Application of ethyl Chloroformate Derivatization for Gas Chromatography–Mass Spectrometry Based Metabonomic Profiling

By: Y. Qiu, M. Su, Y. Liu, M. Chen, J. Gu, J. Zhang, W. Jia

Qiu, Y., Su, M., Liu, Y., Chen, M., Gu, J., Zhang, J., & Jia, W. (2007). Application of ethyl chloroformate derivatization for gas chromatography-mass spectrometry based metabonomic profiling. *Analytica Chimica Acta*, *583*, 277-283.

Abstract:

A new combined gas chromatography and mass spectrometry (GC–MS) method has been developed suitable for the urine sample treatment in aqueous phase with ethyl chloroformate (ECF) derivatization agents. The method has been extensively optimized and validated over a broad range of different compounds and urine samples. Analysis of test metabolite derivatives, containing spiked standards, or rat urine exhibited acceptable linearity, satisfactory intra-batch precision (repeatability) and stability, relative standard deviations (R.S.D.) less than 10 and 15% within 48 h, respectively. The quantification limits were 150–300 pg on column for most metabolites. Recovery of several representative compounds, at different concentrations, ranged from 70 to 120%, with R.S.D. better than 10% for rat urine. We were able to generally eliminate potentially confounding variables such as medium complexity, different urea concentrations, and/or derivatization procedure variability. Metabonomic profiling of 1,2-dimethylhydrazine (DMH)-induced precancerous colon rat urine using GC–MS with ECF derivatization was performed to evaluate the proposed method. The analytical variation of the method was smaller than the biological variation in the rat urine samples, proving the suitability of the method to analyze differences in the metabonome of a living system with perturbed metabolic network. Thus, the proposed GC–MS analytical method is reliable to analyze a large variety of metabolites and can be used to investigate human pathology including disease onset, progression, and mortality.

Article:

INTRODUCTION

Systems biology, commonly involving genomics, proteomics, and metabonomics, is emerging as a vital component of life science research [1]. The integration of these “-omics” are likely to provide novel insights and understanding of life's complexity [2]. Metabonomic, which is generally defined as the multi-parametric metabolic responses of a living system to pathophysiological stimuli or genetic modification [3], is an effective tool for toxicological studies on pharmacologically active agents, for disease biomarker screening, and for characterization of biological pathways [4-8]. To accomplish these types of screens, metabonomic analysis focuses on metabolites, small molecules less than 1000 Da, in a single

cell, biofluids, or tissue. Utilizing widely ascribed multivariate data analysis methods such as principal component analysis (PCA) [9], partial least square (PLS) [10], and artificial neural networks (ANN) [11,12], we are able to elucidate significant differences and locate the possible "biomarkers" responsible for such variance within a huge number of endogenous metabolites.

Metabonomic studies require sensitivity, quantifiable data, and robustness of analytical methodology, which generally employ such techniques as nuclear magnetic resonance (NMR) [4,7,13-16], high-performance liquid chromatography–mass spectrometry (LC–MS, and LC–MS–MS) [17-21], capillary electrophoresis–mass spectrometry (CE–MS) [12,22,23], and gas chromatography–mass spectrometry (GC–MS) [24-28]. However, none of these analytical techniques could truly gather all the endogenous metabolites present in the biological samples but combinations of analytical techniques facilitate a more comprehensive analysis [29]. In this study, we "harvest" the information contained in the rat urine metabolite fragmentations detected with a GC–MS method consisting of electron impact (EI) ionization and single quadrupole mass analyzer. This method provides the necessary data to identify possible metabolites present, which can be further validated if appropriate standards are utilized. Since many metabolites are unstable at separation temperatures or not volatile, derivatization prior to GC–MS detection is a common prerequisite. Unlike trimethylsilyl derivatization agents which only work in nonaqueous phase, ECF is reactive in aqueous medium, less expensive, and less time-consuming [26-28] during the derivatization process. To our knowledge, a GC–MS method with ECF derivatization technique has not been previously used in the metabonomics context, and therefore we embarked on a study to determine if the methodology was appropriate for analysis of endogenous metabolites from dimethyl hydrazine (DMH)-induced, precancerous colon rat's urine [30]. The new GC–MS procedure with ECF derivatization has been optimized to effectively capture and analyze a range of metabolites involving organic acids, amines, amino acids, fatty acids, and amino-alcohols, without water exclusion during derivatization.

