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Short Communication

Chromatographic diagnosis of maple syrup urine disease by measuring the L-alloisoleucine/L-phenylalanine ratio in dried blood spots

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ABSTRACT

A high-performance ligand-exchange chromatography with ultraviolet detection method for confirmation diagnosis of maple syrup urine disease (MSUD) was developed that relies on the determination of branched-chain amino acids (BCAAs) and Phe levels in blood. The dynamic ranges for the BCAAs and Phe were 50–1000 μM (r^2 = 0.9982–0.9996) and 74–873 μM (r^2 = 0.9992) from a dried blood spot, and the BCAA detection limits (S/N = 3) were 0.43–1.91 μM. The mean recoveries of BCAA for intra- and inter-day assays were 92.1–103.0%. The ranges of alloisoleucine (Allo-Ile)/Phe ratio were ND–0.04 and 1.5–2.4 for PKU and MSUD patient samples, respectively. The lowest ratio (1.5) of the MSUD samples was 37.5 times higher than the highest ratio (0.04) of the PKU samples. Therefore, the Allo-Ile/Phe ratio was very useful biomarker for confirmation diagnosis of MSUD.

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1. Introduction

Majority of inherited metabolic disorders (IMDs) are autosomal recessive genetic disorders that can induce severe irreversible complications through the accumulation of toxic metabolites by congenital metabolic enzyme deficiencies. These disorders are classified based on accumulated metabolites, such as carbohydrates (e.g., galactosemia), amino acids (e.g., phenylketonuria, PKU; maple syrup urine disease, MSUD), and organic acids (e.g., propionic aciduria). They can be diagnosed by determining the accumulated metabolites in the urine, plasma or blood [1–3]. Maple syrup urine disease (OMIM 248600) is a kind of IMDs caused by enzymatic defects, which effect the metabolic pathways of the branched chain amino acids (BCAAs). This enzymatic deficiency leads to elevated plasma levels of branched-chain L-amino acids (BCAAs) such as L-valine (Val), L-leucine (Leu), L-isoleucine (Ile), and L-alloisoleucine (Allo-Ile). The worldwide incidence of MSUD is approximately 1 in 185,000 [4]. Patients with MSUD display feeding intolerance, vomiting, lethargy, a urine odor reminiscent of maple syrup, and if left untreated, can progress to irreversible mental retardation, seizures, coma, and possibly death.

If the confirmation diagnosis of MSUD is made early, peritoneal dialysis, hemodialysis, or dietary BCAA restriction can be an effective treatment. Early diagnosis is crucial for the normal development of MSUD patients, to prevent irreversible brain damage [4–6]. Typically, MSUD is diagnosed by determining the blood concentration of each BCAA or the total Leu (tLeu; Leu + Ile + Allo-Ile) [5]. In particular, Allo-Ile has been used as a pathognomonic marker for confirmation diagnosis of MSUD [6,7]. The ratio of tLeu/L-phenylalanine (Phe) or Val/Phe has also been used as a significant parameter for confirmation diagnosis of MSUD [5,8]. However, these methods are not sufficient to reduce the false-positive ratio that may misdiagnose other amino acid metabolic disorders as MSUD.

Diverse methods have been developed to diagnose MSUD by determining blood or dried blood spot BCAA levels, including bacterial inhibition assays (BIAs) [9], HPLC [10–12], capillary electrophoresis (CE) [13], GC [8], and tandem mass spectrometry (MS/MS) [14,15]. The HPLC method is commonly used for confirmation diagnosis of MSUD, but requires sample derivatization for high sensitivity and a long analysis time to separate Allo-Ile and Ile. Recently, the combination of LC and MS/MS for underivatized amino acid separation and detection has been developed. It is

