GC-MS with Ethyl Chloroformate Derivatization for Comprehensive Analysis of Metabolites in Serum and its Application to Human Uremia

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Abstract:

An optimized method based on GC-MS with ethyl chloroformate derivatization has been developed for the comprehensive analysis of endogenous metabolites in serum. Twenty-two reference standards and serum samples were used to validate the proposed method. The correlation coefficient was higher than 0.9900 for each of the standards, and the LOD varied from 125 to 300 pg on-column. The analytical equipment exhibited good repeatability (RSD<10%) for all of the standards. Both the repeatability and the within-48-h stability of the analytical method were satisfactory (RSD<10%) for the 18 metabolites identified in the serum samples. Mean recovery was acceptable for the 18 metabolites, ranging from 70% to 120% with RSDs of less than 10%. Using the optimized protocol and a subsequent multivariate statistical technique, complete differentiation was achieved between the metabolic profile of uremic patients and that of age- and sex-matched normal subjects. Significantly decreased levels of valine, leucine, and isoleucine and increased levels of myristic acid and linoleic acid were observed in the patient group. This work demonstrated that this method is suitable for serum-based metabolic profiling studies.

Article:

INTRODUCTION

Following the completion of the Human Genomic Project, tremendous advances in high-throughput “-omics” sciences such as genomics, proteomics, and metabonomics (also called metabolomics and metabolic profiling) have been made in recent years [1–3]. Metabonomics is a newly developed technology for the quantitative measurement of dynamic multiparametric metabolic changes of living systems exposed to pathophysiological stimuli or genetic modifications [4]. The footstone of metabonomics is to reliably measure as many metabolites as possible in biological samples, including body fluids (e.g., urine, serum, amniotic fluid, cerebrospinal fluid), single cells and tissues of given organisms or plants, using modern high-throughput analytical instruments. The main analytical techniques usually involve nuclear magnetic resonance (NMR) [5–8], liquid chromatography–mass spectrometry (LC-MS and LC-

MS-MS) [9–11], capillary electrophoresis–mass spectrometry (CE-MS) [12, 13], and gas chromatography–mass spectrometry (GC-MS) [14, 15]. GC-MS has long been used for metabolic profiling study due to its high sensitivity, reliability and the ease of metabolite identification. As most endogenous metabolites exhibit little or no volatility in biological samples, suitable sample preparation—and derivatization in particular—is the prerequisite for a GC-MS assay. Trimethylsilyl (TMS) derivatization agents, such as bis(trimethylsilyl) trifluoroacetamide (BSTFA) and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) containing 1% trimethylchlorosilane (TMCS), have been frequently applied in GC-MS-based metabolic profiling studies [16, 17]; however, TMS derivatization only proceeds in nonaqueous media, and the entire reaction procedure is very labor-intensive and time-consuming [15]. As an effective alternative to TMS, ethyl chloroformate (ECF) is a suitable derivative agent for a broad array of low molecular weight metabolites (generally, MW <1000 Da) including organic acids, amines, amino acids, and aminoalcohols that are present in biological matrices [18–20]. Recently, we successfully applied an optimized two-step ECF derivatization protocol to GC-MS-based urinary metabonomic studies. Compared to previous work [19], the currently proposed two-step ECF derivatization for GC-MS analysis exhibited better derivatization efficiency and provided more useful information [15], a crucial aspect in metabonomic studies. However, this approach has not yet been assessed when used for metabonomic studies of serum samples.

Uremia is a clinical syndrome characterized by abnormal accumulations of certain compounds, which are normally secreted into the urine by the kidneys in healthy subjects [21]. If these retained compounds interfere with physiological functions, they can result in different levels of renal toxicity. Moreover, uremia commonly develops with many complications, such as hypertension, dyslipidemia, diabetes, etc. [22], each of which results in devastating effects on patients. Due to a lack of sufficient donor kidneys, the current treatment for uremia is usually still dialysis. Thus, a comprehensive understanding of uremic solutes and their toxic effects will make dialysis more safe and rational. However, due to the analytical challenges presented by complex biological matrices and the extreme variability of this syndrome, the study of significant alterations of certain endogenous metabolites closely associated with human uremia remains incomplete.

In this study, we describe an optimized ECF derivatization procedure for the comprehensive analysis of serum samples using GC-MS. The developed method was extensively validated using a large set of standards with varied chemical properties as well as complex serum samples. Furthermore, the optimized method was employed to profile and characterize the significantly altered metabolites in uremic human serum. This study will extend our previously optimized two-step ECF derivatization for the global analysis of urine samples using GC-MS, and provide a complementary measure for identifying metabolites associated with human pathology, leading comprehensive understanding and effective treatments of complex human diseases.

