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USE OF BIOMARKERS IN DIAGNOSIS BY ISOTOPIC DILUTION AND GAS-CHROMATOGRAPHY -MASS SPECTROMETRY

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Abstract
Small volumes of 20 µl of plasma or blood spots were used for neonatal blood screening for diagnosis of phenylketonuria and other metabolic diseases. The blood samples were derivatized as trifluoroacetylbutyl esters and analyzed by gas chromatography coupled with mass spectrometry in the SIM mode. Regression curves for some standard amino acids are used for quantitative determination of valine, leucine, proline, phenylalanine and tyrosine. The method is a rapid tool for diagnosis of some neonatal diseases. Samples were separated on a Rtx-5MS capillary column, 30 m x 0.25 mm, 0.25µm film thickness, using a temperature program from 50^{o}C(1min), then 6^{o}C/min to 100 ^{o}C, 4o^{o}C/min to 200 ^{o}C, 20^{o}C/min to 300 ^{o}C in scan mode or 50^{o}C(1min), then 20^{o}C/min to 310 ^{o}C in the SIM mode. The following conditions were followed: transfer line temperature: 250^{o}C, injector temperature:200 ^{o}C; ion source temperature 250 ^{o}C; Splitter: 10:1. Electron energy was 70eV and emission current, 100µA. Quantitative determination of the amino acids of interest was made in the SIM mode by selecting the highest ions of the mass spectrum and the m/z 155 for the internal standard, ^{15}N-labelled glycine
Keywords: biomarker, diagnosis, amino acids.

1.Introduction

Gas chromatography-mass spectrometry (GC-MS) is an indispensable method for diagnosing inborn error of metabolism and is widely recognized for its effectiveness in related fields [1]. Phenylketonuria (PKU) is a metabolic disease usually caused by phenylalanine hydroxylase deficiency which causes an increase of phenylalanine in plasma and a decreased tyrosine, a specific pattern of blood amino acids. The normal catabolism of phenylalanine in mammals requires its conversion in tyrosine in liver. Maple syrup urine disease (MSUD), other metabolic disease, could be diagnosed by some branched amino acids determination in blood [2]. Newborn amino acids determination by gas chromatography–mass spectrometry is a useful method for diagnosis of inborn errors in metabolism [3]. PKU is general screened by a bacterial inhibition assay (BIA) of elevate blood phenyalanine levels on newborn filter paper samples of

blood specimens. There are many chromatographic methods for screening PKU, but also mass spectrometry as electrospray mass spectrometry, ESI-MS-MS.

A rapid method by profiling some amino acids and their quantitative determination, a minim invasive method, using 20 µl of blood was adapted. Volatile derivatives of amino acids are analyzed in very small volumes of plasma or whole blood by using filter paper blood specimens and SIM-GC-MS technique. PKU relies on the amino acid profiling by mass spectrometry detection of phenylalanine. Neonatal screening for phenylketonuria and other aminoacidemia by GC-MS is low cost method [1].

2. Method and samples

Chemicals and samples

Acetyl chloride was purchased from Fluka, trifluoroacetic anhydride was obtained from Merck (Darmstadt, Germany), ion exchange resin Dowex 50W-X8 50–100 mesh was purchased from Fluka. Amino acids standards were purchased from Sigma.

Derivatization

Amino acids in blood samples or standard samples were derivatized as trifluoroacetyl butyl ester derivative**.** Derivatization was followed in two steps, in screw-cap tubes. Dry samples were esterified with 0.1 ml butanol:acetyl chloride, 4:1, v/v) for 30 min at 100 °C. The excess reagent was removed with a stream of nitrogen. The amino group was acetylated with 100 µl trifluoacetic anhydride (TFAA) at 100 °C for 30 min. After cooling, the excess reagent was removed under nitrogen at ice temperature and ethyl acetate was added.

The method is useful for diagnosis of metabolic diseases as PKU by determination of phenylalanine (Phe) and tyrosine (Tyr) in blood and maple syrup urine disease (MSUD) by determination of valine (Val), leucine (Leu) and proline (Pro) in blood. The analysis of the five amino acids in blood samples by GC-MS is useful in the diagnosis of the both diseases.

