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Gas chromatography–mass spectrometry method for determination of phenylalanine and tyrosine in neonatal blood spots

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Abstract

In this paper we developed a simple, rapid and sensitive method for the quantitative analysis of phenylalanine (Phe) and tyrosine (Tyr) in dried blood spots of newborns by gas chromatography–mass spectrometry (GC–MS). Phe and Tyr in blood samples were reacted with *N*-methyl-*N*-(*tert.*-butyldimethylsilyl)trifluoroacetamide at 120 °C for 30 min and their corresponding single derivatives were obtained. Phe and Tyr were determined by measurement of their derivatives by GC–MS in the selected ion monitoring mode. Contents of Phe and Tyr in blood spots were calculated by external standard method. The ratio of Phe to Tyr was used for neonatal screening for phenylketonuria. The present method only took a few minutes to perform and required minimal sample preparation. In addition it provided low detection limits of 1.2 μmol l^{-1} for Phe and 1.6 μmol $l^{-1}$for Tyr.

Keywords: Phenylalanine; Tyrosine

1. Introduction

Phenylketonuria (PKU) is a fairly common autosomal recessive disease, usually caused by a deficiency of phenylalanine hydroxylase [1–3]. 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. 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 Phe levels collected on newborn filter paper samples [4]. Since that time, high-performance liquid chromatography (HPLC), micellar electrokinetic chromatography and laser-induced fluorescence, flurometry and ion-exchange chromatography were used for screening neonatal PKU [5–14]. Recently, tandem mass spectrometry (MS–MS) has been reported as a powerful diagnostic tool in patients with PKU [15–18]. Chace and Millington [18] demonstrated that the reduction of false positive samples was achieved through higher accuracy of measurements of Phe and Tyr concentrations in patients with PKU.

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Gas chromatography–mass spectrometry (GC–MS) is a simple and rapid technique with high-power resolution, which has been applied to screening for inborn errors of metabolism (IEM) including PKU by simultaneous determination of amino acids and organic acids in urine [19,20]. Kaiser et al. [21] and Zoomezely et al. [22] reported that 20 protein amino acids in normal blood modified by *n*-butanol and trifluroacetic anhydride were analyzed by GC–flame ionization detection (FID). In our previous study, five amino acids with aminoacidemias in blood samples of patients modified by *n*-butanol and trifluroacetic anhydride were determined by GC–MS [23]. However the derivatization reactions included two steps (esterification+acylation), which were time-consuming and tedious. Recently bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and *N*-methyl-*N*-(*tert*.-butyldimethylsilyl)trifluoroacetamide (MTBSFA) were introduced to modify amino acids, which made amino and carboxyl groups of amino acids simultaneous silylated in a single step. BSTFA was used to modify amino acids and organic acids in urine in clinical diagnosis [19,20]. MTBSFA could provide more stable and single amino acid derivatives under more convenient reaction conditions than BSTFA. Molnar-Perl and Katona investigated derivatization reactions of protein amino acids with three different silylation agents and the fragmentation patterns of the derivatives were achieved [24]. However the optimum derivatization conditions for the amino acids were not obtained.

In this paper, derivatization reactions of 10 protein amino acids including Phe and Tyr with MTBSFA were investigated and the optimum derivatization temperature and time were obtained. Phe and Tyr in blood samples were modified by MTBSTFA under the optimum derivatization conditions of 120 °C and 30 min. They were determined by measuring selected ion peak areas of their derivatives. PKU was diagnosed on basis of content ratio of Phe to Tyr in neonatal blood spots.

2. Materials and methods

2.1. Chemicals, standards and samples

All chemicals were of analytical grade or better. MBSTFA was obtained from Merck. L-Phenylaline (Phe), L-tyrosine (Tyr), L-alanine (Ala), L-glycine (Gly), L-methionine (Met), L-serine (Ser), L-threonine (Thr), L-asparagine (Asp), L-glutamine (Gln) and L-glutmic acid (Glu) were obtained from Sigma. Standard and GC calibration solutions spanning the concentration range for Phe and Tyr from 5.0 to 160.0 μmol l^{-1} were made by dissolving the appropriate amino acid in water. Standard solutions with a concentration of 40 μmol l^{-1} for each amino acid were prepared for the determination of the optimum derivatization temperature and time.

