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Evaluation of an automated LC-MS/MS system for analyzing hydrophilic blood metabolites

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Introduction

Recently, metabolomics has been developed and applied to a variety of research fields, such as the food science, agriculture, engineering, and medical fields. In the medical research field, metabolomics is used to search for novel metabolite biomarkers of a variety of diseases and elucidate pathogenic mechanisms, etc., and there have been a considerable number of metabolite biomarker studies. As a step toward the practical use of metabolite biomarkers, a simple and quick automated sample preparation method involving metabolite extraction and metabolite measurement should be developed. In this study, we assessed whether the plasma levels of metabolites could be quantitatively measured using a fully automatic pre-treatment system for LC/MS that can be connected online to an LC/MS device.

Methods and Materials

Reagents: Acetonitrile (LC/MS grade), formic acid (LC/MS grade) and methanol (MeOH; LC/MS grade) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 2-Morpholinoethanesulfonic acid (MES), which was employed as an internal standard of primary metabolites, was purchased from Sigma Aldrich. L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine were acquired from Sigma Aldrich (MO, USA). Isotopically labeled L-valine (D_8), L-leucine ($^{13}C_6$), L-isoleucine (D_{10}), L-tyrosine ($^{13}C_9$, ^{15}N), and L-phenylalanine (D8) were purchased from Cambridge Isotope Laboratories (MA, USA). Commercially available pooled plasma (Kohjin-Bio Co., Saitama, Japan), which was collected using EDTA-Na as an anticoagulant, was utilized as human plasma, and pooled plasma with the same lot number was used for all experiments.

Manual method: 20 µL of plasma (N=5) were mixed with 230 µL of MeOH containing 10 µM isotopically labeled L-valine, L-leucine, L-isoleucine, L-tyrosine, L-phenylalanine and MES as internal standards. Next, the mixture was shaken at 1,200 rpm for 30 min at room temperature, before being passed through an ultrafiltration filter (Amicon Ultra 0.5-mL centrifugal filters, Ultracel-3K). The mixture was then centrifuged at 14,000 g for 60 min at 4°C, and the collected solution was subjected to the LC/QqQMS-based analysis of L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine.

Automatic method: The automatic method used to analyze L-valine, L-leucine, L-isoleucine, L-tyrosine, L-phenylalanine levels was performed using an CLAM-2000 (Shimadzu Corporation, Kyoto, Japan). In the CLAM-2000, MeOH containing 10 µM isotopically labeled L-valine, L-leucine, L-isoleucine, L-tyrosine, L-phenylalanine and MES (as internal standards) was added into the solvent container, and 20 µL of plasma (N=5) were also applied into another tubes. By running the CLAM-2000, 20 µL of plasma were automatically mixed with 230 µL of MeOH and the internal standards, before the resultant mixture was shaken at 1,900 rpm for 30 min at room temperature. Then, the mixture was automatically subjected to suction filtration for 90 sec, and the filtered solution was transferred to an SIL-30AC autosampler online, before being subjected to LC/QqQMS analysis.

