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SHORT COMMUNICATION

Target metabolic profiling analysis of free amino acids in plasma as EOC/TBDMS derivatives by GC-SIM-MS

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ABSTRACT: Metabolic profiling analysis of free amino acids (AAs) in plasma (20 μL) was performed by gas chromatography–mass spectrometry in selected ion monitoring mode after ethoxycarbonyl/tert-butyldimethylsilyl derivatives. Characteristic fragment ions, including $[M - 57]^+$ ions, permitted sensitive and selective detection of most of the AAs in the presence of co-extracted carboxylic acids, including free fatty acids, at much higher levels. The overall method was linear ($r \geq 0.9991$), reproducible (relative standard deviation = 2.3–8.8%) and accurate (relative error = –7.3–7.7%) with detection limits of 0.01–1.9 ng/mL. A total of 18 AAs, 15 protein AAs and three nonprotein AAs were quantitatively screened in a normal human plasma sample. This selective and simple method using a minimal sample volume was effective for the quantitation of plasma free AAs. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: target metabolic profiling analysis; gas chromatography–mass spectrometry in selected ion monitoring mode; ethoxycarbonyl/tert-butyldimethylsilyl derivatives; free amino acids; plasma

INTRODUCTION

The amino acid (AA) equilibrium in plasma reflects AA intake, changes in nitrogen handling by organs and altered AA metabolism. Recently, the development of various methods for the accurate and rapid metabolic profiling analysis of AAs has become an important issue in the clinical field. In our previous report, a two-phase ethoxycarbonyl (EOC) reaction in alkaline solution with ethyl chloroformate (ECF) in the dichloromethane phase, with a subsequent tert-butyldimethylsilyl (TBDMS) reaction, was effective for the assay of clinically important AAs and carboxylic acids in a single run by gas chromatography (GC) (Paik and Kim, 2004; Paik *et al.*, 2005).

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Abbreviations used: AA, amino acid; ECF, ethyl chloroformate; EOC, ethoxycarbonyl; MTBSTFA, *N*-methyl-*N*-(tert-butyldimethylsilyl) trifluoroacetamide; TBDMS, tert-butyldimethylsilyl.

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The present study was undertaken to determine AAs in plasma by employing our previous GC method (Paik and Kim, 2004). However, when the method was applied to plasma samples, a more selective detection device, such as GC-SIM-MS, was required because of co-extracted endogenous metabolites, including neutral compounds, lipids such as sterols and carboxylic acids including fatty acids.

These metabolites are present in biological samples at much higher concentrations and interfered with the resolution of individual AAs in GC analyses (Paik *et al.*, 2005). Thus, we investigated optimal conditions for selective and sensitive GC-SIM-MS analysis and then the method was validated using these optimal conditions. Finally, the previous method (Paik and Kim, 2004) combined with the optimized GC-SIM-MS analysis was applied to accurately quantify AAs in a human plasma sample.

EXPERIMENTAL

Chemicals and reagents. The 18 AAs, 3,4-dimethoxybenzoic acid, which was used as an internal standard (IS), and ECF were purchased from various vendors, such as 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 of 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. The stock solution of each AA was made up at 10 μg/μL in 0.1 M HCl. Standard working solutions of 0.01 and 0.5 μg/μL were then prepared by diluting each stock solution with 0.1 M HCl. 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 0.05 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 120°C (2 min) and programmed to rise to 240°C at 5°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 AA 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 the electron multiplier was set to 300 V higher than that in the scanning mode (1200 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 AA standards. All GC-SIM-MS runs were performed in triplicate.

Sequential EOC/TBDMS derivatives and validation for AA assays in plasma. A mixed standard solution containing 18 AAs at varying amounts (0.05–5.0 μg/mL) and IS (0.5 μg/mL) was used for reaction optimization tests. These were added in 1 mL of distilled water then adjusted to pH = 12 with 5.0 M NaOH. Each spiked sample was subjected to *N*-EOC derivatization, with a sequential TBDMS reaction, as described elsewhere (Paik and Kim, 2004). Briefly, a two-phase EOC reaction was performed in one step by vortex-mixing (5 min) an aqueous phase with ECF (20 μL) present in the dichloromethane phase (1.0 mL). The reaction mixture was extracted with diethyl ether (3.0 mL) and ethyl acetate (2.0 mL) sequentially, and the combined extracts were evaporated to dryness under a gentle nitrogen stream (40°C). The residue was reacted (60°C for 30 min) with MTBSTFA (20 μL) in toluene (20 μL) for GC-SIM-MS analysis. All samples were individually prepared in triplicate and were analyzed on the

Table 1. Validation data for the assay of the 18 AAs investigated and AA levels in normal pooled plasma by GC-SIM-MS