EXPERIMENTAL

Materials and methods

Ethyl chloroformate (ECF), pyridine, anhydrous ethanol, sodium hydroxide, chloroform, *n*-hexane, and anhydrous sodium sulfate of analytical grade were from the China National Pharmaceutical Group Corporation (Shanghai, China). L-2-chlorophenylalanine (Shanghai Intechem Tech. Co. Ltd., China) was used as an internal standard for batch quality control. All of

the standards (Table 1), which were commercially obtained from Sigma-Aldrich (St. Louis, MO, USA), were prepared in ultrapure water (Milli-Q system, Millipore, Billerica, MA, USA) or ethanol solution (Sigma-Aldrich). Blood samples of 24 uremic patients and 23 age- and sex-matched healthy controls were collected from Shanghai Dongfang hospital (Shanghai, China). The clinical study was approved by the local ethics committee and all of the volunteers gave their written, informed consent. Serum was prepared by centrifugation at 1,400×*g* for 5 min at 4 °C, and stored at −80 °C prior to the GC-MS assay.

Table 1: Linearities, LODs and repeatabilities of the test compounds

Compound	Linear (μg/ml)[a]	*n*	R^{2}, [b]	LOD		Repeatability (%)[e]
				pg on column[c]	S/N ratio[d]	RSD
Amino acids						
Alanine	0.1–12.5	9	0.9990	125	16	3.9
Asparagine	0.5–12.5	7	0.9921	200	27	7.7
Aspartic acid	0.1–12.5	8	0.9946	150	22	4.1
Cysteine	0.25–12.5	8	0.9919	200	26	4.5
Glycine	1–12.5	6	0.9944	300	38	8.9
Isoleucine	0.1–12.5	9	0.9951	125	15	3.1
Leucine	0.1–12.5	9	0.9938	125	10	4.2
Methionine	0.1–12.5	8	0.9931	150	27	4.5
Phenylalanine	0.1–12.5	9	0.9967	125	12	4.8
Proline	1–12.5	7	0.9987	125	31	9.2
Threonine	0.1–12.5	9	0.9953	250	33	4.9
Tyrosine	0.1–12.5	9	0.9909	125	22	4.5
Valine	0.1–12.5	9	0.9990	125	18	4.0
Amine						
Tyramine	0.5–12.5	7	0.9935	200	36	4.3
Organic acids						
Butanedioic acid	0.1–12.5	9	0.9991	125	26	3.1
Citrate	1–12.5	6	0.9912	300	35	4.7
Hexadecanoic acid	0.1–12.5	9	0.9931	250	18	3.9
Malic acid	0.5–12.5	7	0.9960	125	31	2.1
Octadecanoic acid	0.1–12.5	9	0.9902	300	33	4.2
9,12-Octadecadienoic acid	0.5–12.5	7	0.9908	150	37	8.2
Propanoic acid	0.1–12.5	9	0.9982	125	19	2.9
Tetradecanoic acid	0.1–12.5	9	0.9967	150	19	4.1

[a]Stock solutions of test compounds were prepared in distilled water or ethanol at 1 mg/ml and stored at −4 °C. The mixed standard solution was obtained through the addition of a 100-μl aliquot of stock solution in order to obtain a concentration of 100 μg/ml. The linear range for each compound was determined by diluting the mixed standard solution at different concentrations.
[b]The correlation coefficient (*r*-square) was calculated for the concentration range shown.
[c]LOD (limit of detection, pg on column) was obtained by calibrating the injection of the standard solution with a S/N ratio ≥3.0.
[d]S/N ratios were claculated in order to show the peak-to-peak values by TurboMass software.
[e]The repeatability was evaluated by RSD for the 22 test compounds at concentrations of 1 μg/ml for amino acids and organic acids and 5 μg/ml for the amine using six identical samples; the aim was to evaluate the repeatability of the analytical equipment.

ECF derivatization

Each 600-μl aliquot of the diluted serum sample (serum: water = 1:1, v/v) or the mixture of the 22 test standards was added to a glass tube with a screw top and combined with 100 μl of L-2-chlorophenylalanine (0.1 mg/ml), 400 μl of anhydrous ethanol, 100 μl of pyridine and 50 μl of ECF for the first step of the derivatization reaction. The resulting mixture was ultrasonicated at 20.0 °C and 40 kHz for 60 s to accelerate the reaction, and subsequently extracted with 500 μl of *n*-hexane. After the pH of the aqueous layer had been carefully adjusted to 9–10 using 100 μl of NaOH (7 mol/l), the second step of the ECF derivatization procedure was carried out by supplying an additional 50 μl of ECF to the system. The products arising from the two consecutive derivatization steps were vortexed for 30 s and centrifuged for 5 min at 1,400×*g*. After carefully transferring 300 μl of the supernatant to a screw-topped glass vial, an additional 500 μl of *n*-hexane was added to the residue in order to extract the derivatives. A total of 400 μl

of the supernatant was transferred to a screw-topped glass vial, and a combined 700 µl of *n*-hexane extracts were dried with a gentle stream of nitrogen at room temperature. The resulting residue was resolved in 200 µl of chloroform, and a small amount of anhydrous granular sodium sulfate was added to remove any traces of water for the subsequent GC-MS assay.

