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Short communication

Rapid diagnosis of phenylketonuria and other aminoacidemias by quantitative analysis of amino acids in neonatal blood spots by gas chromatography–mass spectrometry

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Abstract

A new method for quantifying specific amino acids in small volumes of plasma and whole blood has been developed. Volatile derivatives of amino acids are analyzed by gas chromatography–mass spectrometry. The method only takes a few minutes to perform and requires minimal sample preparation. The accurate assay of phenylalanine, tyrosine and other amino acids in dried blood spots could be used for neonatal screening for phenylketonuria and other aminoacidemias. Because of the low cost, this neonatal screening method is suited to application in developing countries such as China. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Derivatization, GC; Amino acids

1. Introduction

Phenylketonuria (PKU; McKusick 261600), a fairly common autosomal recessive disease, usually caused by a deficiency of phenylalanine hydroxylase (PHA; E.C. 1.14.16.1) [1]. The normal catabolism of phenylalanine (Phe) in mammals requires its initial conversion to tyrosine (Tyr) in the liver. The enzyme defect leads to a specific pattern of plasma amino acids with increased Phe at normal or decreased Tyr [2]. Newborn screening for PKU relies on the detection of Phe in the filter paper blood specimens obtained prior to discharge. In general, PKU was screened by a bacterial inhibition assay (BIA), which allowed for the easy, rapid screening of elevated blood phenylalanine levels collected on newborn filter paper samples. Since that time, others methods have been used to screen PKU, including gas–liquid chromatography [3], high-performance liquid chromatography [4], ion-exchange chromatography [5], gene diagnosis by capillary electrophoresis [6], fluorometry [7], micellar electrokinetic chromatography and laser-induced fluorescence detection [8], restriction enzyme analysis [9]. Recently, amino acid profiling by electrospray-tandem mass spectrometry (ESI-MS–MS) has been reported as a powerful diagnostic tool in patients with PKU. Chace et al. demonstrated the utility of ESI–TMS in the detection of early discharge neonates and the reduction of false positive samples achieved through higher accuracy of measurements of Phe and Tyr concentrations in

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patients with PKU [10,11]. Because MS–MS is the most advanced, comprehensive technique, it is now possible to detect over 30 inherited metabolic disorders, including PKU, maple syrup urine disease (MSUD), hyperphenylalaninemias (HPA), in time to begin effective management [12–18].

Gas chromatography–mass spectrometry (GC–MS) has been used for analyzing organic acids in urine for chemical diagnosis of metabolic disorders of organic acids since 1966 because it assures high accuracy and sensitivity as well as providing information about multiple compounds simultaneously [19]. Since Tanaka discovered isovaleric academia using GC–MS, many metabolic disorders of organic acids have been found [20,21], and the usefulness of GC–MS has been widely approved [22–24]. Screening of PKU has also been described using GC–MS analyses of *N*-acetyl derivatives in urine [25]. At present, GC–MS is an indispensable method for diagnosing inborn errors of metabolism, and is widely recognized for its effectiveness in related fields [26].

Zoomzely and Gehrke reported that 20 protein amino acids in normal blood modified by *n*-butanol and trifluoroacetic anhydride were quantitatively analyzed by GC–FID [26,27]. In this article we developed and applied GC–MS for the detection of five amino acids with aminoacidemias in newborn blood. The five amino acids, L-phenylalanine, L-tyrosine, L-proline, L-leucine and L-valine were modified by *n*-butanol and trifluoroacetic anhydride. They were determined by measuring select ion peak area of their derivatives. On basis of content of amino acids, we are able to diagnose PKU, MSUD and other aminoacidemias.

2. Materials and methods

2.1. Chemicals, standards and samples

All chemicals were of analytical grade or better. The esterifying agent, 3 *M* HCl, was prepared in *n*-butanol (Merck, Darmstadt, Germany). Trifluoroacetic anhydride was obtained from Merck. L-Phenylalanine (Phe), L-tyrosine (Tyr), L-proline (Pro), L-leucine (Leu) and L-valine (Val) were obtained from Sigma. Standard and GC calibration solutions spanning the concentration range for every amino acid from 5.0 to 160.0 μ*M* were made by appropriate dissolving amino acid in water.

Dried blood samples from newborns were obtained from the neonatal screening center of XinHua Hospital (Shanghai, PR China).