Amino acid	Selected ion (*m/z*)	Linearity,[a] *r*	LOD[b] (ng/mL)	Added (μg/mL)	Precision (% RSD)	Accuracy (% RE)	AA levels in normal plasma (mean ± SD, μmol/L)
Alanine	190, **218**, 260	0.9998	0.03	0.5	8.0	2.8	447.3 ± 10.2
Glycine	176, **204**, 246	0.9996	0.04	0.5	6.0	1.6	312.9 ± 6.9
Valine	144, 218, **246**	0.9998	0.02	0.5	4.9	2.8	197.0 ± 3.1
Leucine	158, 232, **260**	0.9999	0.06	0.5	3.5	–2.2	104.3 ± 3.3
Isoleucine	158, 232, **260**	0.9998	0.2	0.5	2.3	2.8	60.6 ± 1.1
Proline	142, 216, **244**	0.9999	0.01	0.5	4.7	1.9	180.8 ± 5.2
Pipecolic acid	156, 230, **258**	0.9999	0.01	0.5	5.4	1.3	1.0 ± < 0.1
Serine[c]	216, 302, **348**	0.9999	0.03	0.5	4.3	–1.3	161.6 ± 8.8
Threonine[c]	230, 316, **362**	0.9996	0.3	0.5	4.0	0.8	170.9 ± 8.4
Phenylalanine	192, 205, **294**	0.9999	0.01	0.5	5.4	–3.5	40.6 ± 3.0
Aspartic acid	287, 330, **376**	0.9999	0.01	0.5	3.3	7.7	22.8 ± 1.1
4-Hydroxyproline[d]	242, **332**, 374	0.9999	0.8	0.5	7.9	–1.5	41.1 ± 3.1
Glutamic acid	288, 344, **390**	0.9999	0.1	0.5	5.8	2.8	78.5 ± 2.1
Ornithine	142, 287, **333**	0.9991	1.5	0.5	8.8	5.3	55.0 ± 3.2
Glutamine	287, 343, **389**	0.9999	1.9	0.5	6.0	0.4	583.0 ± 1.5
Lysine	**156**, 301, 347	0.9991	0.7	0.5	3.4	–7.3	165.3 ± 10.3
Tyrosine	221, 335, **424**	0.9995	0.3	0.5	3.4	3.5	74.3 ± 1.4
Tryptophan	**130**, 244, 333	0.9988	0.3	0.5	8.7	6.6	31.3 ± 2.1
3,4-Dimethoxybenzoic acid (IS)	165, 195, **239**						

Ultra-2 capillary column (25 m × 0.20 mm i.d., 0.11 mm d_f), from 120°C (2 min) and programmed to 240°C at 5°C/min and finally to 300°C (3 min) at 30°C/min in SIM mode with 100 ms dwell time and 1200 V electron multiplier. Quantitation ions are in bold.

[a] Correlation coefficient.

[b] LOD, limit of detection.

[c] Threonine and serine were calculated as mono-EOC/di-TBDMS derivatives.

[d] 4-Hydroxyproline was calculated as di-EOC/mono-TBDMS derivatives.

All quantitative calculations were based on peak area ratios relative to that of IS (0.5 μg/mL) measured on the Ultra-2 column.

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same day to assess reproducibility, accuracy and linearity. 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 limit of detection (LOD) for each AA was 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 AAs in plasma. Normal plasma samples were collected from 10 healthy adult male and 10 healthy adult female volunteers and pooled. A 20 μL sample of normal pooled plasma containing 0.5 μg of IS was vortex-mixed with acetonitrile (0.1 mL) for 3 min. The mixture was 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 aforementioned EOC/TBDMS reactions prior to GC-SIM-MS analysis.