GC-MS assay

Each 1-µl aliquot of the analyte was injected in a splitless mode into a PerkinElmer gas chromatography system coupled to a TurboMass-Autosystem XL mass spectrometer (PerkinElmer, Waltham, MA, USA). A DB-5MS capillary column coated with 5% diphenyl crosslinked with 95% dimethylpolysiloxane (30 m × 250 µm i.d., 0.25-µm film thickness; Agilent J & W Scientific, Folsom, CA, USA) was used to separate the ECF derivatives. The initial temperature of the oven temperature was held at 80 °C for 2 min, ramped to 140 °C at a rate of 10 °C/min, to 240 °C at a rate of 4 °C /min, to 280 °C at a rate of 10 °C/min, and then held at 280 °C for 3 min. Helium was used as carrier gas at a constant flow rate of 1 ml/min through the column. The temperatures of the ion source and injector were 200 °C and 260 °C, respectively. The electron energy was 70 eV and mass data were collected in a full scan mode (*m/z* 30–550). The dwell time for each scan was set at 100 ms and the solvent delay at 3 min.

Analytical method validation

The linear concentration range and the correlation coefficient (r^2) were obtained for each test standard by using a series of standard solutions at different concentrations. Injections were performed in triplicate for each concentration and the average value was used to establish the calibration curves. Typically, the r^2 value should be greater than 0.9900 within the linear concentration range. The limit of detection (LOD) was determined for each test standard by analyzing the signal-to-noise ratio (*S/N*) provided by the TurboMass interface. The LOD was defined as a *S/N* value of ≥3.0 in this study.

The repeatability of the analytical method was evaluated using six independently prepared samples, and the repeatability of the analytical instrument was found by repeatedly measuring the same sample six times. Sample stability was assessed by measuring the peak areas of the compound identifiable from the serum at different time points: 0 h, 6 h, 12 h, 24 h, 48 h, 60 h, and 72 h.

The recovery was evaluated by adding 18 representative compounds, including organic acids, amino acids and an amine, at three different concentrations of 1 µg/ml, 2.5 µg/ml, and 5 µg/ml to serum samples. In the method validation experiments, the peak area for each test compound was integrated by TurboMass software (v. 4.0, PerkinElmer) and adjusted using the peak area of the internal standard.

Data analysis

Unprocessed GC-MS data files generated by the metabolic profiling of serum samples from 24 uremic patients and 23 normal subjects were converted into NetCDF format via DataBridge (Perkin-Elmer) and processed by custom scripts in MATLAB R2007a (The MathWorks, Natick, MA, USA), during which noise elimination, peak identification and alignment, and exclusion of the internal standard and impurities were automatically performed [*23*]. The resulting three-dimensional matrix consisting of peak indices (retention time (RT)-*m/z* pairs), sample names

(observations), and normalized peak areas (variables) was introduced into the SIMCA-P 11.0 software package (Umetrics, Umeå, Sweden) in order to perform multivariate statistical analyses including principal component analysis (PCA) and orthogonal partial least squares projection to latent structures discriminant analysis (OPLS-DA).

PCA, an unsupervised pattern recognition method, is commonly used to detect the possible outliers and natural clusters among observations [23–25]. In this study, PCA was conducted on mean-centered, pareto-scaled spectral data from the serum samples. The mean-centering procedure subtracts the average spectrum of the datasets, and the pareto-scaling method weighs each variable according to the square root of its standard deviation. One of the main reasons for using the pareto-scaling method is that such procedure is a satisfactory comprise between UV-scaling (scaling to unit variance), which gives equal weight to the baseline noise and individual signals in the entire chromatogram, and no scaling, which could fail to pick out small changes in metabolites with lower concentrations [23]. Additionally, in order to maximize the discrimination of metabolic profiles of uremic patients from those of the healthy controls, an OPLS-DA model—a supervised method capable of removing information unrelated to the response matrix Y (commonly set to 0/1, etc.) from an original matrix X—was utilized accordingly. Variable importance in the projection (VIP) values were obtained for the variables in the OPLS-DA model in order to select the differentially expressed metabolites, and variables with VIP values greater than 1.0 were considered statistically significant metabolites. In parallel, the regression coefficients from the OPLS-DA model were used to look for the up- or downregulations of these significant metabolites in the uremic patient group compared to the control subject group.

Default seven-round crossvalidation was performed using the SIMCA-P software by excluding one-seventh of all the samples from the model in each round in order to validate the model against overfitting. This procedure was repeated in an iterative manner until each sample had been excluded once. The cumulative value of R2Y (R2Ycum) provides an estimate of how well the model fits the Y data, and the cumulative value of Q2Y (Q2Ycum) is an estimate of how well the model predicts the Y data. When R2Ycum and Q2Ycum approach 1.0, it indicates that a robust model with a reliable predictability has been obtained. In order to check the results from the multivariate statistical analyses, a univariate statistical analysis of the *t*-test experiments was subsequently performed. Based on the fold-change rank and *p*-values from the *t*-tests, the differentially expressed metabolites identified from the multivariate statistical analyses were validated at the univariate analysis level. The critical *p*-value for statistical significance in the *t*-tests was set to 0.05 in this study.