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

2.2. Derivatization conditions for 10 protein amino acids with MTBSTFA

A 100-μl volume solution of 10 amino acids (40.0 μmol l^{-1}) was fortified to 1 ml, and the solvent evaporated under an N_2 stream at 40 °C. The residue was reacted with 100 μl of an MBSTFA–acetonitrile (ACN) (1:1, v/v) mixture at 60, 120 and 160 °C with reaction times of 15, 30 and 60 min at each temperature. After the solvent was evaporated to dryness under nitrogen, the derivatives were redissolved with 100 μl methanol.

2.3. Derivation 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. It corresponded to 20 μl of whole blood. A 200-μl volume of 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 100-μl volume of supernatant fluid was transferred to a 1-ml vial and evaporated to dryness under an N_2 stream at 40 °C. The residue was reacted with 100 μl of an MBSTFA–ACN (1:1, v/v) mixture at 120 °C for 30 min. After the solvent was evaporated to dryness under nitrogen, the derivatives were redissolved with 100 μl methanol.

A 100-μl volume each of GC calibration solutions of Phe an Tyr spanning the concentration range from 5.0 to 160 μmol l^{-1} was fortified to 1 ml vial, and the solvent evaporated. The same procedure for derivation and preparation was followed as described above.

2.4. Gas chromatography–mass spectrometry

A Finnigan Voyager GC–MS system was used in the electron impact (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 system. A 1-μl volume of the sample was injected in the splitless mode and the oven temperature program was as follows: initial temperature 70 °C for 2 min, which was increased 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 a temperature of 280 °C. The qualitative analysis was carried out under the full-scan acquisition mode within the 41–500 u range. Quantification was carried out in the selected ion monitoring (SIM) mode. Selected ions were m/z 336 for Phe and 466 for Tyr.

3. Results and discussion

The total ion chromatogram of 10 protein amino acids derivatives obtained at reaction temperatures of 60, 120 and 160 °C with the same reaction time of 30 min is shown in Fig. 1. The retention times of Ala, Gly, Met, Ser, Thr, Phe, Asp, Glu, Gln and Tyr derivatives are 10.4, 10.5, 13.6, 13.7, 13.9, 14.4, 14.8, 15.5, 16.4, 17.6 min, respectively. Single derivatives of Met, Ser, Thr, Phe, Asp were obtained under reaction temperature of 160 °C with a reaction time of 30 min. While other amino acids could not

