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SHORT COMMUNICATION

Simultaneous clinical monitoring of lactic acid, pyruvic acid and ketone bodies in plasma as methoxime/tert-butyldimethylsilyl derivatives by gas chromatography–mass spectrometry in selected ion monitoring mode

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ABSTRACT: Simultaneous determination of lactic acid, pyruvic acid, 3-hydroxybutyric acid and acetoacetic acid for clinical monitoring of lactic acidosis and ketone body formation in human plasma (20 μL) was performed by gas chromatography–mass spectrometry in selected ion monitoring (SIM) mode after generating methoxime/tert-butyldimethylsilyl derivatives. All of the targeted carboxylic acids were detected by characteristic fragment ions, which permitted sensitive and selective identification in the presence of co-extracted free fatty acids and other acidic metabolites at much higher levels. The method was linear ($r \geq 0.9991$), reproducible (% relative standard deviation = 1.2–5.8), and accurate (% relative error = –7.2–7.6), with detection limits of 0.05–1.7 ng/mL. This rapid, accurate and selective method using minimal plasma samples (20 μL) is useful in the clinical monitoring of lactic acidosis and ketone body formation in plasma. Copyright © 2008 John Wiley & Sons, Ltd.

KEYWORDS: lactic acidosis; ketone body; gas chromatography–mass spectrometry in selected ion monitoring mode; methoximation; tert-butyldimethylsilylation; plasma

INTRODUCTION

The levels of pyruvic acid, lactic acid and ketone bodies, including 3-hydroxybutyric acid and acetoacetic acid, play major roles in fuel homeostasis and are increased in plasma in various pathological conditions (Costell *et al.*, 1999; Ewaschuk *et al.*, 2002). Thus, for clinical monitoring, it is important to develop methods for determination of these compounds and the lactic acid/pyruvic acid and 3-hydroxybutyric acid/acetoacetic acid ratios.

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Abbreviations used: CAs, carboxylic acids; KAs, keto-acids; MO-TBDMS, methoxime/tert-butyldimethylsilyl; MTBSTFA, *N*-methyl-*N*-(tert-butyldimethylsilyl)trifluoroacetamide.

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Our previous uterine cervical cancer study indicated that combined methoxime/tert-butyldimethylsilyl (MO/TBDMS) reactions are suitable for profiling analysis of urinary carboxylic acids (CAs), including keto acids (KAs) (Kim *et al.*, 1998). Our recent studies showed that a two-phase ethoxycarbonyl-MO/TBDMS reaction was useful for assaying clinically important amino acids and CAs, including KAs in a single run (Paik and Kim, 2004; Paik *et al.*, 2005). The present study was performed to determine the levels of the targeted CAs (lactic acid, 3-hydroxybutyric acid) and KAs (pyruvic acid, acetoacetic acid) in plasma for clinical monitoring, using our previously developed GC method (Kim *et al.*, 1998; Paik and Kim, 2004).

As fatty acids, acidic metabolites and other interfering molecules are co-extracted from biological samples at much higher concentrations, a more selective detection device, such as gas chromatography–mass spectrometry (GC-MS) in selected ion monitoring (SIM) mode (GC-SIM-MS) was required for resolution of individual CAs. Therefore, we investigated the optimal conditions for accurate, rapid and sensitive GC-SIM-MS analysis, and then used this method to validate the conditions.

In this study, our MO/TBDMS reaction method (Kim *et al.*, 1998; Paik and Kim, 2004) combined with the optimized GC-SIM-MS analysis was applied to accurately quantify targeted CAs in human plasma samples.

EXPERIMENTAL

Chemicals and reagents. Lactic acid, pyruvic acid, 3-hydroxybutyric acid, acetoacetic acid, 3,4-dimethoxybenzoic acid (internal standard, IS), methoxyamine hydrochloride and triethylamine were purchased from Sigma-Aldrich (St Louis, MO, USA). *N*-Methyl-*N*-(tert-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was obtained from Pierce (Rockford, IL, USA). Toluene, diethyl ether, ethyl acetate and dichloromethane (pesticide grade) were purchased from Kanto Chemical (Tokyo, Japan). Sulfuric acid and sodium hydroxide were obtained from Duksan (Seoul, South Korea). All other chemicals were of analytical grade and were used as received.

