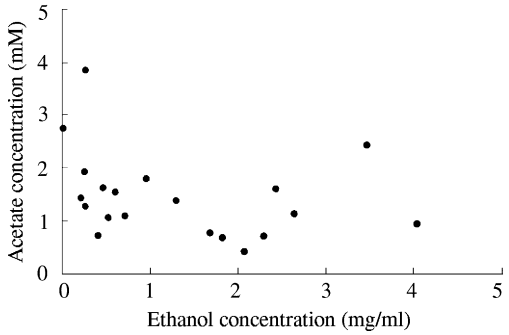

Fig. 4. Correlation of acetate concentrations vs. ethanol concentrations in 20 blood samples from which ethanol was detected.

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