Apparatus

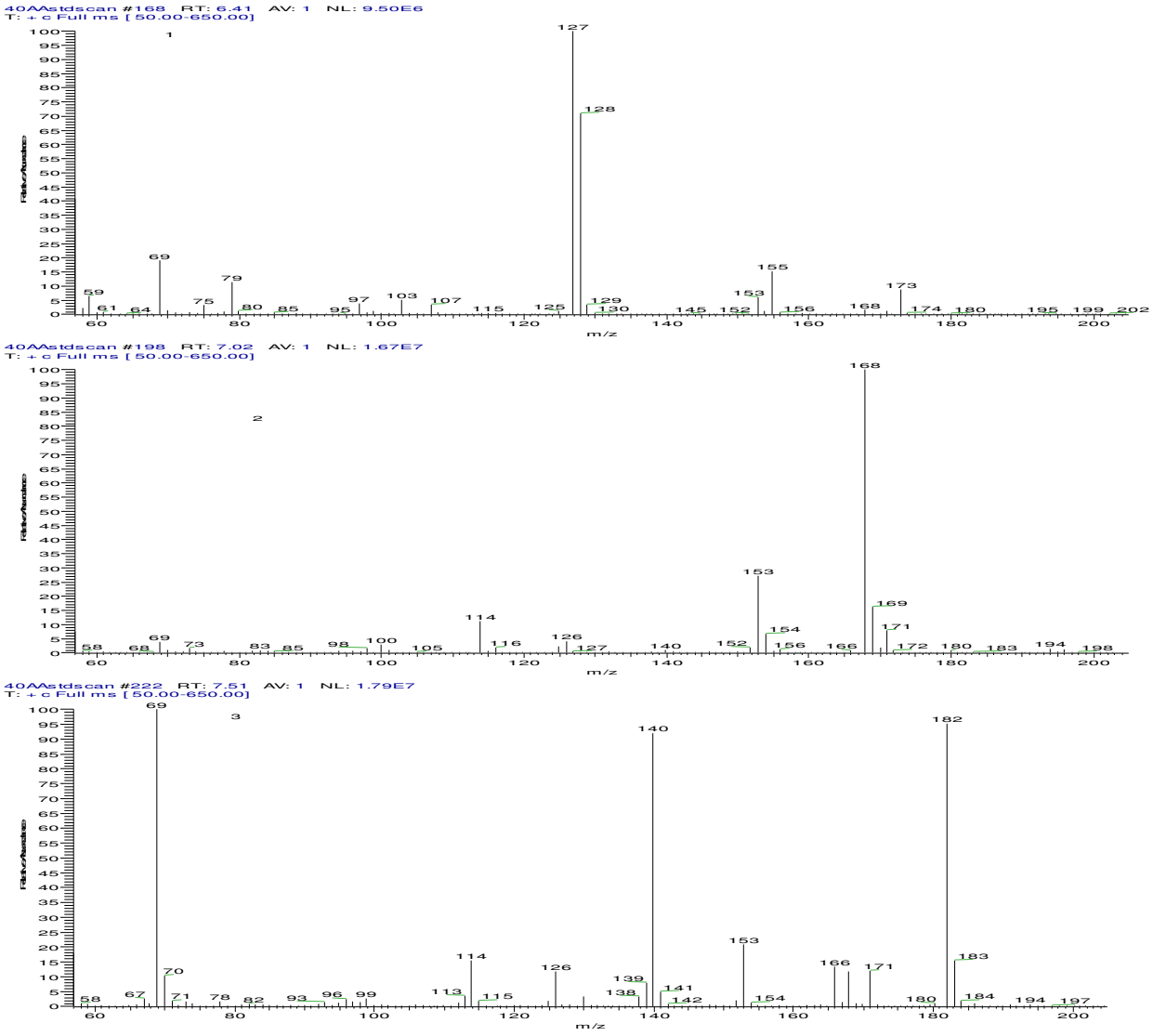
A Trace DSQ ThermoFinnigan quadruple mass spectrometer in the EI mode coupled with a Trace GC was used. The capillary column Rtx-5MS was of 30 m length x 0.25 mm, 0.25µm film thickness, by using a temperature program from 50^{o}C (1 min), then increased to 310^{o}C, at 20 ^{o}C/min, in the selected ion monitoring mode (SIM). Helium (99.9995%) carrier gas had a flow rate of 1 ml/min. The qualitative analysis was carried out in the mass range 50-500 a.m.u.