Preparation of standard solutions. Standard stock solutions of CAs (lactic acid, 3-hydroxybutyric acid) and KAs (pyruvic acid, acetoacetic acid) were prepared at 10 μg/μL in methanol and distilled water, respectively. Standard working solutions of 0.01 and 0.5 μg/μL were prepared by diluting the stock solution with methanol for CAs and distilled water for KAs. The IS stock solution, prepared by dissolving at 10.0 μg/μL in methanol, was used to make an IS working solution of 0.05 μg/μL in methanol. The mixed calibration samples were prepared at five concentrations, ranging from 0.02 to 5.0 μg/mL, by mixing appropriate aliquots of each working solution. All standard solutions were stored at 4°C.

Gas chromatography–mass spectrometry. GC-MS analyses in SIM mode were performed with an Agilent 6890N gas chromatograph interfaced to an Agilent 5975B mass-selective detector (70 eV, electron impact mode) equipped with an Ultra-2 (5% phenyl–95% methylpolysiloxane bonded phase; 25 m × 0.20 mm i.d., 0.11 μm film thickness) cross-linked capillary column (Agilent Technologies, Palo Alto, CA, USA). The temperatures of the injector, interface and ion source were 260, 300 and 230°C, respectively. Helium was used as the carrier gas at a flow rate of 0.5 mL/min in constant flow mode. Samples were introduced in split-injection mode (10:1) and the oven temperature was set initially at 100°C (2 min) and programmed to rise to 145°C at 10°C/min and finally to 300°C (3 min) at 30°C/min. The mass range scanned was 50–600 u at a rate of 0.99 scans/s. In the SIM mode, three characteristic ions for each CAs and KAs were used for peak identification, while one bold ion was selected for quantification (Table 1). A dwell time of 100 ms was chosen for all ions. The relative voltage of electron multiplier was set to 300 V higher than that in the scanning mode (1300 V) for each ion monitored. Each peak in the plasma samples was identified by matching the area ratios of the three ions with those of the CAs and KAs standards. All GC-SIM-MS runs were performed in triplicate.

Sequential MO/TBDMS derivatives and validation for targeted CA assays. A mixed standard solution containing four targeted CAs, including two KAs, at varying concentrations (0.02–5.0 μg/mL) and IS (0.5 μg/mL) were added to 1 mL of distilled water, followed by adjustment to ca. pH 13 with 5.0 M NaOH. The MO reaction of the keto groups was then performed with methoxyamine hydrochloride (1.0 mg) at 60°C for 30 min, followed by washing with diethyl ether (3 mL × 2). The aqueous solution was then acidified (pH ≤ 2.0) with concentrated sulfuric acid, and saturated with sodium chloride. It was then subjected to solvent extraction sequentially with diethyl ether (3 mL) and ethyl acetate (2 mL). After addition of triethylamine (5 μL), the combined extracts were evaporated to dryness under a gentle stream of nitrogen gas (40°C). Toluene (30 μL) as a solvent and MTBSTFA (20 μL) as a silylation reagent were added to the residue containing intact CAs, including MO derivatives of KAs, and the mixtures

Table 1. Validation data and metabolite levels in plasma for monitoring of lactic acidosis and ketone bodies as MO/TBDMS derivatives by GC-SIM-MS