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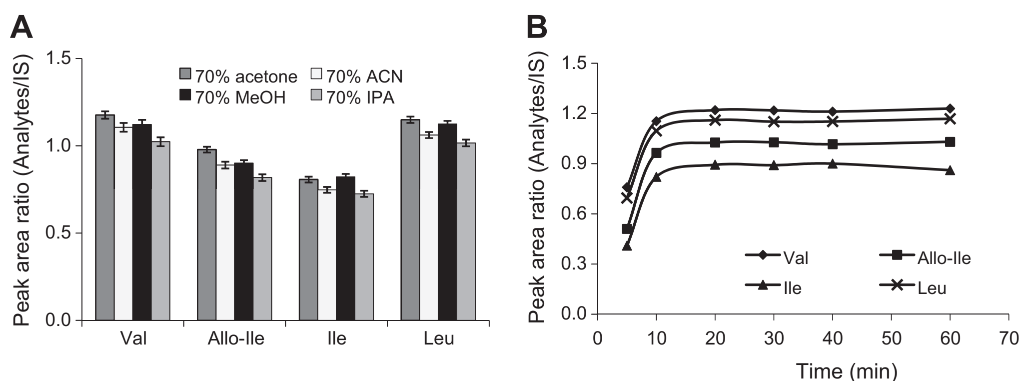

Fig. 1. Optimized conditions for BCAA extraction in dried blood spots. Comparison of the extraction efficiencies of BCAAs in dried blood spots according to the various organic solvents (A), and extraction time for BCAAs in dried blood spots using the optimal extraction condition of 70% acetone (B).

suitable for diagnosis of MSUD with clear separation of BCAAs [7]. Owing to its high throughput capability, the LC–MS/MS method is a powerful approach for determining BCAA levels; however, it requires extremely expensive initial implementation [16].

The present study introduces a practical application for both diagnosis and confirmation of MSUD patient by the ratio of Allo-Ile and Phe using a high-performance ligand-exchange chromatography (HPLEC)-UV method. Ligand-exchange chromatography is the most useful technique for D- and L-amino acid separation, and uses a chiral polymer column where amino acid residues are bound to metal anions [17,18].

To determine the extraction efficiency of BCAAs from dried blood spots, we determined the concentration of BCAAs and Phe, as well as the Allo-Ile/Phe ratio, with confirmed MSUD patient samples. To verify that the new method did not produce false positives, the BCAA and Phe concentrations, as well as the Allo-Ile/Phe ratio, were assessed in confirmed PKU and MSUD patient samples.

2. Materials and methods

2.1. Materials

Val, Allo-Ile, Ile, Leu, L-norleucine (Nle), and copper (II) sulfate ($CuSO_4$) were purchased from Sigma-Aldrich (Seoul, South Korea). Acetone and isopropyl alcohol (IPA) were purchased from J. T. Baker (Phillipsburg, NJ, USA). All other reagents and solvents used were of guaranteed or analytical grade. Water used to prepare solutions was purified using the Automatic Aquarius AW-1001 (Top Trading, Seoul, South Korea) and solvents were filtered through a Millipore membrane filter (type PTFE, pore size 0.45 μm, Billerica, MA, USA). Before injection, all samples were filtered through a disposable syringe filter (type PTFE, pore size 0.20 μm, Advantec MFS, Tokyo, Japan). Nle was used as an internal standard.

2.2. Standard blood spot specimens

Whole blood was collected in EDTA vials from a healthy male adult. Stock solutions containing different concentrations of BCAAs were prepared, and the final concentrations were 50, 150, 400 and 1000 μM in blood. Standard blood spots were produced by pipetting 50 μL of the blood mixtures onto filter paper (type 903; Schleicher & Schuell). Standard blood spots were dried overnight at room temperature and stored in a zippered plastic bag at −20 °C.

For Phe determination, certified standard blood spot containing Phe (74, 243, 414, and 873 μM) were purchased from Bio-Rad Diagnostics Group (Hercules, CA, USA).

2.3. Clinical samples

Dried blood spots collected from Korean newborn babies within 7 days of birth (150 normal, 11 PKU, 3 MSUD) were kindly provided by the Seoul Medical Science Institute (SMSI; Seoul, South Korea), a national depository of samples obtained from general hospitals. All samples were confirmed using BIA [9] or MS/MS [14] (from SMSI). Dried blood spots were sealed in a vinyl pack and stored at −20 °C before analysis.