RESULTS AND DISCUSSION

Derivatization procedure

The derivatization procedure is an indispensable step in the GC-MS analysis of compounds with little or no volatility. In contrast to TMS derivatization, ECF derivatization proceeds in aqueous media and does not necessarily involve the removal of water before and during the reaction, which facilitates large-scale sample preparation and greatly improves batch reproducibility [15]. Secondly, the extraction efficiencies of chloroform and *n*-hexane are comparable, but *n*-hexane was used to extract the derivatives in this study. The major reason for this is that the presence of *n*-hexane in the upper layer of the resulting mixture facilitates solvent removal from the vial.

Thirdly, the dried derivatives were resolvated in 200 µl of chloroform for the GC-MS assay because chloroform has a lower volatility than *n*-hexane and exhibits a better separation in the GC-MS chromatogram for the test samples used in this study. Lastly, it was necessary to adjust the pH to 9–10 with 7 mol/l NaOH during the derivatization process so that groups such as –COOH, $–NH_2$ and –OH could be easily targeted by ECF. Using such a procedure, the 22 mixed standards were clearly separated in the GC-MS total ion current (TIC) chromatogram (Fig. 1), and a total of 18 compounds, including an array of amino acids, organic acids, and an amine, were consistently detected in serum samples using GC-MS spectral library databases of the reference standards available.

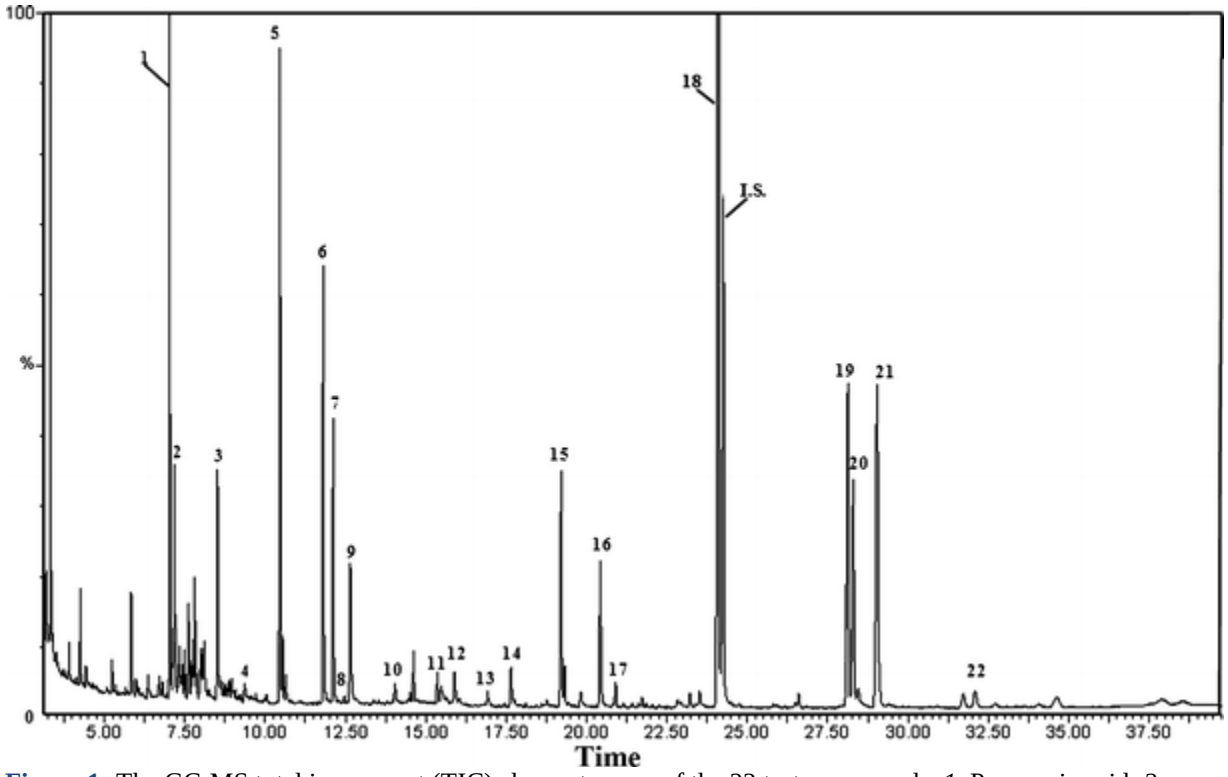

Figure 1: The GC-MS total ion current (TIC) chromatogram of the 22 test compounds. *1*, Propanoic acid; *2*, butanedioic acid; *3*, alanine; *4*, glycine; *5*, valine; *6*, leucine; *7*, isoleucine; *8*, proline; *9*, threonine; *10*, malic acid; *11*, asparagine; 12, aspartic acid; *13*, citrate; *14*, methionine; *15*, tetradecanoic acid; *16*, phenylalanine; *17*, cysteine; *18*, hexadecanoic acid; *19*, tyramine; *20*, 9,12-octadecadienoic acid; *21*, octadecanoic acid; *22*, tyrosine

Analytical method validation

Linearity and LOD. The calibration curve for each test standard was obtained using at least six different concentrations (Table 1). The corresponding concentration range varied from 0.1 to 12.5 µg/ml for most amino acids and organic acids, and from 0.5 to 12.5 µg/ml for amine, with r^2 approaching 1.0000. Exceptions were compounds such as glycine, proline, aspagine, cysteine, malic acid, citrate and 9,12-octadecadienoic acid, which exhibited better linearity at high concentrations, and thus r^2 was calculated for these compounds by excluding lower concentrations. The *S/N* values for most of the test standards were larger than 3.0 (Table 1), allowing the LOD to be individually calculated each compound.