Organic acid	Selected ion (*m*/*z*)	Calibration range (μg/mL)	Linearity r^2 [a]	LOD [b] (ng/mL)	Added (μg/mL)	Precision (%RSD)	Accuracy (%RE)	Levels in normal plasma (mean ± SD, μmol/L) (*n* = 10)
3-Hydroxybutyric acid[c]	161, **185**, 203	0.02–2.0	0.9999	0.07	0.1	5.8	–2.1	23.8 ± 1.2
Pyruvic acid	159, **174**, 216	0.02–2.0	0.9991	0.05	0.1	1.2	7.6	336.2 ± 18.9
Acetoacetic acid[d]	156, 188, **245**	0.02–2.0	0.9997	1.7	0.1	3.1	4.5	96.9 ± 5.7
Acetoacetic acid	116, 188, **230**							
Lactic acid	189, 233, **261**	0.05–5.0	0.9996	1.3	0.1	5.3	–7.2	2154.9 ± 146.9
3,4-Dimethoxybenzoic acid (IS)	165, 195, **239**							
Lactic acid/pyruvic acid								6.4 ± <0.5
Total ketone bodies[e]								120.6 ± <5.2
3-Hydroxybutyric acid/acetoacetic acid								0.2 ± <0.1

Ultra-2 capillary column (25 m × 0.20 mm i.d., 0.11 μm d_f), from 100°C (2 min) and programmed to 145°C at 10°C/min and finally to 300°C (3 min) at a rate of 30°C/min in SIM mode with a 100 ms dwell time, and 1300 V of electron multiplier. Quantification ions are shown in bold.
[a] Correlation coefficient.
[b] LOD, limit of detection.
[c] 3-Hydroxybutyric acids were calculated as mono-TBDMS derivatives.
[d] Acetoacetic acid was calculated as the sum of the syn and anti isomers.
[e] Total ketone bodies are expressed as the sum of the concentrations of acetoacetic acid and 3-hydroxybutyric acid.

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were heated at 60°C for 30 min to form TBDMS derivatives for direct GC-SIM-MS analysis. All samples were prepared individually in triplicate and analyzed on the same day to assess reproducibility, accuracy and linearity of the results. The reproducibility (expressed as a percentage of the relative standard deviation, %RSD) and accuracy (as a percentage of the relative error, %RE) of the method were determined from the calibration samples in triplicate. The limits of detection (LOD) for each CA and KA were estimated based on the lowest concentration giving a signal taken as the sum of the mean blank signal plus three times the standard deviation of the blank signal obtained with three blank measurements.

Sample preparation for assay of targeted CAs in plasma. Normal plasma samples for the assay of targeted CAs were collected from 10 healthy adult male volunteers and pooled. Aliquots of 20 μL of normal pooled plasma containing 0.5 μg IS were vortex-mixed with acetonitrile (0.1 mL) for 3 min. The mixtures were diluted with 0.9 mL of distilled water and centrifuged (15,000 rpm, 15 min) to precipitate proteins. The supernatant layer was subjected to the MO/TBDMS reactions prior to GC-SIM-MS analysis.

RESULTS AND DISCUSSION

Method validation for targeted CA assays

All carbonyl groups in labile pyruvic acid and acetoacetic acid were transformed to stable MO derivatives. After solvent extraction and evaporation, the remaining active acidic groups in the MO derivatives of the KAs and intact CAs were converted to the respective TBDMS derivatives. Lactic acid and pyruvic acid each gave a single peak, whereas two peaks were observed for acetoacetic acid (syn and anti) and 3-hydroxybutyric acid (mono- and di-TBDMS derivatives). The EI mass spectral properties of CAs, including KAs, as MO/TBDMS derivatives were described in our previous report (Paik and Kim, 2004). In SIM mode, three characteristic ions for each compound were selected for peak identification. The $[M - 57]^+$ ions (shown in bold) were formed by the loss of $C(CH_3)_3$ from the molecular ions, and were used for quantification of all compounds (Table 1). The mono-TBDMS derivative of 3-hydroxybutyric acid was selected for quantification because the di-TBDMS derivatives showed much lower reaction yields at trace levels. The quantification of acetoacetic acid was calculated as the sum of the syn and anti isomers. Among the dwell times tested, 100 ms was determined to yield the highest ion abundances for most compounds. The method was found to be very selective for detection of CAs and KAs when the whole procedure of the MO/TBDMS reaction with subsequent GC-SIM-MS analysis was performed for the assay of these compounds in aqueous solution. These compounds were detected simultaneously with good sensitivity and excellent selectivity without any major interference. The detector responses (expressed as peak area ratios) of these compounds showed linearity in the range 0.02–5.0 μg/mL, with regression coefficients >0.9991 (Table 1). This overall linearity showed the suitability of the present method for quantitative determination of these compounds in biological samples. The LODs varied from 0.05 to 1.7 ng/mL. The ranges of precision (%RSD) and accuracy (%RE) for the overall procedure at 0.1 μg/mL in triplicate within one day varied from 1.2 to 5.8 and from –7.2 to 7.6, respectively. The overall precision and accuracy appeared to be satisfactory for quantification of the targeted CAs, including KAs, in biological samples.