EXPERIMENTAL

Materials and methods

Ethyl chloroformate (ECF), pyridine, anhydrous ethanol, sodium hydroxide, chloroform, and anhydrous sodium sulfate were analytical grade from China National Pharmaceutical Group Corporation (Shanghai, China). l-2-chlorophenylalanine (Shanghai Intechem Tech. Co. Ltd., China) was used as an internal quality standard. Standards (Table 1) used for method optimization and validation were obtained from Sigma-Aldrich (St. Louis, MO, USA) and prepared in the ultrapure water from a Milli-Q system (Millipore, USA). Urine samples were collected from male Wistar healthy rats and DMH-induced (1,2-dimethylhydrazine; Sigma-Aldrich) precancerous colon rats (n = 6 for each group, 150 ± 10 g) and stored at −20 °C.

Table 1: Linearity and quantification limit of standards

Compound	Linear range (μg mL^{-1})[a]	*n*	r^{2}[b]	Quantification limit	
				pg on column[c]	S/N ratio[d]
Amino acids					
l-Alanine	0.25–20	8	0.9991	150	50
l-Glycine	0.25–20	8	0.9997	150	40
l-Valine	0.25–20	8	0.9992	150	33
l-Leucine	0.25–20	8	0.9997	150	49
l-Isoleucine	0.25–20	8	0.9983	150	14
l-Threonine	0.25–20	8	0.9946	150	9.8
l-Proline	0.25–20	8	0.9993	150	50
l-Aspartic acid	0.25–20	8	0.9908	150	63
l-Methionine	0.25–20	8	0.9989	150	20
l-glutamine acid	1–20	6	0.9992	150	9.8
l-Phenylalanine	0.25–15	7	0.9960	150	38
l-Cysteine	0.25–20	8	0.9979	150	24
l- Lysine	0.5–20	7	0.9832	600	9.7
l-Tyrosine	0.25–20	8	0.9946	150	14
Asparagine	1–20	7	0.9992	300	18
Glutamine	5–20	4	0.9799	3000	17.2
Amines					
n-Butylamine	0.5–40	8	0.9951	300	98
Putrescine	0.25–15	7	0.9929	150	30.8
Tyramine	0.5–40	8	0.9966	300	23
Dopamine	1–40	7	0.9814	300	10.2
Organic acids					
Fumaric acid	0.5–20	7	0.9951	150	42
Malate	0.25–20	8	0.9992	150	17
Succinate	0.25–20	8	0.9947	150	533
Butenedioic acid	0.5–20	7	0.9964	150	45
Phenylacetic acid	0.25–20	8	0.9976	150	9.2

[a]Each standard stock solution of test compounds was carefully prepared in the distilled water (1 mg mL^{-1}) and stored at −4 °C. The spiked standard solution was accordingly obtained by the addition of each aliquot of stock solution above involving 100 μL of each amino acid, 100 μL of each organic acid, and 200 μL of each amine. So the spiked standards concentration was about 30 μg mL^{-1} for organic acids or amino acids, and 60 μg mL^{-1} for amines. Different volumes of the spiked standard solution were diluted into 600 μL of water for linear range determination.
[b]Regression coefficients were calculated for linearity ranging at the concentration listed here.
[c]Qualification limit (pg on column) is the lowest calibration standard injected with a S/N ratio ≥9.
[d]S/N ratio calculation was carried out to display the peak-to-peak values by TurboMass software.

Derivatization

Each 600-μL aliquot of standard mixture or diluted urine sample (urine: water = 1:1, v/v) was added to a screw-top glass tube. After adding 100 μL of l-2-chlorophenylalanine (0.1 mg mL^{-1}), 400 μL of anhydrous ethanol, and 100 μL of pyridine to the urine sample, 50 μL of ECF was added for first derivatization at 20.0 ± 0.1 °C. The pooled mixtures were sonicated at 40 kHz for 60 s. Subsequently, extraction was performed using 300 μL of chloroform, with the aqueous layer pH carefully adjusted to 9–10 using 100 μL of NaOH (7 mol L^{-1}). The derivatization procedure was repeated with the addition of 50 μL ECF into the aforementioned products. After the two successive derivatization steps, the overall mixtures were vortexed for 30 s and centrifuged for 3 min at 3000 rpm. The aqueous layer was aspirated off, while the remaining chloroform layer containing derivatives were isolated and dried with anhydrous sodium sulfate and subsequently subjected to GC–MS analysis.