2.2. Derivation of standards and samples

Dried blood spots on filter paper were prepared by punching out a 8.0-mm diameter circle into a 1-ml vial with a standard paper punch. It 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. It was then centrifuged at 15 000 *g* for 20 min. A volume of 100 μl of supernatant fluid was transferred to a 1-ml vial, evaporated to dryness under a N_2 stream at 40 °C. The residue was reacted with 3 *M* HCl–*n*-butanol at 100 °C for 30 min. After the solvent was evaporated to dryness under nitrogen, the butyl ester of amino acids was derivatized with trifluoroacetic anhydride at 100 °C for 30 min. Finally, the derivatives was evaporated to dryness under a N_2 stream at 40 °C, then redissolved with 100 μl methanol.

A volume of 100 μl of GC calibration solutions of amino acids spanning the concentration range from 5.0 to 160 μ*M* were placed in a 1-ml vial and the solvent evaporated. The procedure for derivation and preparation as described above was followed.

2.3. GC–MS

A Finnigan Voyager gas chromatograph–mass spectrometer (GC–MS) was used in the EI mode. Analytes were separated using an HP-5MS capillary column of 30 m×0.25 mm with a phase thickness of 0.25 μm from Superlco, 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 70 °C for 2 min, 1 min splitless time, to 300 °C at 15 °C min^{-1}, 300 °C was maintained for 10 min. Helium (99.999%) carrier gas had a flow-rate of 1 ml min^{-1}. The detector was set at the 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 oper-

ated in the SIM mode. Selection ions: 91 for Phe, 166 for Pro, 168 for Val, 182 for Lev and 203 for Tyr.

3. Results

Amino acids in blood sample were reacted with 3 *M* HCl–*n*-butanol, then the butyl ester derivatives were reacted with trifluoroacetic anhydride to convert the free amino group to corresponding *n*-acetyl derivative. The two reactions were rapid, complete, and led to a single product. The final derivatives of the amino acids provided excellent sensitivity for the detection of amino acids in blood by GC–MS. The retention times of Val, Leu, Pro, Phe and Tyr derivatives were 6.0, 6.7, 7.7, 9.7 and 10.5 min, respectively. SIM was used to determine the sensitivity and detection limits for the analysis of these derivatives.

The EI mass spectra of Val, Pro, Leu, Phe and Tyr derivatives with *n*-butanol and trifluoroacetic anhydride is shown in Fig. 1. The fragment ions at m/z 166, 168, and 182 corresponding to the loss of butylester from the molecule ion of Pro, Val and Leu derivatives ($[M-COOC_4H_9]^+$) were selected for the SIM experiment. The fragment ion m/z 91 $[C_6H_5CH_2]^+$ and m/z 203 $[CF_3COC_6H_4CH_2]^+$, characteristic ions of Phe and Tyr derivatives, respectively, were used for quantitative analysis. The SIM (m/z 91, 166, 168, 182 and 203) chromatogram of blood sample is shown in Fig. 2.

A calibration curve at concentrations of 5.0–160 μ*M* for each of these five amino acids was achieved. The regression lines and the equations for each amino acid tested showed an excellent relationship between the signal (selected ion peak area, *y*) and amino acid concentration (*x*, μ*M*) (Table 1).

The detection limits of Phe, Tyr, Val, Pro and Leu were 1.5, 0.8, 1.9, 2.4 and 1.6 μ*M*, respectively. The detection limits were below the physiologically normal ranges for Phe, Tyr, Val, Pro and Leu.

The analytical recoveries of added Phe, Tyr, Pro, Val and Leu from blood were determined in triplicate at concentrations of 10, 40 and 160 μ*M*. The respective mean values obtained were 98, 96, 104, 102 and 95% at 10 μ*M*; 96, 94, 106, 102 and 110% at 40 μ*M*; 102, 103, 97, 105 and 106% at 160 μ*M*.

The precision of the assay was calculated by replicate analysis of the same blood sample by the complete analytical procedure for blood spots described in Material and Methods. These RSD values representing the within-assay variation, were 5.6% for Phe, 2.3% for Tyr, 3.7% for Val, 5.1% for Pro, 4.6% for Leu and 4.1% for Phe:Tyr ratio ($n=10$). The calibration curves for Phe, Tyr, Val, Pro and Leu determined for the same sample on different occasions within 1 month, representing the inter-assay variation, were 3.6, 2.8, 4.8, 7.9 and 9.4%, respectively ($n=8$). The absolute concentrations of Phe, Tyr, Val, Pro and Leu were 119, 204, 172, 106 and 132 μ*M*, respectively.

