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Diagnosis of maple syrup urine disease by determination of L-valine, L-isoleucine, L-leucine and L-phenylalanine in neonatal blood spots by gas chromatography–mass spectrometry

Chunhui Deng[a,*], Yonghui Deng[b]

[a]*Department of Chemistry, Fudan University, Shanghai 200433, China*
[b]*Department of Macromolecules, Fudan University, Shanghai 200433, China*

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Abstract

A novel method was developed for the diagnosis of maple syrup urine disease (MSUD) by the determination of L-valine, L-leucine, L-isoleucine and L-phenylalanine in dried blood spots of newborns by gas chromatography–mass spectrometry (GC–MS). The four amino acids were extracted from blood samples by methanol and derivatized by *n*-butanol and trifluroacetic anhydride under optimum reaction conditions. The corresponding single derivatives of the four amino acids were obtained under the optimum conditions. Their contents in blood samples were analyzed quantitatively by measurement of their derivatives by GC–MS in selected ion monitoring mode. MSUD can be diagnosed on the basis of the ratio of the total content of L-leucine and L-isoleucine to that of L-phenylalanine. The present method only took a short time to perform and required minimal sample preparation, which provided low detection limits and a relative standard deviation of less than 5.0%. The derivatization reactions of the four amino acids, L-valine, L-isoleucine, L-leucine and L-phenylalanine, with *n*-butanol and trifluroacetic anhydride were investigated and the optimum reaction conditions, including reaction time and temperature, were obtained and used for the determination of the amino acids in plasma samples.

Keywords: Maple syrup urine disease; Valine; Isoleucine; Leucine; Phenylalanine

1. Introduction

When there is a lack of enzymes associated with an amino acid metabolic pathway, amino acids and their precursors abnormally accumulate in the body. As a result, amino acid concentrations increase in blood and urine. If this is a genetic defect, it causes major intellectual disturbance in humans. If a neonatal diagnosis is made, appropriate treatment can be given. Therefore, it is important to develop a simple and accurate method to determine amino acids in biological samples [1]. Numerous screening methods have been published to determine amino acids in blood or urine, including the bacterial inhibition assay (BIA), high-performance liquid chromatography (HPLC), micellar electrokinetic chromatography and fluorometric assay, ion-exchange chromatography and tandem mass spectrometry [2–9]. Recently, a gas chromatography–mass spectrometry (GC–MS) method was developed for the determination of amino acids in blood and urine, and was

*Corresponding author. Fax: +86-21-6564-3140.
E-mail address: dengchunhui@163.com (C. Deng).

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applied to screening for inborn errors of metabolism (IEM) [10–15]. In our previous studies, a GC–MS method was developed for the diagnosis of phenylketonuria by quantitative analysis of phenylalanine and tyrosine in neonatal blood samples [16,17].

Maple syrup urine disease (MSUD) is an autosomal recessive disorder of the metabolism of the branched-chain amino acids (BCAA) L-leucine, L-isoleucine and L-valine and their corresponding branched-chain keto acids (BCKAS). The disorder is caused by a severe deficiency in the activity of the branched chain α-keto acid dehydrogenase complex (BCKD; EC 1.2.4.1) [18]. Recently, Tavares et al. found that extracellular glutamate levels may be increased in MSUD and that excitotoxicity may be involved in the neuropathology of this disorder [19]. It was also found that cytoskeletal disorganization may be one of the factors associated with the neurodegeneration characteristic of MSUD disease [20]. A marked increase of serum and urine concentrations of BCAAs and BCKAs is the biochemical hallmark of the disorder. Patients with MSUD predominantly present severe neurological symptoms, including psychomotor delay or mental retardation, hypotonia, lethargy, coma and generalized convulsions. Although the pathophysiology of the neurologic dysfunctions of MSUD is poorly known, there is a large body of evidence associating the defective leucine metabolism and the neurologic symptoms of these patients [21,22]. MSUD is diagnosed by the determination of L-leucine, L-isoleucine and L-valine in neonatal blood. HPLC, enantioselective multidimensional capillary gas chromatography–mass spectrometry (enantio-MDGC–MS) and tandem mass spectrometry (MS–MS) have been applied to the diagnosis of MSUD [23–26]. MS–MS is the most powerful tool for the diagnosis of MSUD because of its high throughput and accuracy. It has become the gold standard for investigations of MSUD in developed countries. However, the MS–MS instrument is very expensive, and hospitals in developing countries cannot afford it. The price of a GC–MS instrument is much lower than that of the MS–MS instrument, therefore it is desirable to develop a GC–MS method for the diagnosis of MSUD.