GC–MS analysis

The derivatized extracts were analyzed with a PerkinElmer gas chromatograph coupled with a TurboMass-Autosystem XL mass spectrometer (PerkinElmer Inc., USA). A 1-μL extract aliquot of the extracts was injected into a DB-5MS capillary column coated with 5% diphenyl cross-

linked 95% dimethylpolysiloxane (30 m× 250 μm i.d., 0.25-μm film thickness; Agilent J&W Scientific, Folsom, CA) in the split mode (3:1). Either the injection temperature or the interface temperature was set to 260 °C; and the ion source temperature was adjusted to 200 °C. Initial GC oven temperature was 80 °C; 2 min after injection, the GC oven temperature was raised to 140 °C with 10 °C min^{-1}, to 240 °C at a rate of 4 °C min^{-1}, to 280 °C with 10 °C min^{-1} again, and finally held at 280 °C for 3 min. Helium was the carrier gas with a flow rate set at 1 mL min^{-1}. The measurements were made with electron impact ionization (70 eV) in the full scan mode (*m*/*z* 30–550).

Data analysis

All the GC–MS raw files were converted to CDF format via DataBridge (PerkinElmer Inc., USA), subsequently processed by the XCMS toolbox (http://metlin.scripps.edu/download/) using XCMS's default settings with the following exceptions: xcmsSet (full width at half-maximum: fwhm = 4; S/N cutoff value: snthresh = 8, max = 20), group (bw = 5) [31,32]. The resulting table (TSV file) was exported into Matlab software 7.0 (The MathWorks, Inc.), where normalization was performed prior to multivariate analyses. The resulting three dimensional matrix involving peak index (RT-*m*/*z* pair), sample names (observations), and normalized peak area percent was introduced into Simca-P 11.0 Software package (Umetrics, Umeå, Sweden), which utilizes principal components analysis (PCA) to display the metabolic information visually. Additionally, the majority of the metabolites detected were identified by Turbomass 4.1.1 software (PerkinElmer Inc., USA) coupled with commercially available compound libraries: NIST, Wiley, and reference compounds available.

RESULTS

Derivatization procedure

As previously published [28], ECF derivatization is favored because of two advantages. First, ECF derivatization proceeds in the aqueous medium and thus does not necessarily remove water before reaction, which facilitates batch preparation and improved reproducibility. Secondly, two-step derivatization protocols have satisfactory derivatization efficiencies and enhanced data collection, i.e. more peaks were detected in urine samples, and each peak area of the 25 representative test compounds belonging to different chemical classes (amino acids, organic acids, and amines) were significantly expanded (data now shown). Notably, adjusting the pH with NaOH is crucial for the further derivatization probably because these molecules are more soluble and reactive in the aqueous medium [33]. Also, derivatization time, temperature, and GC–MS parameters (see Section 2) were chosen not solely upon the 25 reference compounds but also in consideration of the urine samples.

Linearity and quantification limit

The linearity of response was determined by using spiked standards at different concentrations ranging from 0.25 to 20 μg mL^{-1} for amino acids or organic acids and from 0.5 to 40 μg mL^{-1} for amines. A calibration curve for each test compound was adjusted using an internal standard. For most of the compounds investigated, the coefficients of determination (*r*-squares) generally approached 1.000 (Table 1). Exceptions were compounds such as glutamine, l-lysine, putrescine and dopamine, which had nonlinear regression at lower or higher concentrations. The final adjusted *r*-squares were achieved by excluding such data.

In this paper, we determined each compound's quantification limit by analyzing the signal-to-noise ratio (S/N) provided by TurboMass interface, where the greatest height of the signal range above the mean noise value is divided by the variance. From Table 1, all the S/N values are higher than 9.0, allowing the individual calculation of each compound's quantification limit. Occasionally, the actual quantification limit was lower than the lowest spiked concentration, which had a higher S/N value. In this case, the lowest spiked concentration was used as the quantification limit, e.g. succinate.

Reproducibility and stability

We used both the spiked standard solution and urine sample from a typical healthy rat to investigate reproducibility. After the correction with internal standard, the relative standard deviations (R.S.D.) of peak area for each standard spiked and standards (endogenous metabolites) detected in rat urine were comparable, R.S.D. below 10%. The results were repeated by two different operators. However, several derivatives such as fumaric acid, glutamine, and succinate could not be detected in the urine predominately due to the detection limits. Additionally, standard compounds with varying physical and chemical properties were selected for the stability assay. For most spiked compounds, the R.S.D. were better than 10% within 36 h and less than 15% within 48 h (Fig. 1). l-Glutamine acid and asparagine were suspected to be unstable metabolites because of RSDs higher than 15%, after 60 h. The GC–MS sensitivity appeared to decrease after about 60 samples (~48 h); therefore, each batch analysis was performed within 2 days.