Repeatability, stability and recovery. The analytical equipment exhibited satisfactory repeatabilities for the 22 test compounds, with RSDs of better than 10% (Table 1). The analytical method gave good repeatability for the 18 test compounds identified in serum samples, with RSDs of less than 10% (Table 2). Similar results were obtained by three different operators in our laboratory.

Table 2: Repeatabilities, stabilities and recoveries of the test compounds

Compound	Repeatability (%) [a]	Stability (48h, %)[b]	Recovery (%)[c]	
	RSD	R.S.D	Average	RSD
Amino acids				
Alanine	3.2	5.9	114.2	7.1
Aspartic acid	4.3	6.2	98.3	4.3
Cysteine	4.9	8.3	81.4	8.6
Glycine	9.9	8.0	70.1	9.2
Isoleucine	3.8	3.9	86.3	8.1
Leucine	4.6	4.1	101.9	4.3
Methionine	4.7	7.6	82.2	5.5
Phenylalanine	6.9	7.1	76.7	6.8
Proline	9.3	9.2	70.5	9.1
Threonine	3.9	5.2	101.3	8.3
Valine	4.7	3.4	98.1	6.5
Organic acids				
Butanedioic acid	4.2	3.9	72.3	9.5
Hexadecanoic acid	4.6	7.9	82.5	5.2
Octadecanoic acid	4.9	9.7	116.3	8.7
9,12-Octadecadienoic acid	5.9	9.8	81.2	5.9
Propanoic acid	3.7	4.8	71.2	4.0
Tetradecanoic acid	4.5	8.6	106.4	2.8
Amine				
Tyramine	4.7	9.0	71.9	8.3

[a]Repeatability was determined using six different samples for the 18 test compounds (at concentrations of 1 μg/ml for amino acids and organic acids and 5 μg/ ml for the amine); the aim was to evaluate the repeatability of the analytical method.
[b]Stability over 48 h was measured for the 18 compounds at concentrations of 1 μg/ml for amino acids and organic acids and 5 μg/ ml for the amine.
[c]Mean recovery was obtained for the 18 test compounds (using three replicate samples at three different concentrations).

In addition, the stabilities of most of the 18 test compounds in the serum were fairly acceptable, with RSDs of less than 10% within 48 h (Table 2). It is worth noting that the RSDs of certain compounds such as asparagine and 9,12-octadecadienoic acid were greater than 15% after 60 h. Moreover, the overall sensitivity appeared to decrease when the GC-MS was used normally over 48 h. Therefore, all of the samples from each batch were analyzed within 48 h, and maintenance of the equipment was carried out after every 48-h analysis.

The mean recoveries of the 18 test compounds ranged from 70 to 120%, with RSDs of less than 10% (Table 2), which is acceptable for the global analysis of endogenous metabolites in serum samples.

In summary, the proposed method offered a reliable and stable measurement approach for a wide array of metabolites with diverse chemical characteristics, which aided the global analysis of metabolites in serum samples from certain pathophysiological states.

Clinical application. There is an urgent need for the comprehensive analysis of uremic solutes with varied physiochemical characteristics before the extensive application of therapeutic strategies such as dialysis and kidney transplantation. To this end, we applied a global metabolic profiling technology to unbiasedly identify the crucial metabolites closely associated with human

uremia. The approach used employed a combination of a GC-MS assay with modern multivariate statistical techniques. Fifty compounds (about 56%) out of the 90 peaks detected in the serum samples were identified by comparing their mass fragmentation with those from GC-MS spectral databases, including those of NIST and Wiley, using Turbomass 4.1.1 software (PerkinElmer). Eighteen of these compounds were verified by reference standards available in this work (Table 3).

Table 3: List of compounds identified in the serum samples using GC-MS spectral library databases