Compared with other inherited metabolic diseases such as PKU, MSUD is a rare genetic disorder with an incidence of about 1:80 000 in China. In general, the HPLC method is used for the diagnosis of MSUD. However, the HPLC method requires complex sample preparation and a long analysis time. In this work, we developed a GC–MS system with derivatization reagents *n*-butanol and trifluroacetic anhydride for the diagnosis of MSUD. L-Valine, L-leucine, L-isoleucine and L-phenylalanine in blood spots were extracted by 0.1% HCl–methanol and derivatized by *n*-butanol and trifluroacetic anhydride. The four amino acids were determined by measuring the peak areas of their corresponding single derivatives. The ratio of the total content of leucine and isoleucine to that of L-phenylalanine was used for the diagnosis of MSUD. Derivatization reactions of the four amino acids were investigated and the optimum conditions, including reaction temperature and time, were obtained.

2. Materials and methods

2.1. Chemicals, standards and samples

All chemicals were of analytical grade or better. Trifluroacetic anhydride and *n*-butanol were obtained from Merck. L-Phenylalanine, L-valine, L-leucine and L-isoleucine were obtained from Sigma. Standard and GC calibration solutions spanning the concentration range for the four amino acids from 10.0 to 1000.0 μmol L^{-1} were prepared by dissolving the amino acids in water. A standard solution in water with a concentration of 50 μmol L^{-1} of each amino acid was prepared and stored at −10 °C until used for investigation of the derivatization reactions of the four amino acids with *n*-butanol and trifluroacetic anhydride.

Dried blood samples of newborns were obtained from Fuzhou Hospital (Jiangxi Province, China).

2.2. Investigation of the derivatization reaction of L-phenylalanine, L-valine, L-leucine and L-isoleucine with n-butanol and trifluroacetic anhydride

A volume of 100 μL of a solution of the four amino acids (50.0 μmol L^{-1}) was placed in a 1 mL vial, and the solvent evaporated under a stream of N_2 at 40 °C. The residue was reacted with 100 μL

n-butanol at 80, 100, 120 and 150 °C with reaction times of 20, 30, 40 and 60 min at each temperature. After evaporation of the solvent to dryness under a stream of N_2 at 40 °C, the butyl derivatives were reacted with 100 μL of a mixture of trifluroacetic anhydride and acetonitrile (1:1, v/v) at 100 °C for 30 min. The derivatives were evaporated to dryness under nitrogen at 40 °C and redissolved in 100 μL methanol.

2.3. Derivatization of standards and samples

Dried blood spots on filter paper were prepared by punching out an 8.0-mm diameter circle into a 1-mL vial with a standard paper punch. This corresponded to 20 μL of whole blood. A volume of 200 μL 0.1% HCl–methanol was added to the vial at 4 °C for 60 min and then centrifuged at 15 000 *g* for 20 min. A volume of 100 μL of the supernatant fluid was transferred to a 1 mL vial, and evaporated to dryness under a N_2 stream at 40 °C. The residue was reacted with 100 μL of 3 *M* HCl–*n*-butanol at 120 °C for 30 min. After the solvent was evaporated to dryness under nitrogen, the butyl esters of the amino acids were derivatized by trifluroacetic anhydride at 100 °C for 30 min. Finally, the derivatives were evaporated to dryness under a N_2 stream at 40 °C and redissolved in 100 μL methanol.

GC calibration solutions of the four amino acids from 10.0 to 1000 μmol L^{-1} were prepared by dissolving the amino acids in water. A volume of 100 μL calibration solution spanning the concentration range from 10.0 to 1000 μmol L^{-1} was placed in a 1 mL vial, and the solvent evaporated under a N_2 stream at 40 °C. The same procedure for derivatization and preparation was followed as described above.