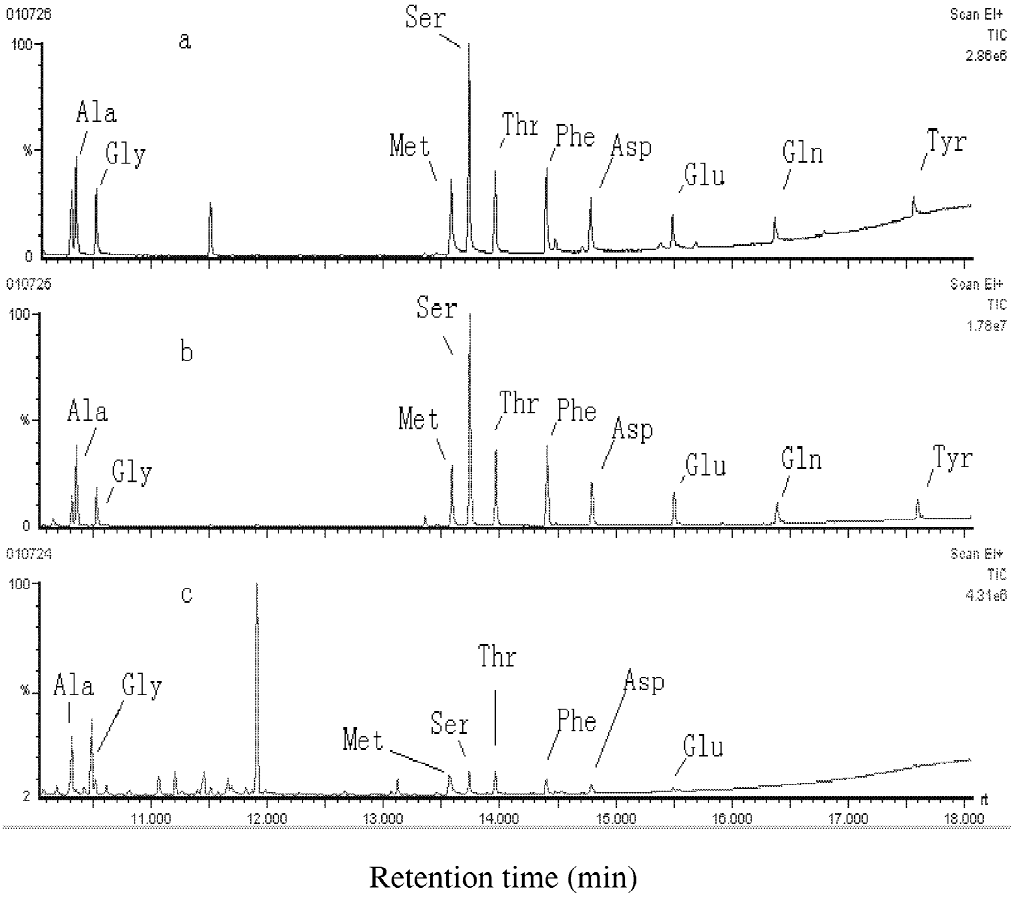

Fig. 1. The total ion chromatogram of 10 amino acid derivatives obtained at three reaction temperatures (a, 60 °C; b, 120 °C; c, 160 °C) with the same reaction time of 30 min.

Table 1
Peak areas of amino acids derivatives obtained at reaction temperature of 120 and 60 °C with the same reaction time of 30 min

Amino acid	Peak area		Ratios of peak areas (120/60 °C)
	60 °C	120 °C	
Ala	2 098 747	11 318 130	5.6
Gly	1 783 690	55 393 245	30.5
Met	2 333 070	10 241 384	4.3
Thr	2 238 958	12 991 126	6.6
Phe	2 096 185	12 680 043	63
Asp	1 331 467	7 130 559	55
Glu	1 055 492	5 339 558	53
Gln	772 449	4 360 620	5.7
Ser	4 955 223	32 395 214	5.9
Tyr	674 274	3 601 604	5.4

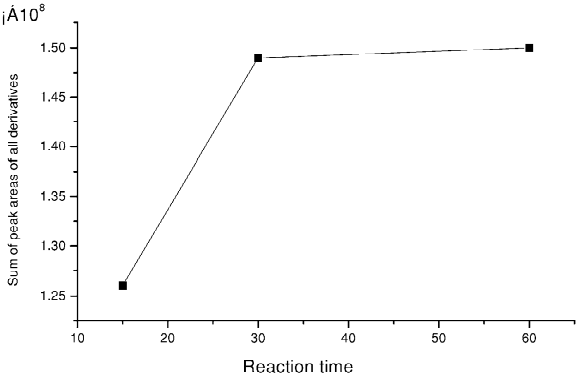

Fig. 2. Effect of reaction time on sum of peak areas of all 10 amino acid derivatives.

produce single derivatives under the same reaction conditions, single derivatives of all 10 amino acid could be obtained at 120 or 60 °C with a reaction time of 30 min. Peak areas of each derivative obtained at the two different reaction temperatures are shown in Table 1. The ratios of peak areas of each derivative obtained at 120 °C to that of 60 °C showed that a larger amount of each derivative could be produced at 120 °C, which suggested that a reaction temperature of 120 °C was optimum. The optimum reaction time was found by varying the derivatization reaction time from 15 to 60 min with the same reaction temperature of 120 °C. Fig. 2 shows the results in graphic form, expressed as the sum of the peak areas of all 10 amino acid derivatives obtained at 120 °C with reaction times of 15, 30 and 60 min. It showed that the amino acids were completely modified by MTBSTFA in 30 min. The optimum derivatization conditions (120 °C, 30 min) for amino acids were obtained and used to modify Phe and Tyr in blood spots.

The EI mass spectrometric data of Ala, Gly, Met, Ser, Thr, Phe, Asp, Glu, Gln and Tyr derivatives are shown in Table 2. All amino acid derivatives could produce a fragment peak $[M–C_4H_9]^+$ such as m/z 336 for the Phe derivative and m/z 466 for the Tyr derivative. These characteristic ions could be used for the quantitative analysis of amino acids in blood and urine. Characteristic ions of m/z 336 for Phe and 466 for Tyr were used to determine Phe and Tyr in neonatal blood spots.