Key	RT (min)[b]	*m/z* [c]	Compounds	Rev[d]
1[a]	7.030	45, 74, 29, 56, 117	2-Hydroxypropanoic acid	890
2[a]	7.197	101, 129, 73, 55, 147	Butanedioic acid	797
3[a]	8.592	116, 72, 70, 44, 29	Alanine	955
4[a]	8.863	102, 74, 57, 45, 29, 85	Glycine	793
5[a]	10.448	144, 101, 116, 72, 55	Valine	786
6[a]	11.784	158, 102, 43, 58, 72	Leucine	912
7[a]	12.091	158, 102, 129, 69, 74	Isoleucine	921
8[a]	12.461	142, 70, 98, 114, 41	Proline	905
9[a]	12.870	145, 117, 102, 73, 45	Theronine	770
10	14.446	88, 101, 73, 157, 228	Dodecanoic acid	851
11[a]	15.902	188, 142, 116, 70, 56	Aspartic acid	628
12	16.021	203, 157, 115, 87, 43	1,2,3-Propanetricarboxylic acid	939
13[a]	17.618	61, 114, 129, 175, 74	Methionine	913
14	18.724	202, 156, 128, 84, 56	Glutamic acid	856
15[a]	19.052	88, 101, 158, 256, 211	Tetradecanoic acid	917
16	19.672	57, 68, 82, 222, 240	Hexadecanal	961
17[a]	20.306	91, 176, 192, 220, 265	Phenylalanine	866
18[a]	21.309	74, 102, 220, 132, 174	Cysteine	480
19	21.408	88, 101, 157, 225, 270	Pentadecanoic acid	890
20	21.675	43, 57, 97, 110, 208	Hexadecanenitrile	977
21	23.111	88, 152, 194, 236, 282	9-Hexadecenoate	909
22	23.231	88, 152, 194, 236, 282	11-Hexadecenoic acid	948
23[a]	23.778	88, 101, 157, 241, 284	Hexadecanoic acid	971
24	23.980	226, 210, 74, 125, 154	Fenclonine	672
25	24.407	57, 68, 82, 222, 250	Octadecanal	971
26	25.143	88, 101, 157, 255, 298	Heptadecanoic acid	824
27[a]	27.146	55, 67, 81, 211, 280	9,12-Octadecadienoic acid	914
28	27.511	67, 81, 220, 262, 308	Linoleate	928
29	27.632	88, 180, 222, 264, 310	Oleate	960
30	27.752	88, 180, 222, 264, 310	9-Octadecenoate	971
31	27.954	79, 95, 108, 261, 306	9,12,15-Octadecatrienoic acid	731
32	27.988	91, 131, 146, 192, 248	Benzyl-L-glutamate	665
33[a]	28.210	88, 101, 157, 269, 312	Octadecanoic acid	907
34	28.624	156, 56, 84, 102, 128	Lysine	609
35	30.317	88, 101, 157, 283, 326	Nonadecanoic acid	779
36	30.839	79, 91, 203, 217, 332	5,8,11,14-Eicosatetraenoic acid	956
37	30.980	79, 91, 201, 215, 330	5,8,11,14,17-Icosapentaenoate acid	944
38	31.282	79, 93, 135, 150, 334	11,14,17-Eicosatrienoic acid	861
39	31.717	81, 95, 150, 291, 336	11,14-Eicosadienoic acid	904
40	31.814	81, 95, 262, 292, 338	11-Eicosadienoic acid	884
41	31.971	59, 72, 126, 264, 281	9-Octadecenamide	955
42[a]	32.147	107, 135, 192, 264, 74	Tyrosine	749
43	32.388	88, 101, 157, 297, 340	Eicosanoic acid	796
44	32.605	59, 72, 128, 240, 281	Octadecanamide	924
45	33.895	130, 117, 203, 101, 73	Tryptophan	589
46	34.475	67, 79, 91, 262, 356	4,7,10,13,16,19-Docosahexaenoic acid	923
47	37.543	81, 145, 247, 353, 368	Cholesta-3,5-diene	926
48	38.894	81, 135, 247, 366, 351	Cholesta-4,6-dien-3-ol	933
49	39.168	81, 147, 260, 353, 368	Cholest-5-en-3-ol(3β)-acetate	935
50	42.896	81, 145, 275, 368, 386	26-Nor-5-cholesten-3β-ol-25-one	896

[a]Compounds were verified using the reference compounds available; the remaining compounds were identified by the commercial compound libraries from NIST and Wiley in Turbomass 4.1.1 software (PerkinElmer).

[b]RT, retention time

[c]Typical ion fragmentations for the compound.

[d]The *Rev* values express the closeness of the matches of the spectra with NIST and Wiley library spectra; the ideal value for *Rev* is 999 in the Turbomass interface.

Visual examination of the representative GC-MS total ion current (TIC) chromatograms showed obvious differences between uremic patients and healthy controls (Fig. 2), but the inherent complexity of the GC-MS chromatograms made direct comparison between groups impractical. Hence, multivariate statistical analyses, including PCA and OPLS-DA, were used to extract meaningful information from the complex biological samples. An initial 3D-PCA scores plot (Fig. 3) showed that there were no outliers among the subjects and that the subjects were naturally clustered into two broad classes. The resulting groupings were in good agreement with their clinical profiles and were verified by the clear separation in the PC1 of OPLS-DA model (Fig. 4), which was constructed from one predictive and two orthogonal components. The R2Ycum of 0.826 and the Q2Ycum of 0.771 demonstrated the high robustness of the OPLS-DA model. The *p*-values of the *t*-tests for all metabolites that gave VIP values of more than 1 in the OPLS-DA were less than 0.05 in this study. Based on the VIP values, the results of the *t*-tests and the GC-MS spectral libraries, we identified two fatty acids, myristic acid (tetradecanoic acid) and linoleic acid (9,12-octadecadienoic acid), and three significantly decreased amino acids, valine, leucine and isoleucine, that were present at significantly raised levels in the uremic patients as compared to the normal subjects. The changes in the levels of these compounds were considered metabolic responses to the uremic syndrome (Table 4).