Quantitative analysis was performed in the SIM mode by using the ions selected from the trifluoroacetyl butyl ester derivatives mass spectra: m/z 155 for ^{15}N-glycine (^{15}N-Gly), used as internal standard, m/z 168 for valine, m/z 182 for leucine and isoleucine, m/z 166 for proline, m/z 91 and 148 for phenylalanine and m/z 203, 260, 316 for tyrosine or m/z 107, 164, 220 for chloroacetyl tyrosine (Fig 1). 20µg/ml of the internal standard was added at each sample.

Written informed consents were obtained from each subject parent prior to this study.

3. Results and Discussions

The mass spectra of the amino acids used for profiling determination are presented in fig. 1.



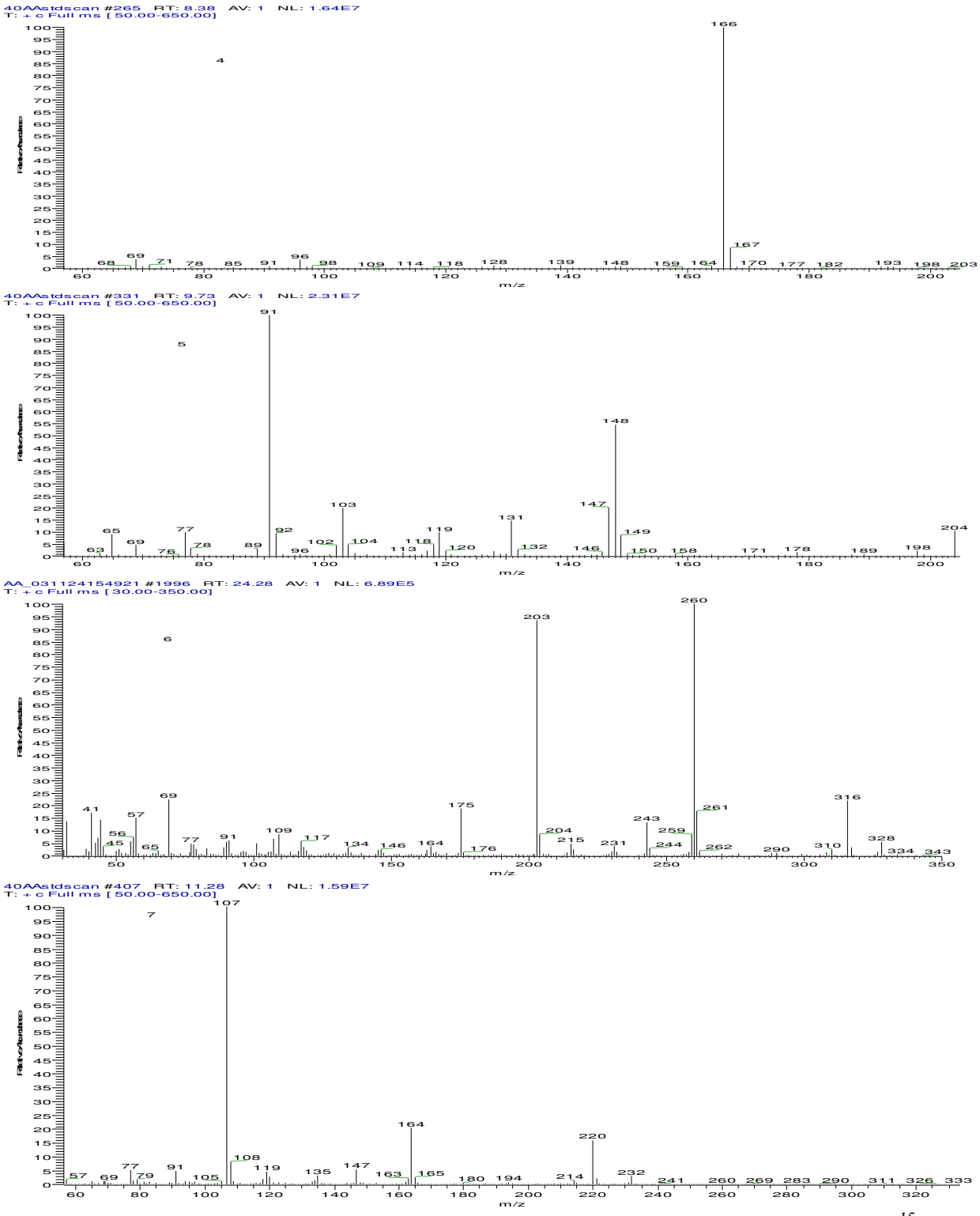


Fig. 1. The mass spectra of the amino acids as trifluoroacetic butyl ester derivatives:1: ^{15}N Gly; 2: Val; 3:Leu; 4: Pro; 5: Phe; 6: Tyr; and 7. Chloroacetyl Tyr

The GC-MS was used in the scan mode in the range 50-500 a.m.u. for qualitative determination of the amino acids to obtain the mass spectrum of each amino acid of interest (Fig. 1) and to select the important ions for the SIM mode. The peaks in the mass spectra of the five important amino acids tested in the two metabolic disease are: m/z 168 for Val, m/z 182 (the third important peak) for Leu, m/z 166 for Pro, m/z 91, 148 for Phe and m/z 203, 260, 316, m/z 107, 164, 220 for Tyr, as shown in fig. 1.

The chromatogram of separation of the standard amino acids is presented in Fig. 2. For Val, Leu and Pro, the important ions selected in the SIM experiment from the trifluoroacetyl butyl esters derivatives mass spectra correspond to the loss of butyl ester from the molecular ion $[M - COOC_4H_9]^+$