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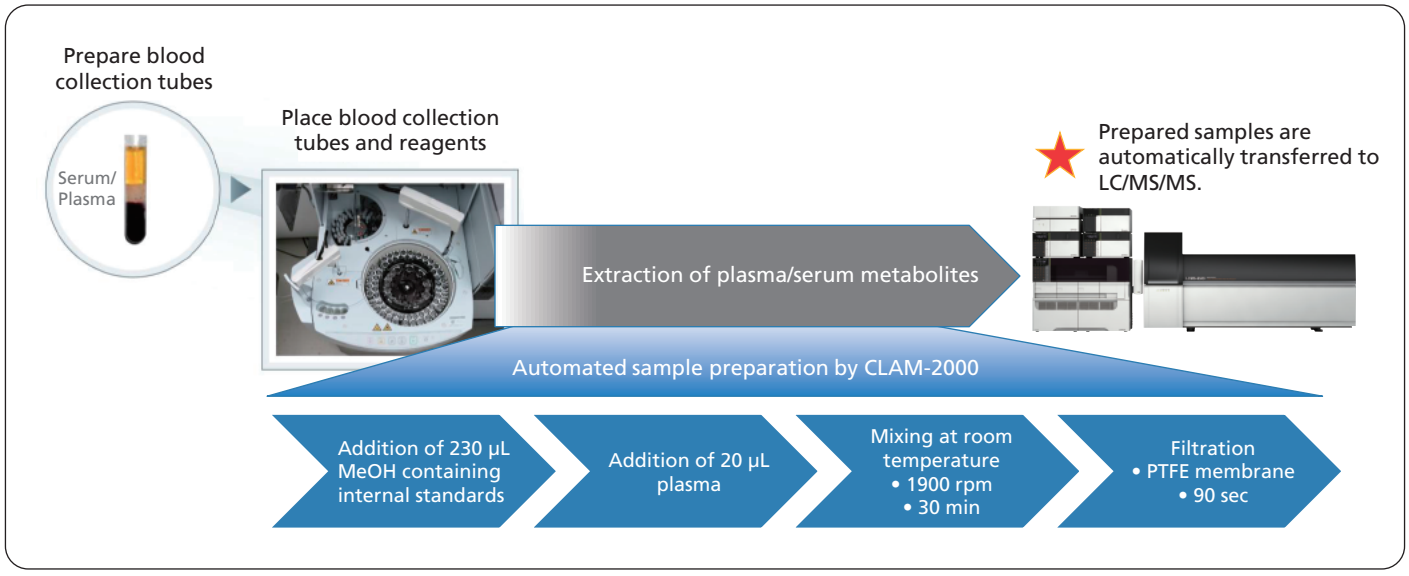

Figure 1. Workflow for analysis of metabolites using fully automated sample preparation LC/MS/MS system

HPLC conditions	
Column	: Discovery HS F5 2.1 mm × 150 mm, 3.0 µm
Mobile phase A	: 0.1% Formic acid/Water
Mobile phase B	: 0.1% Formic acid/Acetonitrile
Time program	: B conc. 25%(5 min) - 35%(11 min) - 95%(15 min) - 95%(20 min) - 0%(20.01-25 min)
Injection vol.	: 1 µL
Flow rate	: 0.25 mL/min.
Column temperature	: 40°C
MS conditions (LCMS-8040)	
Ionization	: ESI (Positive/Negative)
Nebulizing Gas Flow	: 2.0 L/min.
Drying Gas Flow	: 15.0 L/min.
DL temperature	: 250°C
Block Heater Temperature	: 400°C

For the analysis of primary metabolites except L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine, LC/MS/MS Method Package for Primary Metabolites (Shimadzu Corporation, Kyoto, Japan) was used. The multiple reaction monitoring (MRM) transitions of the native and stable isotopes of L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine are shown in Table 1.

An amino acid analysis of plasma samples with the same lot number was also performed by SRL (Tokyo Japan), and the plasma concentrations of L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine (µM) were measured. The Fischer ratio was calculated based on the quantitative results. The resultant data are shown in the 'Reference concentration (µM)' (Table 2).

Table 1. The MRM transitions of native and stable isotope molecules of L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine

Product name	Precursor ion (*m/z*)	Product ion (*m/z*)
L-valine	118.1	72.15
L-tyrosine	182.1	136.1
L-isoleucine	132.1	86.2
L-leucine	132.1	86.05
L-phenylalanine	166.1	120.1
L-valine (D_8)	126.2	80.15
L-tyrosine ($^{13}C_9$, ^{15}N)	192.2	145.2
L-isoleucine (D_{10})	142.25	96.15
L-leucine ($^{13}C_6$)	138.15	91.15
L-phenylalanine (D_8)	174.2	128.2

Results and Discussion

The utility of the CLAM-2000 as an automatic pre-treatment system for analyzing hydrophilic blood metabolites was evaluated in the present study (Table 2). In this experiment, stable isotopes corresponding to the 5 targeted native metabolites; i.e., L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine, were used for the quantitative analysis because the quantitative performance of MS is affected by various factors, such as ion suppression, and stable isotopes are required to obtain detailed quantitative information about the targeted molecules. The targeted metabolites included branched-chain and aromatic amino acids, and the Fischer ratio was calculated based on the quantitative results. In a comparison between the automatic method involving the CLAM-2000 and the manual method, the quantitative results, including the data regarding the Fischer ratio, obtained using the two methods were almost the same. In addition, these quantitative results were almost the same as those acquired by SRL. The measurement stability of each method was also high, and the metabolites' RSD% values were very low (<6%). Regarding the Fisher ratio data obtained using the two methods, the associated RSD% values were <1.5%. Regarding the metabolites except L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine, the measurement stability of the automatic method is higher than that of the manual method (Table 3). These results suggest that the CLAM-2000 could be used for automatic pre-treatment during the analysis of hydrophilic blood metabolites.