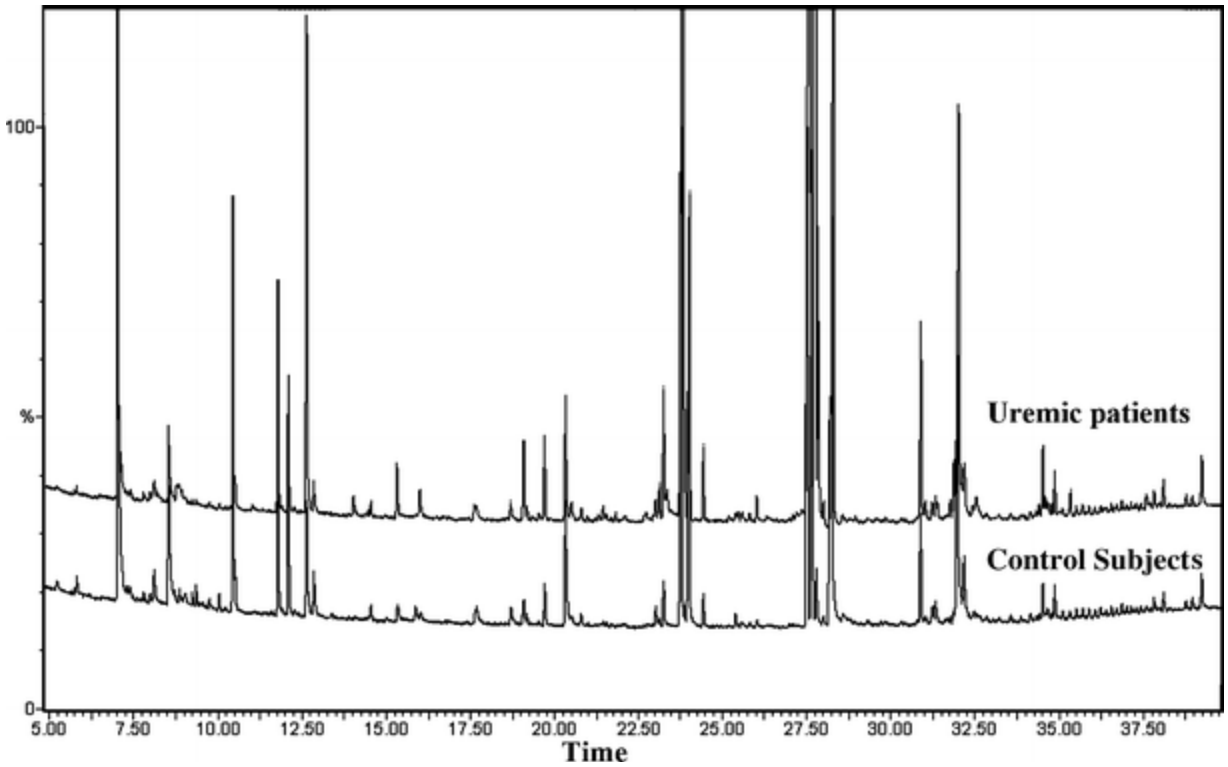

Figure 2: GC-MS TIC chromatograms of typical serum samples from different groups (the control subjects group and the uremic patients group)

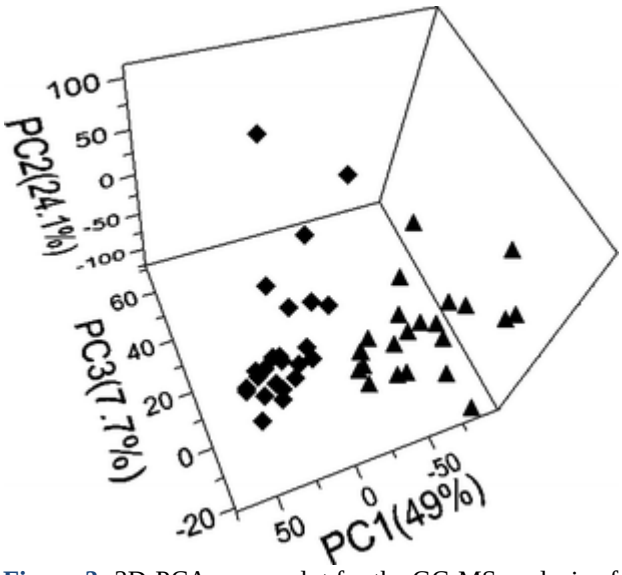

Figure 3: 3D-PCA scores plot for the GC-MS analysis of serum samples from uremic patients and normal subjects. Each symbol represents an individual subject (*triangles*, the control group; *diamonds*, the uremic group)