2.4. Sample preparation

Two 3.2-mm (diameter) discs of dried blood spot and 5 μL of 200 μM Nle (internal standard) were placed in each well of a 96-well microtiter plate. To each well, 95 μL of 70% (v/v) acetone was added and the plates were sealed with adhesive film. Extraction was performed by gentle agitation on a shaker for 15 min. The samples were centrifuged for 10 min at 12,400 *g*. Eighty microliters of the supernatant were evaporated to dryness in a vacuum rotary evaporator. The residue was dissolved in 80 μL of water, filtered through a disposable syringe filter, and then injected into the HPLC system.

2.5. Chromatography

The HPLC equipment consisted of a Model Nanospace SI-2/3001 pump, 3002 UV/Vis Detector, 3004 column oven, and 3023 autosampler (Shiseido, Tokyo, Japan). Chromatographic separation was performed using a ligand-exchange column (Chirex 3126 (D)-penicillamine, 150 mm × 2 mm I.D.; Phenomenex, Torrance, CA, USA) connected to an in-line filter unit (0.2 μm pore size, Shiseido). The mobile phase was comprised of a mixture of 2 mM $CuSO_4$ and IPA (98:2). The flow-rate was 0.4 mL/min, the injection volume was 10 μL, and the column oven temperature was 35 °C.

3. Results

3.1. Sample preparation

To extract BCAAs from dried blood spots, 70% solutions of acetone, acetonitrile, methanol, and IPA were tested [19]. Among these, 70% acetone showed the best extraction value with stable reproducibility (Fig. 1A). The extraction of BCAAs from the dried blood spot was optimal at 15 min (Fig. 1B).

3.2. Separation

Using a mixture of 2 mM $CuSO_4$ and IPA (98:2, v/v) as a mobile phase, four BCAAs and Nle (internal standard) were completely

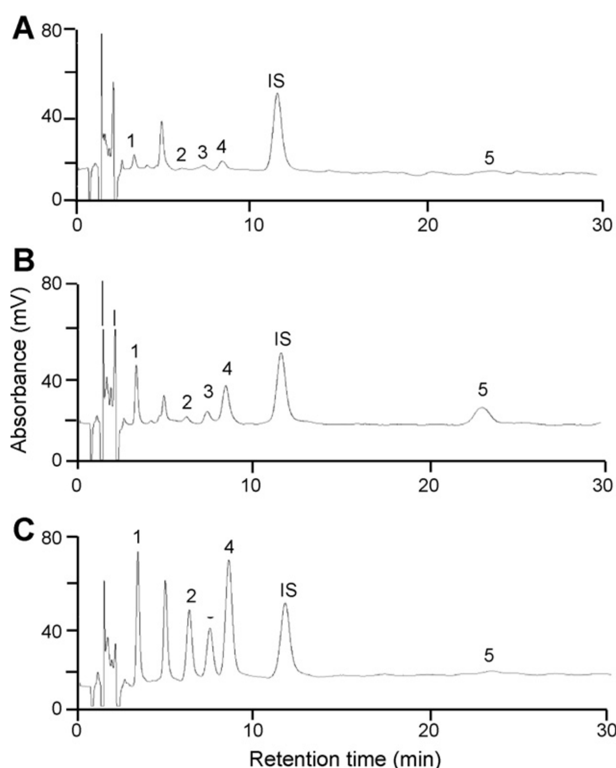

Fig. 2. Typical chromatograms obtained from dried blood spot specimens. Normal (A), PKU (B), and MSUD (C) patient dried blood spots. Peaks: (1) Val; (2) Allo-Ile; (3) Ile; (4) Leu; (5) Phe and IS, internal standard (Nle).

separated in isocratic elution within 15 min. This mobile phase condition also completely separated the four BCAAs and Nle from 40 free amino acids (standard mixture), Phe, and other unknown peaks in the blood (chromatograms shown in Supplementary material). Fig. 2 shows the typical chromatograms of normal (A), PKU (B), and MSUD (C) samples.

With the exception of LC–MS/MS for Allo-Ile determination, current methods to determine blood BCAA and Phe levels require specific derivatization steps. This study reports a newly developed HPLEC-UV method to determine the BCAA, Allo-Ile, and Phe levels of MSUD patient samples without any derivatization steps.