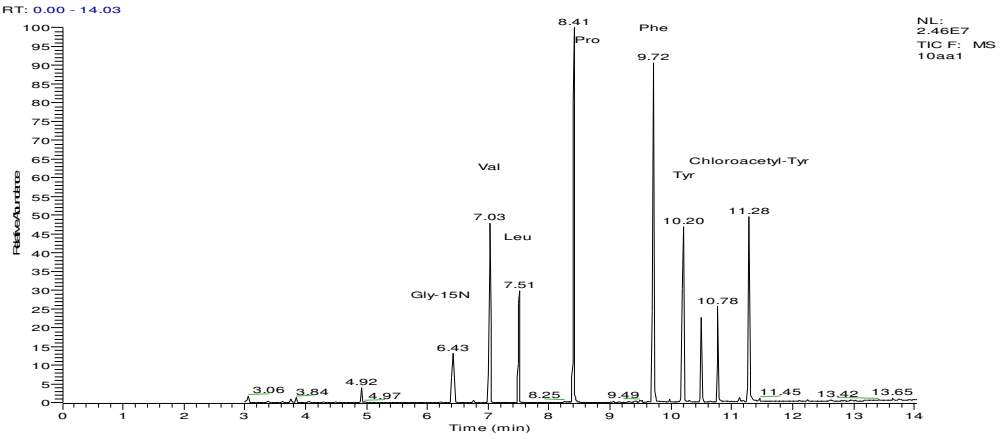


Fig. 2 The chromatogram of separation of the selected triflouoroacetyl butyl esters amino acid standards (^{15}N-Gly:6.43 min; Val: 7.03 min; Leu :7.63 min; Pro: 8.41 min; Phe: 9.71min; Tyr: 10.18 and 11.3 min for chloroacetyl-Tyr).

The method was validated in the range 0-40µg/ml. The calibration curves were obtained by injecting standard solutions containing amino acids in concentration of 5, 10, 15, 20 and 40 µg/ml with 20 µg/ml of ^{15}N-Gly added to each standard solution. Linearity of the method was calculated by representing the ratio of selected ion peak area for each amino acid and the internal standard versus the amino acid standard concentrations, in µg/ml. The regression curves obtained were: Val: y = 0.3632x + 0.3284, r=0.994; Leu: y = 0.1073x + 0.2082, regression coefficient r=0.996; Pro: y = 0.3646x + 0.9485, r=0.992; Phe: y = 0.3428x + 0.9374, r=988; Tyr: y = 0.5117x + 0.1273, r=0.98. Precision was studied by injecting in triplicate the standard solutions of 5, 10 and 20 µg/ml. RSD was obtained between 0.37-33% for 10 µg/ml, 11.5- 24 % for 10µg/ml and 12 -

20 for 20 µg/ml. Fig. 2 shows the aminoacids identified by using the standards mass spectra (Fig.1) and the NIST library. It was found that Tyr is formed after derivatezion in two molecules with elution time at 10.18 and 11.30min. The comparison between a blood sample and standard samples is compared in Fig.3.