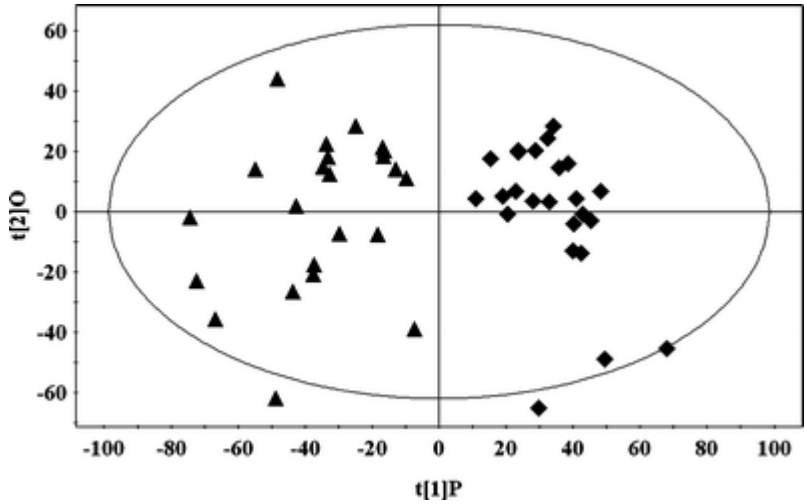

Figure 4: OPLS-DA for the GC-MS analysis of serum samples from uremic patients and normal subjects. (*triangles*, the control group; *diamonds*, the uremic group)

Table 4: List of the significant metabolites identified from OPLS-DA modeling of the metabolic profiles of uremic patients and health control objects

RT (min)	*m/z* [a]	Metabolites	*P* (*t*-test)	Fold change
10.448	144, 101, 116, 72, 55	Valine	0.022	−1.871
11.784	158, 102, 43, 58, 72	Leucine	0.039	−1.648
12.091	158, 102, 129, 69, 74	Isoleucine	0.0161	−2.890
19.052	88, 101, 158, 256, 211	Tetradecanoic acid	0.002	+8.690
27.146	55, 67, 81, 211, 280	9,12-Octadecadienoic acid	0.033	+7.516

[a]Typical ion fragmentations for the compound.

Fold change is based on a two-tailed Student's *t*-test, and it expresses the difference in concentrations between the uremic patient group and the control subject group; “+”and “-” indicate increased and decreased concentrations of metabolites, respectively, in the uremic patients versus the control subjects.

An abnormal accumulation of fatty acids such as myristic acid and linoleic acid was observed in serum samples from uremic patients in this study. The oxidation and metabolism of linoleic acid yields many biologically active compounds such as linolic acid monoepoxides and the corresponding diol metabolites through cytochrome P450. The accumulation of these metabolites in the kidney could induce mitochondrial dysfunction and cause cell death, which are usually responsible for acute kidney impairment [26, 27]. Myristic acid exhibited a protective effect on renal necrosis in rats fed a methyl-deficient diet; however, it should be investigated whether the level of myristic acid in the uremic patient serum increased as a metabolic compensation for uremia-related pathological variations. Numerous studies have reported that carnitine plays an indispensable role in the oxidation and metabolism of fatty acids, thus, the lineloic acid and myristic acid disorders observed in uremic patient sera probably reflect the depletion of carnitine [28], which has significant consequences for patients with myocardial diseases and kidney failure.

In addition to the accumulated metabolites identified in the uremic patient sera, significantly decreased concentrations of certain branched-chain amino acids (BCAAs) including valine, leucine and isoleucine were observed compared to those of normal subjects. The decreased concentrations of these three amino acids in uremic patients presumably resulted from their increased oxidation, which often serves as an alternative energy source.

Despite the fact that these preliminary results, such as the accumulation of fatty acids and the depletion of amino acids, were obtained from a relatively small-scale clinical study, the findings are rational and in good agreement with conventional studies that centered mainly on individual or certain metabolites [29–31]. Therefore, the proposed GC-MS-based metabolic profiling technology is able to characterize the metabolic disturbances associated with human uremia, and so it potentially provides a holistic therapeutic strategy for uremic patients. Further studies will be initiated in order to identify more of the various metabolites responsible for uremia in a large-scale clinical study using GC-MS in combination with other analytical techniques such as NMR and UPLC-QTOF-MS, and to evaluate the therapeutic efficacies of different dialysis prescriptions.

CONCLUSION

The major characteristics of the analytical method reported here is a simple and efficient ECF derivatization procedure coupled with the conventional use of high-throughput GC-MS. The

proposed method exhibited satisfactory linearity and 48-h stability, and acceptable analytical method and equipment repeatability, thus facilitating the global analysis of a broad array of metabolites present in complex serum samples. This method was successfully applied in a serum-based metabonomic study on human uremia, and preliminary results allowed the identification of several significant differences in metabolic function between uremic patients and normal subjects. These results, combined with the simplicity, reliability, and repeatability of the protocol, indicate that this optimized method can be widely applied to metabonomic studies of serum samples.

ABBREVIATIONS

GC-MS	Gas chromatography–mass spectrometry
LOD	Limit of detection
RSD	Relative standard deviation

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