2.4. Gas chromatography–mass spectrometry

A Finnigan Voyager gas chromatograph–mass spectrometer (GC–MS) was used in 70 eV EI mode. Analytes were separated using a HP-5MS capillary column of 30 m×0.25 mm with a phase thickness of 0.25 μm from Supelco, which was inserted directly into the ion source of the MS. A volume of 1 μL of the sample was injected in the splitless mode and the oven temperature program was as follows: initial temperature 50 °C for 2 min, which was increased to 300 °C at 15 °C min^{-1}, and 300 °C was maintained for 10 min. Helium (99.999%) carrier gas at a flow-rate of 1 mL min^{-1} was used. The detector was set at a temperature of 280 °C. The qualitative analysis was carried out under full-scan acquisition mode within the 41–500 a.m.u. range. Quantification was operated in the selected ion monitoring (SIM) mode. Selected ion: *m*/*z* 148 and 91 for L-phenylalanine, *m*/*z* 166 and 153 for L-valine, *m*/*z* 182 and 140 for L-leucine, and *m*/*z* 182 and 153 for L-isoleucine.

2.5. Recovery and precision

Ten microliter standard solutions (10, 50, 200 μmol L^{-1}) were added to three blood samples. Derivatization was carried out under the optimum conditions. Recoveries were obtained by comparing their real values with those calculated by the external standard method. Precision of the assay was calculated by replicate analyses of the same blood sample using the complete analytical procedure.

3. Results and discussion

L-Valine, L-leucine, L-isoleucine and L-phenylalanine were reacted with *n*-butanol and the butyl esters of the amino acids were derivatized by trifluroacetic anhydride. The free hydroxyl and amino groups were modified by *n*-butanol and trifluroacetic anhydride, respectively. The second step of the reaction under the reaction conditions of 100 °C and 30 min is very rapid and complete [16], therefore the first step of the reaction is a bottleneck for the derivatization of the four amino acids. The optimum reaction conditions for the four amino acids with *n*-butanol were determined from the sum of the peak areas of their derivatives obtained under different butyl ester reaction conditions and the same acetyl reaction conditions. The results in Fig. 1 show that the acetyl reaction conditions of 120 °C and 30 min are optimum.

The total ion chromatogram of neonatal blood spots is shown in Fig. 2. The retention times of the L-valine, L-leucine, L-isoleucine and L-phenylalanine derivatives were 8.241, 8.983, 9.086 and 11.579 min,

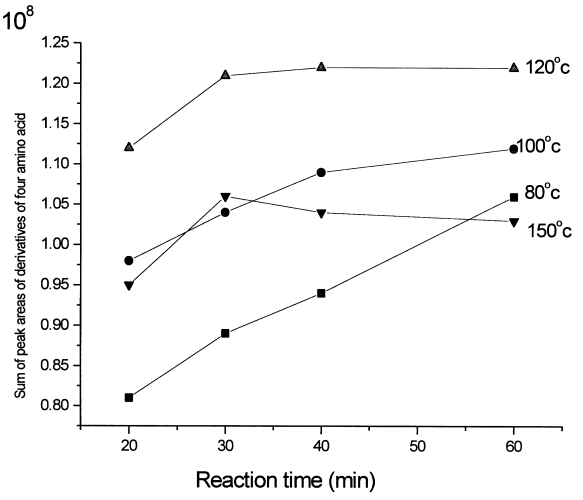

Fig. 1. Effect of reaction temperature and time on the sum of the peak areas of the amino acid derivatives.

respectively. Single derivatives of L-valine, L-leucine, L-isoleucine and L-phenylalanine were obtained under the optimum reaction conditions of 120 °C and 30 min for the butyl ester reaction and 100 °C and 30 min for the acetyl reaction. To our knowledge, SIM can improve the sensitivity of analysis of amino acid derivatives.