Levels of targeted CAs in normal plasma

The MO/TBDMS method was applied to aliquots of 20 μL of pooled normal plasma. Figure 1 shows SIM

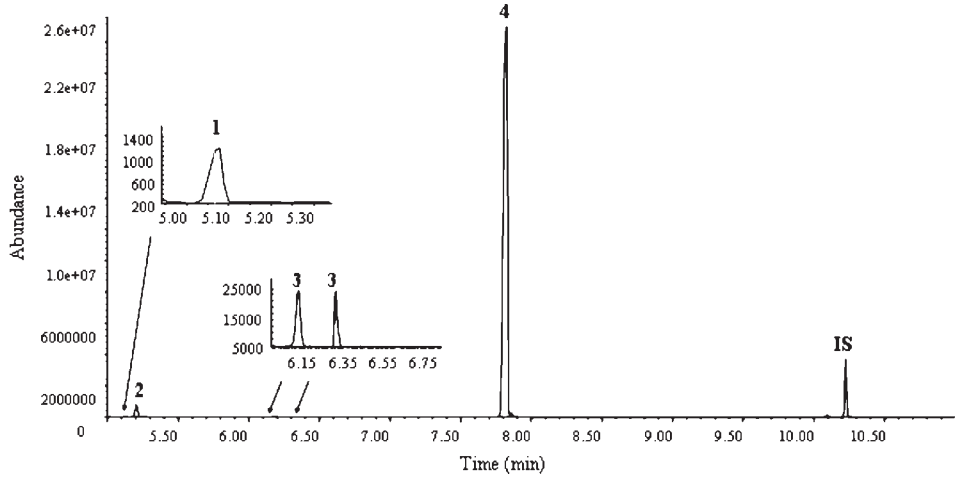

Figure 1. SIM chromatogram of targeted CAs as MO/TBDMS derivatives in normal pooled plasma (20 μL): **1**, 3-hydroxybutyric acid as mono-TBDMS derivative; **2**, pyruvic acid; **3**, acetoacetic acid (syn and anti isomers); **4**, lactic acid. IS-3,4-dimethoxybenzoic acid.

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chromatograms of these compounds in plasma. The concentration of lactic acid was 2154.9 ± 146.9 μmol/L and that of pyruvic acid was 336.2 ± 18.9 μmol/L. The lactic acid–pyruvic acid ratio was 6.4 ± <0.5. The levels of 3-hydroxybutyric acid and acetoacetic acid and the 3-hydroxybutyric acid–acetoacetic acid ratio were 23.8 ± 1.2 and 96.9 ± 5.7 μmol/L and 0.2 ± <0.1, respectively (Table 1). Total ketone bodies (120.6 ± <5.2 μmol/L) were expressed as the sum of the concentrations of acetoacetic acid and 3-hydroxybutyric acid. The mean levels of these compounds in plasma were similar to those reported previously (Hoffmann *et al.*, 1989; Vassault *et al.*, 1991).

The method described here involving MO/TBDMS reaction and subsequent GC-SIM-MS is advantageous for quantification of targeted CAs, including KAs, in which it allows the accurate and selective detection of these compounds, even in the presence of high concentrations of co-extracted interfering metabolites. This method will be useful for simultaneous rapid screening and clinical monitoring of abnormal states such as lactic acidosis and ketosis.

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