Phe and Tyr in blood samples were modified with MTBSTFA at 120 °C for 30 min. The derivatization

Table 2
EI mass spectrometric data of 10 protein amino acids derivatives

Amino acid	Base peak (m/z)	$[M–C_4H_9]^+$ (m/z)	Other fragment ion (m/z)
Ala	73	246	147, 158, 232
Gly	73	260	147, 218
Met	73	320	147, 218, 292
Thr	73	404	303
Ser	73	390	147, 288, 362
Phe	73	336	147, 234, 302
Asp	73	418	147, 302
Glu	73	432	147, 272, 330
Gln	73	431	147, 198
Tyr	73	466	302

reactions were rapid, complete, and led to a single product, which was proved by analysis of the reaction products of standard Phe and Tyr with MTBSTFA by GC–MS and HPLC. The MTBSTFA derivatives of Phe and Tyr could provide excellent sensitivity for the detection of Tyr and Phe in blood by GC–MS. SIM was used to determine the sensitivity and detection limits for the analysis of their derivatives. The fragment ions at m/z 336, 466, corresponding to the loss of *tert*.-butyl from the molecule ion of Phe and Tyr derivatives ($[M-C_4H_9]^+$) were selected for the SIM experiment. Fig. 3 shows the total ion chromatogram and SIM (m/z 336, 466) chromatogram of a blood sample.

A calibration curve at concentrations of 5.0 to 160 μmol l^{-1} for each of the two 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, μmol l^{-1}). For Phe $y=734\,681x-56\,281$, $r^2=0.9947$; for Tyr $y=573\,026x+32\,640$, $r^2=0.9912$. The detection limits of Phe and Tyr were 1.2 and 1.6 μmol l^{-1}, respectively. The detection limits were below the physiologically normal ranges for Phe and Tyr.

The analytical recoveries of added Phe and Tyr from blood were determined in triplicate at concentrations of 10, 40, 160 μmol l^{-1}. The respective mean values obtained were 95 and 92% at 10 μmol l^{-1}; 93 and 97% at 40 μmol l^{-1}; 105 and 91% at 160 μmol l^{-1}.

Precision of the assay was calculated by replicate analysis of the same blood sample by the complete analytical procedure for blood spots described in Materials and Methods. These relative standard deviation (RSD) values representing the within-assay variation, were 7.2% for Phe, 2.7% for Tyr and 4.6% for the Phe/Tyr ratio ($n=5$). The calibration curves for Phe and Tyr determined for the same sample on different occasions within 1 month, representing the inter-assay variation, were 3.9 and 2.2%, respectively ($n=6$). The absolute concentrations of Phe and Tyr were 139 and 154 μmol l^{-1}, respectively.

Phe and Tyr in blood samples were calculated by peak area of their selected ions on the basis of the calibration curve of each amino acid with the external standard method. The results of the quantitative analysis for Phe and Tyr in neonatal blood sample are shown in Table 3. The present result in PKU positive patients: Phe and Phe/Tyr molar