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Table 2. Comparison between the manual and automatic methods for analyzing branched-chain and aromatic amino acids

Product name	Pre-treatment	Concentration (μM)	SD	RSD (%)	Reference concentration (μM)
L-valine	Manual method	210.6	8.19	3.89	215
	Automatic method	200.2	6.65	3.32	
L-leucine	Manual method	132.8	7.20	5.42	127
	Automatic method	126.4	4.30	3.40	
L-isoleucine	Manual method	65.6	3.38	5.15	69
	Automatic method	66.8	2.81	4.22	
L-tyrosine	Manual method	71.8	2.37	3.30	67
	Automatic method	67.8	2.58	3.80	
L-phenylalanine	Manual method	54.6	1.81	3.32	55
	Automatic method	54.5	1.97	3.61	
Fischer ratio	Manual method	3.24	0.048	1.47	3.4
	Automatic method	3.22	0.033	1.03	

Table 3. Comparison between the measurement stability of manual and automatic methods

	The number of detected metabolites	
Method	Manual method	Automatic method
Total	45	46
0%<RSD%£20%	33 (73.3%)	40 (87.0%)
20%<RSD%£50%	11 (24.4%)	6 (13.0%)
50%<RSD%	1 (2.2%)	0 (0%)

To the best of our knowledge, this is the first study in which the CLAM-2000 was utilized for metabolomics. The CLAM-2000 is a fully automatic pre-treatment device for LC/MS, and it can be connected online to an LC/MS system. Therefore, metabolome analysis using the CLAM-2000 might be suitable for measuring larger numbers of serum/plasma samples, because CLAM-2000 has no manual step leading to the decreased accident error by hand working, and moreover CLAM-2000 automatically can do the metabolite extraction and the following measurement of 60 serum/plasma samples in one batch. However, there are some issues related to our CLAM-2000-based procedure that remain to be evaluated. For example, the extracted solutions are directly transferred into an autosampler, but it might be better to dilute the extracted solutions with H_2O to reduce the percentage of organic solvent in the solution because a higher percentage might lead to column flooding and poor chromatography. In addition, removal of lipids from the extracted solutions may be also needed for the stable measurement. If these problems could be resolved, metabolome analysis using the CLAM-2000 could become more practical.

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Table 4. Metabolites detected by using automated sample preparation

Amino acids				
Asymmetric dimethylarginine	Alanine	Arginine	Asparagine	Aspartic acid
Citrulline	Cysteine	Cystathionine	Cystine	Dimethylglycine
Glutamine	Glutamic acid	Glycine	Histidine	Hydroxyproline
Isoleucine	Leucine	Lysine	Methionine	Methionine-sulfoxide
Ornitine	Phenylalanine	Proline	Symmetric dimethylarginine	Serine
Threonine/Homoserine	Tryptophan	Tyrosine	Valine	
Organic acids				
cis-Aconitate	Citrate	Creatine	Isocitrate	Lactate
Malate	Pantothenate	Pyruvate	Uric acid	
Nucleosides and Nucleotides				
Adenosine	Guanosine	Inosine	Thymidine	Uracil
Uridine	AMP			
Others				
Carnitine	Kynurenine	Adenine	Choline	Acetylcarnitine
Creatinine				

Conclusions

- The use of the CLAM-2000 as a fully automatic pre-treatment system for LC/MS-based metabolomics facilitates the identification of metabolite biomarker candidates, the validation of metabolite biomarker candidates, and the practical use of metabolite biomarkers.
- Further optimization of our method for CLAM-2000-based metabolome analysis is necessary.

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