The EI mass spectra of L-valine, L-leucine, L-isoleucine and L-phenylalanine derivatives are shown in Fig. 3. L-Valine, L-leucine and L-isoleucine derivatives produced a fragment peak $[M-COOC_4H_9]^+$ at m/z 168 for L-valine derivative and at m/z 182 for both L-leucine and L-isoleucine derivatives. The fragment ions at m/z 140 $[CF_3CONHCHCH_3]^+$ and 182 for leucine, at m/z 153 $[CF_3CONHCHCHCH_3]^+$ and 182 for isoleucine, and at m/z 153 $[CF_3CONHCHCHCH_3]^+$ and 168 for valine were used for SIM experiments. The fragment ions at m/z 91 $[C_6H_5CH_2]^+$ and 148 $[C_6H_5CH_2CH_2NHCO]^+$ for L-phenylalanine were used for SIM experiments. The characteristic ions at m/z 168, 153 for L-valine, m/z 182, 140 for L-leucine, m/z 91, 148 for L-phenylalanine and m/z 182, 153 for L-isoleucine were used for the SIM experiments to determine the four amino acids in neonatal blood spots.

The four amino acids in blood samples were modified with *n*-butanol and trifluroacetic anhydride. The derivatization reactions were rapid, complete, and led to a single product, which was demonstrated by the analysis of the reaction products of standard amino acids by GC–MS and HPLC [16,17]. The derivatives of the four amino acids provided excellent sensitivity for the detection of L-valine, L-leucine, L-isoleucine and L-phenylalanine in blood samples by GC–MS. SIM was used to determine the sensitivity and detection limits for the analysis of the derivatives. The fragment ions at m/z 148, 91 for L-phenylalanine, m/z 168, 153 for L-valine, m/z 182, 140 for L-leucine and m/z 182, 153 for L-isoleucine were selected for the SIM experiment. Fig. 4 shows the SIM (m/z 91, 140, 153, 168, 182) chromatogram of the blood sample.

A calibration curve at concentrations of 10.0 to 1000 μmol L^{-1} for each of the four amino acids was constructed. The regression lines and the equations for each amino acid tested showed an excellent relationship between the signal (select ion peak area, y) and amino acid concentration (x, μmol L^{-1}) (Table 1). The detection limits of the amino acids were 1.8, 2.5, 2.8 and 3.3 μmol L^{-1}, respectively. The detection limits were below the physiologically normal ranges for L-valine, L-leucine, L-isoleucine and L-phenylalanine.

The analytical recoveries of the amino acids from blood were determined in triplicate at concentrations of 10, 50 and 200 μmol L^{-1}. The respective mean values obtained were 97, 95, 93 and 104% at 10 μmol L^{-1}, 97, 102, 91 and 99% at 50 μmol L^{-1} and 101, 94, 102 and 104% at 200 μmol L^{-1}.

Precision of the assay was calculated by replicate analyses of the same blood sample by the complete analytical procedure for blood spots described in Materials and methods. The relative standard deviation (RSD) values representing the within-assay variation were 3.4% for valine, 3.6% for leucine, 4.1% for isoleucine, 4.6% for phenylalanine, and 3.9% for the (leucine + isoleucine)/phenylalanine ratio ($n=5$). The calibration curves for valine, leucine, isoleucine and phenylalanine determined for the same sample on different occasions within 1 month, representing the inter-assay variation, were 2.4, 2.8, 3.3 and 3.8%, respectively ($n=6$). The absolute concentrations of amino acids were 145, 141, 168 and 189 μmol L^{-1}, respectively.

The four amino acids in blood samples were determined by the peak areas of their selected ions