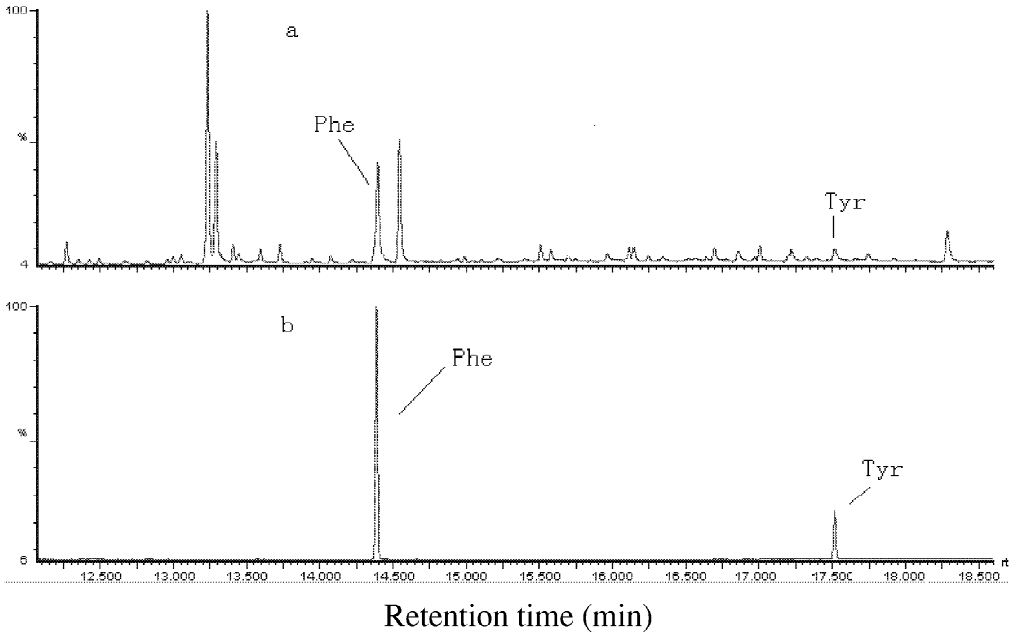

Fig. 3. The total ion chromatogram (a) and SIM chromatogram (b) of blood sample.

Table 3
Quantitative analysis for Phe and Tyr in neonatal blood spots by GC–MS

Sample	Phe (μmol l^{-1})	Tyr (μmol l^{-1})	Phe/Tyr
PKU1	741	109	6.8
PKU2	1092	154	7.0
PKU3	353	108	3.2
PKU4	785	186	4.2
Control (1)	129	167	0.78
Control (2)	187	212	0.89
Control (3)	176	195	0.90
Control (4)	97	154	0.63
Tyrosinemia (1)	153	549	0.28
Tyrosinemia (2)	148	672	0.22

significantly higher were consistent with what has been reported with other extensively used techniques for screening and for diagnoses confirmation, such as the bacterial inhibition assay, flurometric assay, enzymatic assay and recently tandem mass spectrometry. GC–MS combined with MTBSTFA could be applied to screening for neonatal PKU. Tyr in blood with higher tyrosinemia was consistent with that obtained by flurometric assay. So the method could be used for simultaneous diagnosis of PKU and tyrosinemia.

The main methods for the diagnosis of PKU included BIA, HPLC, MS–MS and ion-exchange chromatography. The BIA method is an excellent screening technique and the cost of the method to screen for PKU was much lower than that of other methods, but it is semiquantitative and had a false positive rate of up to 5% [4]. The BIA method is still the primary method for screening PKU in China. Fluorometric assay provided a sensitivity of 30 μmol l^{-1} and a false positive rate of 0.6% [5,6]. Ion-exchange assay is lengthy and complex and requires expensive dedicated equipment and specially trained personnel [14]. MS–MS has excellent resolution power [16,18]. It is effective in determining several amino acids at once, and had a sensitivity of 3 μmol l^{-1} for Phe and 10 μmol l^{-1} Tyr, and a sensitivity of 100% with 99.9% specificity and a significantly reduced repeat analysis rate. However MS–MS instrumentation is too expensive for low budget hospitals, it is not widely applied to the screening of PKU in developing countries. Conversely, GC–MS is a simple, rapid and less expensive technique with high-resolution power [19,20,23]. MTBSTFA was used to modify Phe and Tyr, which made the amino and carboxyl group simultaneously silylated in a single step, so the sample preparation was simple and required little time. The present method had a sensitivity of 1.2 μmol l^{-1} for Phe and 1.6 μmol $l^{-1}$for Tyr. In this method PKU was diagnosed on the basis of the ratio of Phe to Tyr in blood sample and reduction false positive samples was achieved.

4. Conclusion

The optimum reaction conditions (120 °C, 30 min) were used to modify Phe and Tyr in blood samples, and the derivatization reactions were rapid, complete and single derivatives could be obtained. Phe and Tyr could be further determined by measuring their corresponding derivatives. The SIM mode was used to improve the detection limits and sensitivity. The present method for the quantitative analysis of Phe and Tyr in neonatal blood sample is simple, rapid and sensitive, which makes it suitable for simultaneous screening for PKU and tyrosinemia.

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