Recovery of standards in rat urine

To further validate our methodology to analyze complex endogenous metabolites, each 300-μL aliquot of 20, 50, 100, and 200 μg mL^{-1} of 14 standard solutions were spiked to 300 μL in pooled urine, prior to ECF-derivatization. Importantly, these compounds were specifically selected from different species with various characteristics, e.g. fumaric acid, an organic acid, was not detected in the urine, while amino acids, typical of l-leucine, was quantitatively detected in a relative higher amount. In general, extraction recovery was calculated through an adjusted linear curve, and the mean recovery of all these compounds with different concentrations ranged from 70 to 120% with R.S.D. better than 10% (Table 2). Considering the environment variation, each 300 μL of the test compounds was mixed with 300 μL urine from a healthy male volunteer. However, the results are comparable to rat urine (data not shown). In addition, urea did not disrupt our derivatization or extraction, suggesting urea does not react with the derivatization reagent.

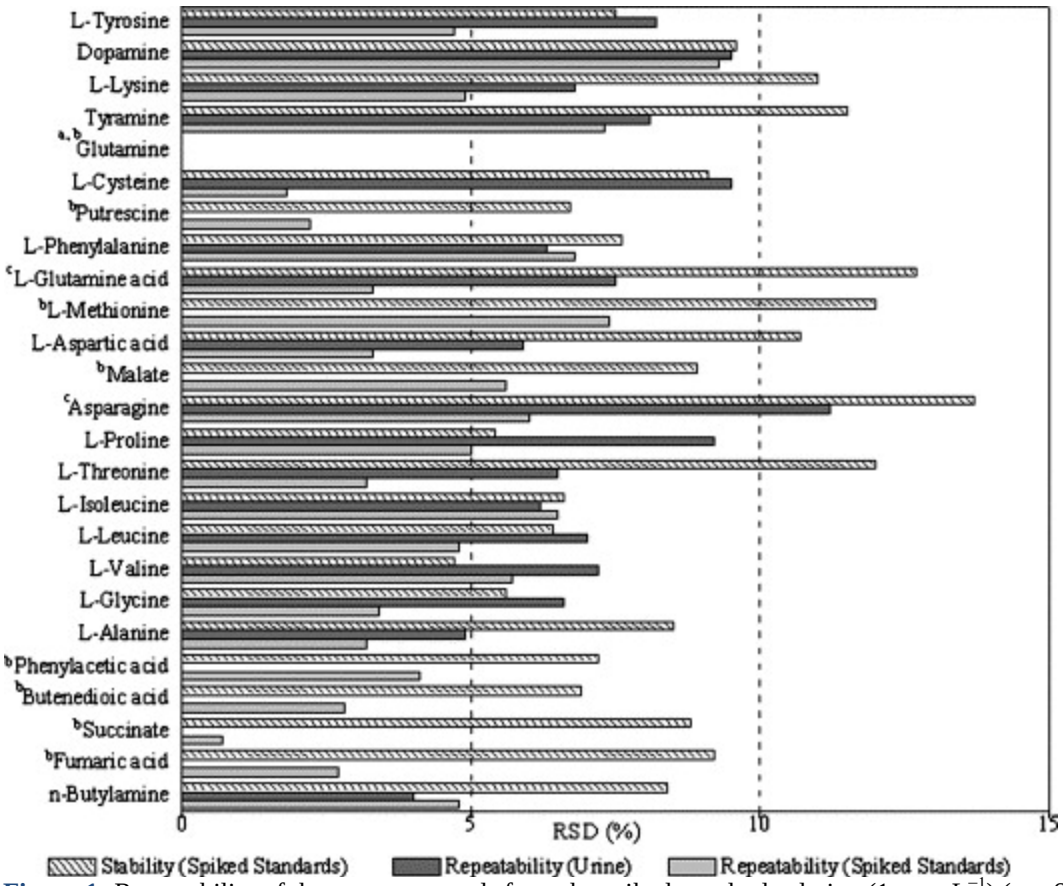

Figure 1: Repeatability of the test compounds from the spiked standard solution (1 μg mL^{-1}) (n = 6) and detected from the urine samples (n = 6); stability of the test compounds added (n = 12). [a]Not detected in standard solution; [b]not detected in the urine sample; [c]unstable compounds, R.S.D. of the corrected response was slightly higher than 15 after 60-h determination.