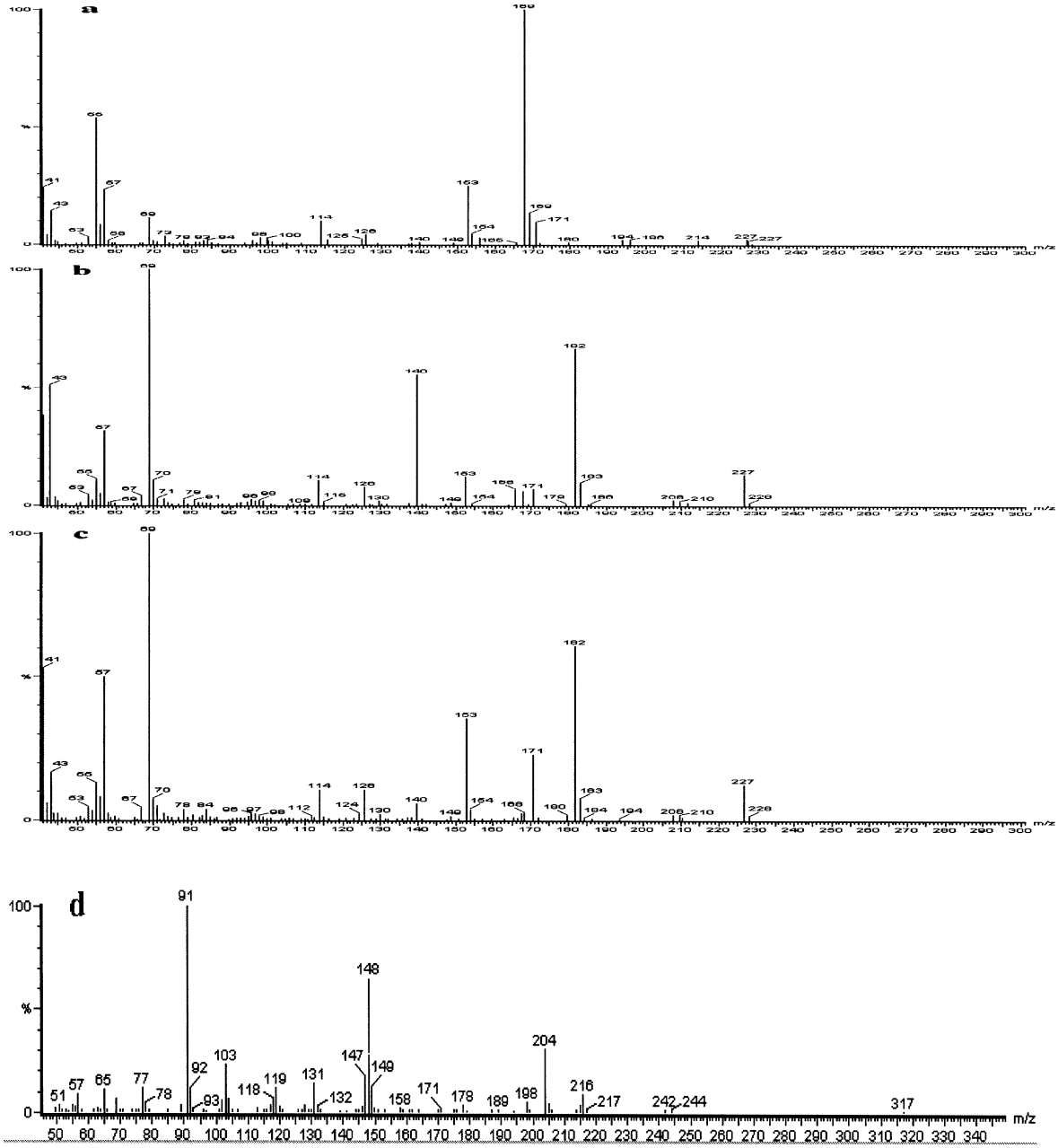

Fig. 2. Mass spectra of the derivatives of L-valine (a), L-leucine (b), L-isoleucine (c) and L-phenylalanine (d).

on the basis of the calibration curve for each amino acid with the external standard method. The result of the quantitative analysis of amino acids in neonatal blood samples is shown in Table 2. The present result for MSUD-positive patients of significantly higher L-leucine, L-isoleucine and L-valine levels is consistent with reports using other techniques for screening and for diagnostic confirmation, such as HPLC and tandem mass spectrometry. Chace et al. demonstrated that the ratio of the L-leucine and