3.3. Validation

A calibration curve was generated using standard blood spots containing 4 different BCAA concentrations (50, 100, 400, and 1000 μM). The calibration curve of the peak area ratio (analyte/IS) versus analyte concentration was linear from 50 to 1000 μM. A calibration curve for Phe on standard blood spot was also generated, with a range of 74–873 μM. The final concentration ranges for an unknown injected sample were 3.13–62.5 μM for BCAAs, and 4.63–54.7 μM for Phe.

The equations and correlation coefficients (r^2) of calibration curves for BCAAs and Phe on standard blood spot were as follows: Val, $y = 0.0005x + 0.0128$, $r^2 = 0.9994$; Allo-Ile, $y = 0.0005x + 0.0041$, $r^2 = 0.9996$; Ile, $y = 0.0004x - 0.0049$, $r^2 = 0.9994$; Leu, $y = 0.0005x + 0.0077$, $r^2 = 0.9982$; Phe, $y = 0.0012x - 0.0821$, $r^2 = 0.9992$. The mean SDs for the slope and intercept were 0.0001 and 0.0055, respectively ($n = 6$). The limits of detection (LOD) and limits of quantification were as follows: Val, 0.86 and 2.16 μM; Allo-Ile, 0.76 and 1.91 μM; Ile, 1.14 and 2.29 μM; Leu, 1.14 and 2.29 μM; Phe, 1.21 and 3.03 μM, respectively. The HPLEC-UV method was relatively sensitive compared to other methods (according to the Allo-Ile LOD values:

Table 1
BCAA and Phe concentrations determined by use of the LEC-UV method, and the percentages of false-positive and flase-negative of healthy control newborns, PKU-positives and MSUD-positives.

	n	Val			Ile			Leu			Allo-Ile			Phe		
		Mean (range)	False-positive[b]	False-negative[c]	Mean (range)	False-positive	False-negative	Mean (range)	False-positive	False-negative	Mean (range)	False-positive	False-negative	Mean (range)	False-positive	False-negative
Controls	150	101 (3–351)	0%	–	43 (ND[d]–84)	0%	–	151 (36–224)	0%	–	1 (ND–3)	0%	–	63 (2–200)	1%	–
PKU	11	474 (385–567)	45%	–	187 (95–400)	73%	–	547 (415–825)	100%	–	6 (ND–13)	64%	–	459 (249–673)	–	0%
MSUD	3	614 (401–940)	–	33%	358 (101–668)	–	33%	958 (429–1856)	–	0%	238 (137–304)	–	0%	131 (92–188)	33%	–
Cut-off value (μM)[a]		480			105			230			5			182		

[a] Cut-off value: cut-off value for MSUD-positive of Val, Ile, Leu, Allo-Ile, and cut-off value for PKU-positive of Phe.
[b] False-positive percentage when use the cut-off value for MSUD and PKU diagnosis.
[c] False-negative percentage when use the cut-off value for MSUD and PKU diagnosis.
[d] ND, not detected.

Table 2
Concentration ratios of tLeu/Phe, Val/Phe and Allo-Ile/Phe measured in PKU and MSUD patient dried blood spot samples.

Samples	tLeu/Phe	Val/Phe	Allo-Ile/Phe
MSUD			
1	11.6	4.4	1.5
2	10.4	4.5	2.4
3	22.0	7.3	2.4
PKU			
1	0.9	0.7	0.01
2	2.0	1.3	0.01
3	2.4	1.8	ND[a]
4	1.4	1.0	ND[a]
5	3.8	1.8	0.02
6	1.2	0.7	0.02
7	1.5	1.0	0.02
8	1.9	1.2	0.04
9	2.0	1.2	0.01
10	1.4	0.9	ND[a]
11	1.0	0.6	0.01

[a] ND, not detected.

HPLEC-UV, 0.76 μM; CE, 0.014 μM; GC–MS, 1.8 μM; MS/MS, 2.0 μM; and LC–MS/MS, 1.0 μM).