4. Discussion

Phe, Tyr, Val, Pro and Leu in blood samples were calculated by peak area of their selected ions on basis of calibration curve of each amino acid with exterior standard (Table 2). The present result in PKU positive patients: Phe and Phe:Tyr molar significantly higher are consistent with other extensively used techniques for screening and for diagnoses confirmation, such as the bacterial inhibition assay, fluorometric assay, enzymatic assay and, recently, tandem mass spectrometry.

The BIA method is an excellent screening technique and the cost of the method for screening for PKU is much lower than that of other methods, but it is semiquantitative and a false positive rate of up to 5%. Fluorometric assay provide a sensitivity of 30 μ*M* and a false positive rate of 0.6%. Ion-exchange assay is lengthy and complex and requires expensive dedicated equipment and specially trained personnel. MS–MS has excellent resolution power. It is good at measuring several amino acids at once, which has a sensitivity of 3 μ*M* for Phe and 10 μ*M* Tyr, and a sensitivity of 100% with 99.9% specificity and a significantly reduced repeat analysis rate. However, since the MS–MS instrument is too expensive for low budget hospitals, it is not widely applied to diagnosis aminoacidemias including PKU and MSUD in developing countries. Conversely, GC–MS is simple, rapid, less expensive and a high resolution power, which can measure several amino acids at once. The analysis time of amino acids by