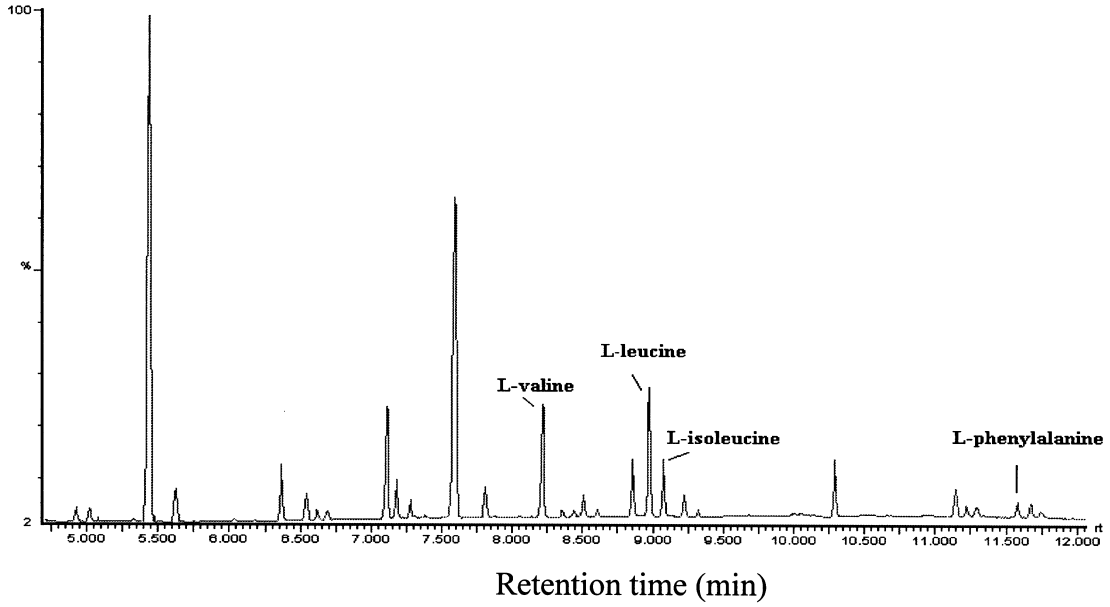

Fig. 3. Total ion chromatograms of neonatal blood spots.

L-isoleucine content to L-phenylalanine could be applied to the diagnosis of MSUD [22]. The results in Table 2 show that the ratio of the total content of L-leucine and L-isoleucine to L-phenylalanine in patients with MSUD is more than 8.0, while the ratio in normal blood is less than 4.0, which is similar to the results obtained by MS–MS [22]. It was found that the ratio of L-valine to L-phenylalanine in