Table 2: Recovery of several typical standards[a]

Compounds	Recovery (%)[b]	
	Average	R.S.D.
n-Butylamine[c]	79.3	5.1
Tyramine[c]	74.1	6.0
Dopamine[c]	84.1	5.5
l-Valine[d]	83.4	5.1
l-Leucine[d]	74.5	3.0
l-Isoleucine[d]	82.7	3.2
Malate[e and f]	76.2	4.6
Fumaric acid[e and f]	80.2	7.0
l-Cysteine[d]	99.9	9.2
l-Tyrosine[d]	82.9	2.6
l-Proline[d]	80.0	6.0
Asparagine[d]	110.9	4.9
l-Aspartic acid[d]	110.5	2.4
l-Phenylalanine[d]	74.1	1.1
l-Glutamine acid[d and g]	88.3	6.3
l-Methionine[d and f]	110.5	5.4

[a]The standards were screened for recovery calculation based on their various characteristics and concentration in urine. [b]Mean Recovery was obtained by 12 determinations (three parallel samples at four different concentrations) with an internal standard. [c]Amines group. [d]Amino acids group. [e]Organic acids group. [f]Not commonly detected in a typical urine sample. [g]Unstable metabolites.

Application

DMH-induced precancerous colon rat model tested the potential application of the GC–MS method, with ECF derivatization, for the body fluid analysis. We analyzed rat urine samples obtained from healthy or DMH-treated rats (n = 6 for each group) (DMH was IP given twice with one week interval. Samples were collected seven weeks after treatment). Using our optimized GC–MS analysis protocol, we identified about 200–250 different urine metabolites, with 87 of them present in the library or confirmed via standard compounds (data not shown). The R.S.D. of the added internal standards were less than 5% and the mean recovery was about 10%, indicating the GC–MS method was reproducible. In the Fig. 2, the total ion chromatograms (TIC) of urine samples from two groups (the healthy control versus DMH-induced precancerous colon group) are illustrated. Visual examination of the TICs shows a major difference with each other, typically in the zoomed range. The GC–MS data were processed via XCMS software prior to PCA. PCA scores plot (Fig. 3) of GC–MS data describes the general differences between two different groups at the end point of the study. To obtain a vivid view in the results from multivariate data analysis, univariate plots were made. In these error-bar plots (Fig. 4), the mean and standard deviation were plotted for each group. Such a plot may help explore the nature of biological disturbance reflected by these latent biomarkers. For instance, the mean concentration of tryptophan is higher in the DMH-induced group than in the healthy group, indicating that the biological pathway related to tryptophan may be disturbed to some extent, while the concentration of 4-hydroxybenzeneacetic acid is somewhat higher in the healthy group than in the drug group. The mechanism of DMH-induced pathological change may be elucidated based on the up- or down-regulated metabolic pathways reflected by these biological alterations.

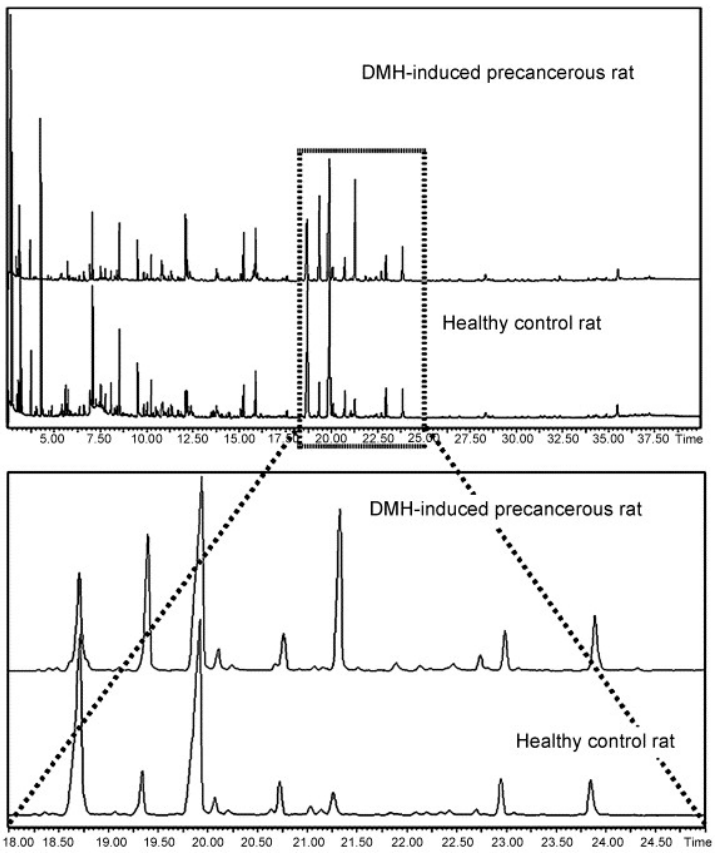

Figure 2: Full scan GC–MS TIC of typical urine samples from different group (DMH-induced vs. healthy control).