RESULTS AND DISCUSSION

Determination of optimal GC-SIM-MS conditions and method validation

The EI mass spectral properties of the 18 AAs as N-EOC/TBDMS derivatives were discussed in our previous report (Paik and Kim, 2004). In SIM mode, three characteristic ions, including the $[M - 57]^+$ ion, resulting from the loss of $C(CH_3)_3$, for each AA were selected for peak identification, and one bold ion was used for quantification (Table 1). The mono-EOC/di-TBDMS derivatives of serine and threonine, and the di-EOC/mono-TBDMS derivatives of 4-hydroxyproline, were selected for quantification because the mono-EOC/mono-TBDMS and mono-EOC/di-TBDMS derivatives, respectively, were produced with low reaction yields. For most AAs, the $[M - 57]^+$ ion was used for quantification, except for lysine (*m/z* 156) and tryptophan (*m/z* 130). Among the dwell times tested, 100 ms was determined to yield the highest ion abundances for most AAs. This was found to be very selective in detecting AAs when the whole procedure of EOC/TBDMS derivatization with subsequent GC-SIM-MS analysis was validated for the assay of AAs in aqueous solution. The 18 AAs were simultaneously detected with good sensitivity and excellent selectivity, without major interference. The detector responses (expressed as peak area ratios) of the 18 AAs showed linearity in the range 0.05–5.0 μg/mL, with regression coefficients >0.9991 (Table 1). This overall linearity demonstrated the suitability of the present method for the quantitative assay of AAs in biological samples. The LODs varied from 0.01 to 1.9 ng/mL. The ranges of precision (RSD) and accuracy (RE) for the overall procedure at 0.5 μg/mL in triplicate within one day varied from 2.3 to 8.8% and from –7.3 to 7.7%, respectively. The overall precision and accuracy appeared to be satisfactory for the quantification of AAs in biological samples.

Levels of amino acids in pooled normal plasma

When the present EOC/TBDMS method was applied to pooled normal plasma, the SIM chromatogram of the 18 AAs screened positively in plasma is shown in Fig. 1. Glutamine was the most abundant, followed by alanine, glycine, valine and proline. Pipecolic acid

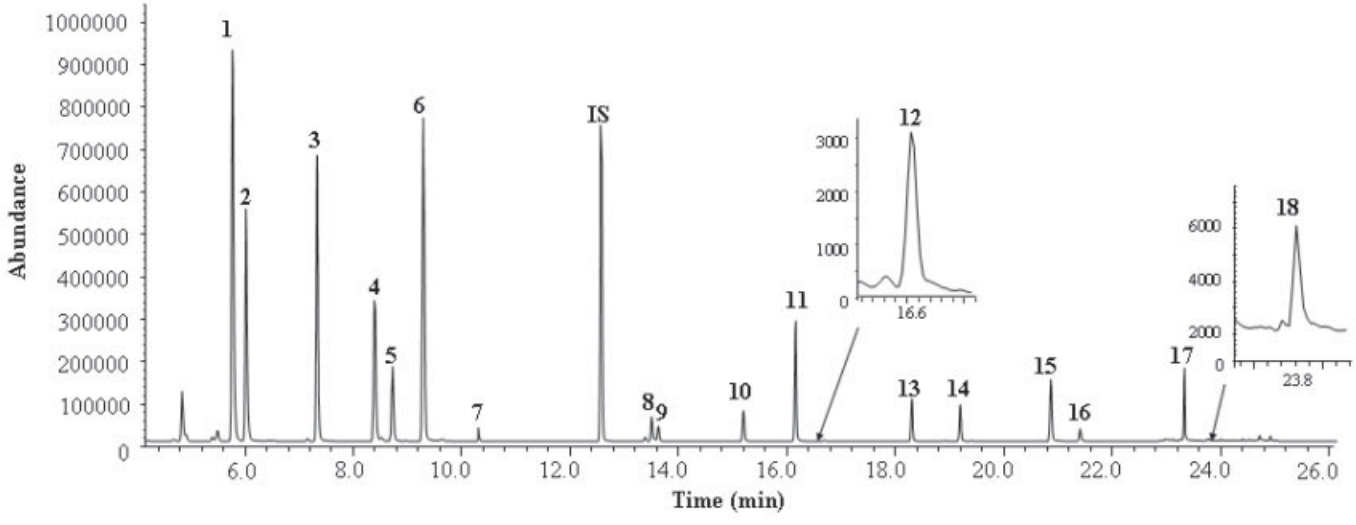

Figure 1. SIM chromatogram of amino acids as ethoxycarbonyl/tert-butyldimethylsilyl derivatives in normal pooled plasma: **1**, alanine; **2**, glycine; **3**, valine; **4**, leucine; **5**, isoleucine; **6**, proline; **7**, pipecolic acid; **8**, serine; **9**, threonine; **10**, phenylalanine; **11**, aspartic acid; **12**, 4-hydroxyproline; **13**, glutamic acid; **14**, ornithine; **15**, glutamine; **16**, lysine; **17**, tyrosine; **18**, tryptophan. IS, 3,4-dimethoxybenzoic acid.

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was the least abundant. These normal mean levels of AAs in plasma were similar to those values reported in the literature (Slocum and Cummings, 1991; Rashed *et al.*, 2001; Chuang *et al.*, 2006).

A major advantage of the present two-phase aqueous EOC derivatization combined with TBDMS derivatization and subsequent GC-SIM-MS method is the accurate and selective detection of AAs, even in the presence of co-extracted interfering metabolites at higher concentrations. The present method will be useful for rapid screening and monitoring for abnormal states in the clinical field.

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