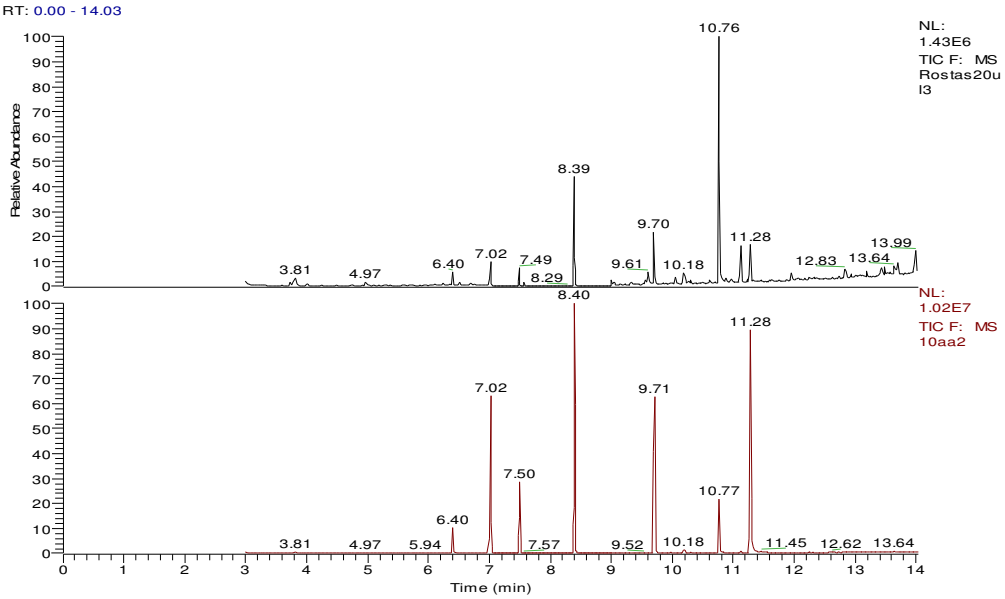


Fig.3 Amino acid screening in the SIM-GC-MS mode for a standard mixture of amino acids and a blood sample.

The amino acid quantitative values were calculated by using 20 µg ^{15}N-Gly blood as internal standard per ml of blood sample. Table 1 shows the comparison of the results obtained for the amino acids average value in newborn blood samples [4] and the values obtained by blood spot analysis for a child (n=3) in our preliminary study.

Table 1 Plasma amino acid concentrations mean values in control newborns [4] and our data

Plasma	Newborn (µM/l)	Newborn (µg/ml)	Child (µM/l)	Child (µg/ml)
Val	136.75	16	28,53	3,34
Leu	72.52	9.5	64,14	7,50
Pro	185.22	21.3	163,88	19,17
Phe	78.79	13	88,06	10,30
Tyr	69.61	12.6	69,65	8,15

Our preliminary results obtained by using only 20 µl of blood sample show good results. PKU diagnosis could be tested by calculating the ratio Phe/Tyr. Diagnosis of MSUD disease will be obtained by calculating the ratio between aliphatic and aromatic amino acids in blood samples.

4. Conclusions

The BIA method is a good screening technique and a low cost method for PKU diagnosis but is a semiquantitative with false positive up to 5% [1]. GC-MS is rapid, simple, less expensive method, which can measure several amino acids at once from very few µl of blood.

Screening of plasma amino acids is the first step in diagnosis of metabolic diseases. Further work is needed for optimization of the analytical method described. The preliminary results showed that GC-MS is a suitable method for PKU diagnosis in neonatal blood samples, either by screening or quantitation of some amino acids. The method is a minim invasive one by using very small quantities of blood. The ratio of aliphatic amino acids to aromatic amino acids could indicate other disorders of the amino acid metabolism such as MSUD..

Acknowledgements

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