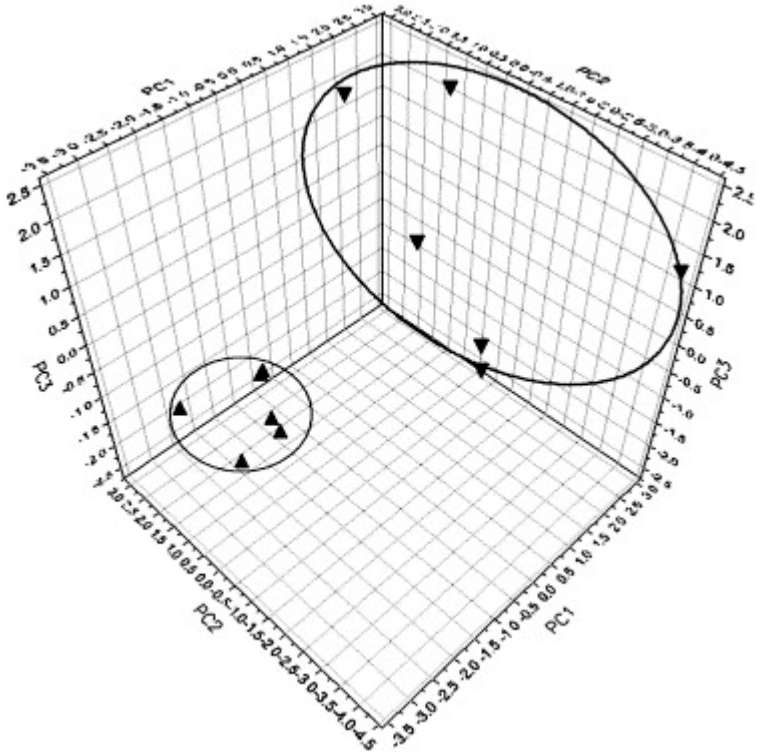

Figure 3: PCA scores plot from the analysis of DMH-induced precancerous colon rats (▽) and healthy control rats (△).

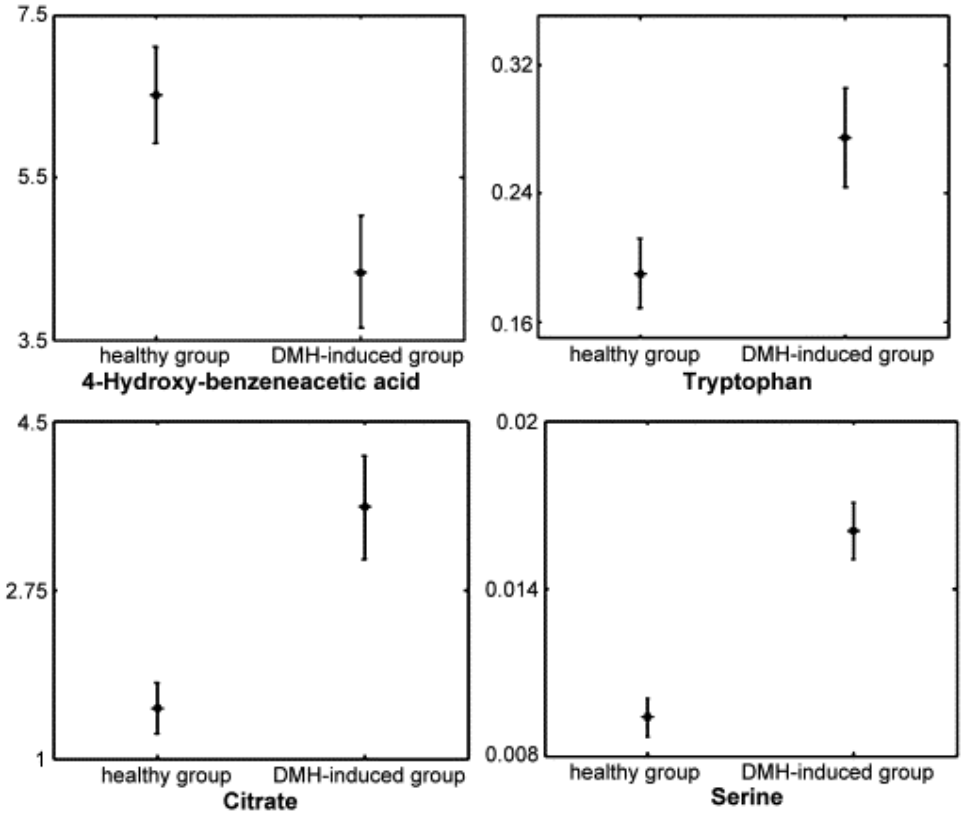

Figure 4: Univariate up- or down-regulation error-bar plots for potential biomarkers responsible for DMH-induced precancerous colon rats. An asterisk (*) represents the mean value, and the error bars.