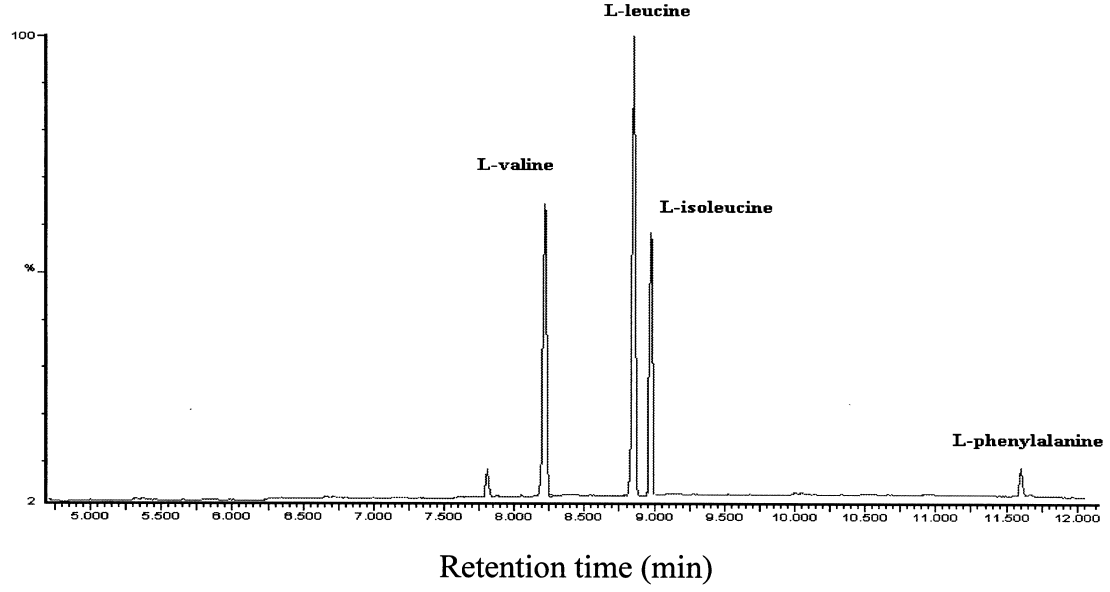

Fig. 4. SIM (m/z 91, 140, 148, 153, 168, 182) chromatograms of neonatal blood spots.

Table 1
Regression lines and equations for the quantitative analysis of the four amino acids

Amino acid	Equation	R^2
L-Valine	$y = 5.83 \cdot 10^5 x - 4.33 \cdot 10^4$	0.998
L-Leucine	$y = 5.14 \cdot 10^5 x + 3.95 \cdot 10^3$	0.994
L-Isoleucine	$y = 4.96 \cdot 10^5 x + 5.12 \cdot 10^4$	0.988
L-Phenylalanine	$y = 4.79 \cdot 10^5 x - 2.60 \cdot 10^4$	0.997

patients with MSUD is more than 2.0, while the ratio in normal blood is less than 1.5. This shows that the ratio of L-valine to L-phenylalanine can also be used for the diagnosis of MSUD.

The BIA method is an excellent screening technique and the cost of the method to screen for MSUD is much less than for other methods, but it is semiquantitative and readily yields false-positive results for MSUD. MS–MS has excellent resolving power [22] and is able to measure several amino acids at once. The ratio of the total content of leucine and isoleucine to that of phenylalanine in neonatal blood was used for the diagnosis of MSUD, therefore it can reduce false-positive results. However, the MS–MS instrument is too expensive for low-budget hospitals, and is not widely applied for the screening of MSUD in developing countries. Conversely, GC–MS is a simple, rapid and less-expensive technique with a high resolving power [16,17]. The present method has a sensitivity of 1.8 μmol L^{-1} for L-valine, 2.5 μmol L^{-1} for leucine, 2.8 μmol L^{-1} for isoleucine and 3.3 μmol L^{-1} for phenylalanine. With this method, MSUD is diagnosed on the basis of the ratio of the contents of leucine and isoleucine to that of phenylalanine in a blood sample and a reduction in false-positive results was achieved.

4. Conclusions

The four amino acids L-valine, L-leucine, L-isoleucine and L-phenylalanine in blood samples were modified by *n*-butanol and trifluroacetic anhydride under optimum reaction conditions, and the corresponding single derivatives of the four amino acids were obtained. L-Valine, L-leucine, L-isoleucine and L-phenylalanine were further determined by measuring their derivatives by GC–MS in the SIM mode, which was used to improve the detection limits and sensitivity. The ratio of the total contents of L-leucine and L-isoleucine to L-phenylalanine was used for the diagnosis of MSUD. In summary, the present method for the quantitative analysis of the four amino acids in neonatal blood samples is simple and sensitive, which makes it very suitable for screening for MSUD.

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Table 2
Contents of L-valine, L-leucine, L-isoleucine and L-phenylalanine in neonatal blood spots

Sample	L-Valine (μmol L^{-1})	L-Leucine (μmol L^{-1})	L-Isoleucine (μmol L^{-1})	L-Phenylalanine (μmol L^{-1})	(Leucine + isoleucine)/ phenylalanine	Valine/ phenylalanine
MSUD						
1	546.2	989.8	828.3	128.9	14.20	4.22
2	486.1	869.0	918.9	201.4	8.89	2.43
3	466.5	912.9	1095.8	224.9	8.96	2.07
Control						
1	261.5	208.6	147.3	189.4	3.26	1.38
2	215.5	198.3	204.6	231.5	1.74	0.92
3	169.3	190.8	246.8	158.5	2.77	1.06
4	208.6	217.1	187.3	223.2	1.81	0.93
5	179.4	298.6	156.2	141.5	3.22	1.27
6	123.8	243.6	109.8	194.6	1.81	0.63

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