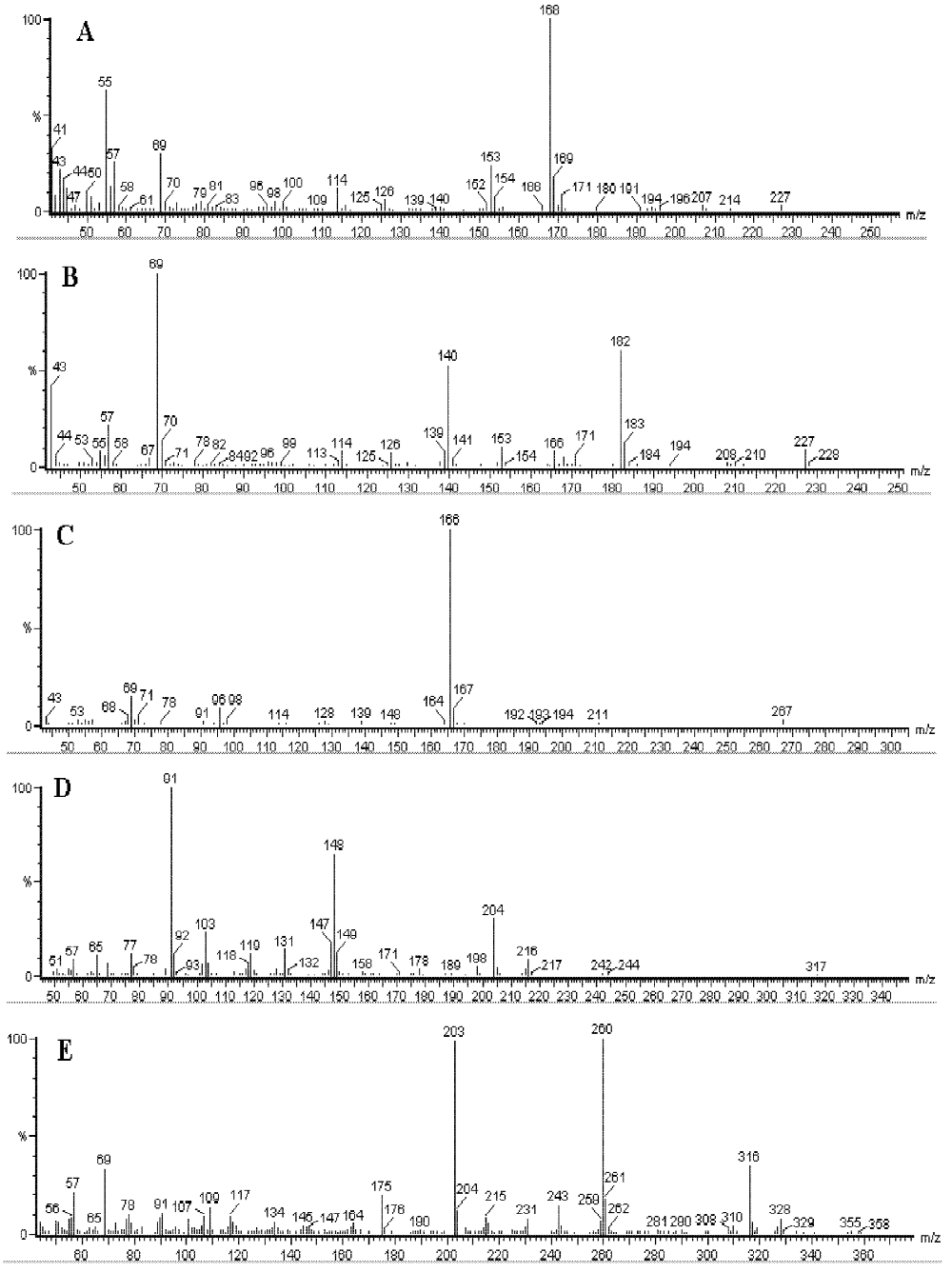

Fig. 1. EI mass spectra of Val (A), Leu (B), Pro (C), Phe (D) and Tyr (E) derivatives.

GC–MS is very short (11 min) and GC–MS has a sensitivity of 1.5 μ*M* for Phe.

In summary, these results demonstrate that GC–MS can be a reliable method for detecting PKU in neonatal blood samples. In addition to PKU, the new method might be expected detect other disorders of

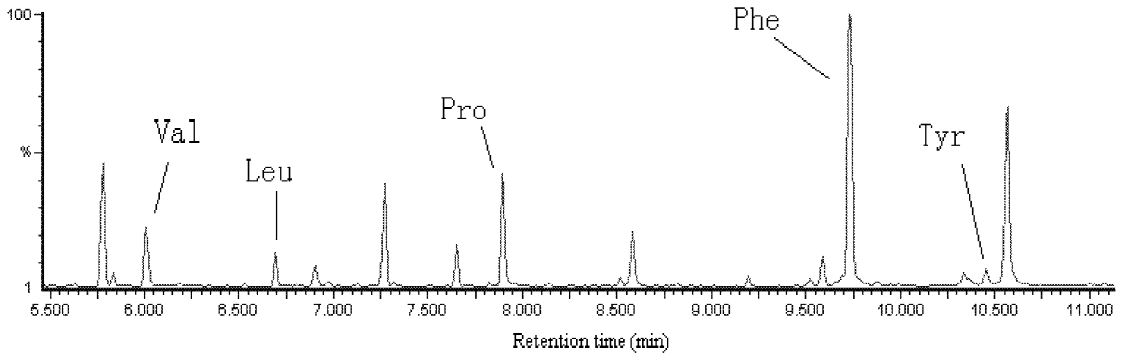

Fig. 2. SIM (m/z 91, 166, 168, 182, 203) chromatogram of blood sample.

Table 1
Regression lines and the equations for each amino acid tested

Amino acids	Equations[a]	R^2
Phe	$y = 6.4 \cdot 10^5 x - 6.5 \cdot 10^3$	0.998
Tyr	$y = 4.8 \cdot 10^5 x + 1.8 \cdot 10^4$	0.998
Pro	$y = 4.4 \cdot 10^5 x - 3.4 \cdot 10^4$	0.996
Val	$y = 4.0 \cdot 10^5 x - 7.3 \cdot 10^3$	0.999
Leu	$y = 4.5 \cdot 10^5 x + 3.2 \cdot 10^4$	0.991

[a] y, Selected ion peak area; x, amino acid concentration (μM).

amino acid metabolism such as maple syrup urine disease (MSUD) and tyrosine. The diagnosis of MSUD can be done on basis of determination of branched amino acids such as Leu and Val in blood by this method. In MUSD blood samples, aliphatic amino acids are present at higher levels than aromatic amino acids and the ratio is >2. Diagnosis of MSUD can be done on basis of the ratio of aliphatic amino acids to aromatic amino acids. Because of low cost of the GC–MS instrument, this method is suited to screen PKU and other disorders of amino acid metabolism in developing countries.

Table 2
Quantitative analysis for Phe, Tyr, Val, Pro and Leu in blood spots by GC–MS

Sample	Val (μM)	Pro (μM)	Leu (μM)	Phe (μM)	Tyr (μM)	Phe:Tyr
Control group						
1	98	102	73	209	261	0.80
2	88	67	52	78	199	0.39
3	104	76	107	177	209	0.84
4	138	164	126	156	224	0.69
5	131	49	87	107	219	0.49
PKU positive						
1	122	108	66	859	234	3.67
2	81	218	102	571	106	5.38
2	115	245	93	683	226	3.02
4	107	212	161	1023	208	4.92
MUSD						
1	356	452	667	113	203	0.54
2	541	284	643	98	217	0.43

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