DISCUSSION

The goal of metabonomic study is to analyze as many metabolites as possible from biological fluids like urine, serum, semen, or tissue. Such a noninvasive approach can facilitate a better understanding of global biological functions and can be help in disease detection. However, development of a comprehensive analytical metabonomic methodology with high reproducibility and stability is difficult due to the technique's extreme sensitivity and possibility of misleading results generated from analytical variations. Combined gas chromatography and mass spectrometry (GC–MS) is one of the most commonly used techniques because of its simplicity, stability and reproducibility.

A new GC–MS method has been developed suitable for the urine sample treatment in aqueous phase with ECF derivatization agents. The method has been extensively optimized and validated over a broad range of different compounds and urine samples. Metabolites of interest in urine samples can generally be classified into three major groups: amino acids, organic acids, and amines. We detected metabolites over a large, linear dynamic range (0.25–20 $\mu g\ mL^{-1}$), and the detection limits were low (~150 pg on column), with the exception of a few compounds such as glutamine and l-lysine which become detectable at 1–2 $\mu g\ mL^{-1}$ with the current single quadrupole mass analyzer.

Most samples for metabonomics studies are large scale batch processing and thus the measurements can be time intensive. Inter- or intra-batch analysis performed within short period of time, i.e. 48 h, should be reasonably stable and reliable. Mean recovery of different concentrations was 70–120%, which indicated reasonable derivatization efficiency. The R.S.D. of mean recovery for these compounds was satisfactory, at lower than 10%, suggesting that the different concentration of urea in the samples exerted little interference with the detection of other metabolites.

XCMS package for metabolite profiling involved three important algorithms: peak detection, peak matching, and retention time alignment algorithms. This provided a comparable function for peak resolution. Since the GC–MS system elution rate was fast, with sharp peaks, the full width at half-maximum (fwhm) was set at 4. The S/N cutoff value (snthresh = 8) was based on the previously mentioned quantification limits.

The analytical variation of the method was small, compared with the biological variation in the rat urine samples, proving the suitability of the method to analyze differences in the metabonome of a living system with perturbed metabolic network. Thus, the proposed GC–MS methodology is reliable to analyze a large variety of metabolites and can be used to investigate human pathology including disease onset, progression, and mortality.

CONCLUSION

We described a convenient, quantitative and precise analytical method using GC–MS with ECF derivatization with a very large application range for the analysis of all metabolites from cells, body fluids, and tissue. The method has been extensively optimized and validated over a broad range of different compounds and urine samples, and practically used for metabolic profiling of urine samples from DMH-induced model rats. It appeared that such a metabonomic technique is able to delineate the characteristic metabolite expression pattern for a global analysis of a living

system with disturbed regulatory network because of its capability and reproducibility in the quantitative analyses of several classes of metabolites, primarily involving amino acids, organic acids, and amines. Based on its simplicity, robust performance and wide applicability, this method is expected to play an important role in the metabonomic study and systems biology.

ACKNOWLEDGEMENTS

This study was financially supported by Shanghai Leading Academic Discipline Project, project no T0301, and by the Key Basic Research Project (project no 05DJ14009) of Shanghai Science and Technology Commission.