The precision and accuracy of intra- and inter-day assays for BCAAs were determined using five different concentrations of standard blood spots (0; blank, 50, 100, 400, and 1000 μM) in five independent experiments on five consecutive days. Mean recoveries and RSDs of the intra-day assay were 89.0–102.7% and 0.7–6.0%, and those of the inter-day assay were 85.8–103.2% and 0.6–8.1%, respectively (data not shown).

3.4. Application to clinical samples

Table 1 shows the concentration values of BCAAs and Phe levels for clinical samples (150 normal, 11 PKU and 3 MSUD). The false-positive and false-negative indicate the percentages of samples which should be under or over the cut-off values of BCAAs for confirmation diagnosis of MSUD (Val, 480 μM; Ile, 105 μM; Leu, 230 μM; Allo-Ile, 5 μM) and Phe for confirmation diagnosis of PKU (Phe, 180 μM) [5,6]. No normal sample showed a BCAA level above the BCAA cut-off value. Among PKU patients ($n = 11$), 6/11 (for Val level), 8/11 (for Ile level), 11/11 (for Leu level), or 6/11 (for Allo-Ile level) samples were above the cut-off value. Among MSUD patients ($n = 3$), 2/3 (for Val level), 2/3 (for Ile level), 3/3 (for Leu level), or 3/3 (for Allo-Ile level) samples were above the cut-off value. PKU-positive patients have elevated BCAA levels as a result of liver damage, and may be misdiagnosed as MSUD-positive if only the BCAA cut-off values are assessed. To avoid misdiagnosis, current methods use the ratio of tLeu/Phe or Val/Phe for confirmation diagnosis of MSUD [5,8]. To confirm the diagnosis of MSUD, we compared the ratios of tLeu/Phe, Val/Phe, and Allo-Ile/Phe in PKU and MSUD patient samples (Table 2), which were 0.9–3.8, 0.7–1.8, and ND–0.04 for PKU respectively, and 10.4–22.0, 4.4–7.3, and 1.5–2.4 for MSUD, respectively. The lowest tLeu/Phe ratio (10.4) for MSUD was 2.7 times higher than the highest tLeu/Phe ratio (3.8) for PKU. Similarly, the ratio ranges of Val/Phe were 0.6–1.8 and 4.4–7.3 for PKU and MSUD patient dried blood spots, respectively. The lowest Val/Phe ratio (4.4) for MSUD was 2.4 times higher than the Val/Phe ratio for PKU. Thus, neither tLeu/Phe nor Val/Phe displayed a large enough difference to clearly distinguish between the two disorders, highlighting the difficulty in diagnosing MSUD using previously available methods. Until now, Allo-Ile has been recognized as a specific pathognomonic marker for confirmation diagnosis of MSUD, but has not been usable because of the difficulty in derivatizing or separating Allo-Ile from other BCAAs in the classical HPLC-UV approach. Through the HPLEC-UV method described here, Allo-Ile and Phe were successfully separated from other BCAAs. This allowed the determination of the Allo-Ile/Phe ratio, which ranged from ND–0.04 and 1.5–2.4 for PKU and MSUD patient samples, respectively. All ratios were >1.5 for MSUD samples, while all values were <0.04 for PKU samples. The lowest ratio (1.5) for the MSUD samples was 37.5 times higher than the highest ratio (0.04) of the PKU samples. Due to the rareness of MSUD, it was difficult to obtain MSUD-positive samples. Therefore, although only a few MSUD samples were measured, the results from the experimental data show that the Allo-Ile/Phe ratio was sufficient enough to distinguish MSUD from PKU without increasing the false-positive rate.

4. Conclusions

A HPLEC-UV method for confirmation diagnosis of MSUD was developed that relies on the determination of BCAA and Phe levels in dried blood spot. The ligand-exchange column suitable for underivatized isobaric amino acids displayed good performance on simultaneous analysis of Allo-Ile, Ile and Leu in dried blood spot samples with a relatively simple sample preparation. Since the Allo-Ile is used clinically as a specific pathological MSUD marker, the clear separation of BCAAs is an important issue. Compared to other diagnostic parameters such as cut-off values of BCAA, tLeu/Phe and Val/Phe, the value of Allo-Ile/Phe ratio displayed a large enough difference to clearly distinguish between MSUD and PKU.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.05.023.

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