REFERENCES

[1] J.K. Nicholson, I.D. Wilson, Nat. Rev. Drug Discovery 2 (2003) 668.
[2] M. Coen, S.U. Ruepp, J.C. Lindon, J.K. Nicholson, F. Pognan, E.M. Lenz, I.D. Wilson, J. Pharm. Biomed. Anal. 35 (2004) 93.
[3] J.K. Nicholson, J.C. Lindon, E. Holmes, Xenobiotica 29 (1999) 1181.
[4] J.T. Brindle, H. Antti, E. Holmes, G. Tranter, J.K. Nicholson, H.W.L. Bethell, S. Clarke, P.M. Schofield, E. McKilligin, D.E. Mosedale, D.J. Grainger, Nature Med. 8 (2002) 1439.
[5] J.C. Lindon, E. Holmes, J.K. Nicholson, Curr. Opin. Mol. Ther. 6 (2004) 265.
[6] J.C. Lindon, E. Holmes, J.K. Nicholson, Expert Rev. Mol. Diagn. 4 (2004) 189.
[7] A.W. Nicholls, J.K. Nicholson, J.N. Haselden, C.J.Waterfield, Biomarkers 5 (2000) 410.
[8] J.K. Nicholson, J. Connelly, J.C. Lindon, E. Holmes, Nat. Rev. Drug Discovery 1 (2002) 153.
[9] M. Chen, L. Zhao, W. Jia, J. Proteome Res. 4 (2005) 2391.
[10] C.L. Gavaghan, I.D. Wilson, J.K. Nicholson, FEBS Lett. 530 (2002) 191.
[11] K.H. Ott, N. Araníbar, B. Singh, G.W. Stockton, Phytochemistry 62 (2003) 971.
[12] M. Sugimoto, S. Kikuchi, M. Arita, T. Soga, T. Nishioka, M. Tomita, Anal. Chem. 77 (2005) 78.
[13] J. Azmi, J. Connelly, E. Holmes, J.K. Nicholson, R.F. Shore, J.L. Griffin, Biomarkers 10 (2005) 401.
[14] J.T. Brindle, J.K. Nicholson, P.M. Schofield, D.J. Grainger, E. Holmes, Analyst 128 (2003) 32.
[15] M.E. Dumas, E.C. Maibaum, C. Teague, H. Ueshima, B. Zhou, J.C. Lindon, J.K. Nicholson, J. Stamler, P. Elliott, Q. Chan, E. Holmes, Anal. Chem. 78 (2006) 2199.
[16] J.R. Espina, J.P. Shockcor,W.J. Herron, B.D. Car, N.R. Contel, P.J. Ciaccio, J.C. Lindon, E. Holmes, J.K. Nicholson, Magn. Reson. Chem. 39 (2001) 559.
[17] M. Chen, M. Su, L. Zhao, J. Jiang, P. Liu, J. Cheng, Y. Lai, Y. Liu, W. Jia, J. Proteome Res. 5 (2006) 995.
[18] R.S. Plumb, C.L. Stumpf, J.H. Granger, J. Castro-Perez, J.N. Haselden, G.J. Dear, Rapid Commun. Mass Spectrom. 17 (2003) 2632.
[19] C. Wang, H. Kong, Y. Guan, J. Yang, J. Gu, S. Yang, G. Xu, Anal. Chem. 77 (2005) 4108.
[20] I.D. Wilson, R. Plumb, J. Granger, H. Major, R. Williams, E.M. Lenz, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 817 (2005) 67.
[21] J. Yang, G. Xu, Y. Zheng, H. Kong, C. Wang, X. Zhao, T. Pang, J. Chromatogr. A 1084 (2005) 214.
[22] T. Soga, Y. Ohashi, Y. Ueno, H. Naraoka, M. Tomita, T. Nishioka, J. Proteome Res. 2 (2003) 488.

[23] S. Ullsten, R. Danielsson, D.Bäckström, P. Sjöberg, J. Bergquist, Bergquist, J. Chromatogr. A 1117 (2006) 87.
[24] O. Fiehn, J.Kopka, P. Dormann,T. Altmann, R.N.Trethewey, L.Willmitzer, Nat. Biotechnol. 18 (2000) 1157.
[25] O. Fiehn, J. Kopka, R.N. Trethewey, L.Willmitzer, Anal. Chem. 72 (2000) 3573.
[26] P. Hušek, J. Chromatogr. B 717 (1998) 57.
[27] P. Hušek, P. Matucha, A. Vránková, P. Šimek, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 789 (2003) 311.
[28] A. Namera, M. Yashiki, M. Nishida, T. Kojima, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 776 (2002) 49.
[29] J.P. Shockcor, S.E. Unger, I.D. Wilson, P.J.D. Foxall, J.K. Nicholson, J.C. Lindon, Anal. Chem. 68 (1996) 4431.
[30] L.K.T. Lam, J. Zhang, Carcinogenesis 12 (1991) 2311.
[31] A. Nordstrom, G. O'Maille, C. Qin, G. Siuzdak, Anal. Chem. 78 (2006) 3289.
[32] C.A. Smith, E.J.Want, G. O'Maille, R. Abagyan, G. Siuzdak, Anal. Chem. 78 (2006) 779.
[33] S.G. Villas-Bôas, D.G. Delicado, M. Åkesson, J. Nielsen, Anal. Biochem. 322 (2003) 134.