ID-GC-MS DIAGNOSIS OF METABOLIC DISEASES

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Abstract. This paper has developed an isotopic dilution gas chromatography-mass spectrometric (ID-GC-MS) rapid method for diagnosing inborn error of metabolism of some neonatal diseases. Small volumes of dry plasma or blood spots were used for neonatal blood screening for diagnosis of phenylketonuria (PKU) and maple syrup urine disease (MSUD) metabolic diseases. The blood samples were derivatized as trifluoroacetyl butyl esters and analyzed by gas chromatography coupled with mass spectrometry in the selected ionization monitoring (SIM) mode. Regression curves for standard amino acids are used for quantitative determination of valine, leucine, proline, phenylalanine and tyrosine, by using ^{15}N-glycine as internal standard. GC-MS analyses were performed on a Trace DSQ ThermoFinnigan quadrupole mass spectrometer coupled with a Trace GC gas chromatograph. Samples were separated on a Rtx-5MS capillary column, 30 m × 0.25 mm, 0.25 μm film thickness, using a temperature program from 50 ^{o}C (1 min), then 20 ^{o}C/min to 310 ^{o}C, in the selected ion monitoring (SIM) mode. The following important ions from the mass spectra of Phe, Pro, Val, Leu and Tyr were used: m/z 91, 148, 204 for Phe, m/z 166 for Pro, m/z 168 for Val, m/z 182 Leu, m/z 203, 260, 316 for Tyr and m/z 155 for the internal standard. 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 70 eV and emission current 100 μA.

Key words: isotopic dilution, blood, amino acids, diagnosis.

INTRODUCTION

Gas chromatography - mass spectrometry technique (GC-MS) demonstrates to be an indispensable method for diagnosing inborn error of metabolism and is widely recognized for its effectiveness in related fields [1, 2]. The normal catabolism of phenylalanine in mammals requires its conversion in tyrosine in the liver. Phenylketonuria (PKU) is a metabolic disease usually caused by phenylalanine hydroxylase deficiency which causes an increase of phenylalanine in the plasma and a decreased tyrosine, a specific pattern of blood amino acids. Maple syrup

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urine disease (MSUD), other metabolic disease, could be diagnosed by some branched amino acids determination in the blood [1]. 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 phenylalanine 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 by using isotopic dilution GC-MS. Volatile derivatives of amino acids are analyzed in very small volumes of plasma or whole blood by using filter paper blood specimens and 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 a low cost method [2].

MATERIALS AND METHODS

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. [^{15}N]-glycine (99%) was produced by chemical synthesis. All other chemicals were from Comchim (Bucharest).

The blood samples were obtained from patients and volunteers from the Pediatric Clinic III Cluj-Napoca. Written informed consents were obtained from each subject parent prior to this study.

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 vials. Dry samples were esterified with 100 µl 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 trifluoroacetic 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 the blood and MSUD by determination of valine (Val), leucine (Leu) and proline (Pro) in the blood. The analysis of the five amino acids in blood samples by GC-MS is useful in the diagnosis of both diseases.

APPARATUS

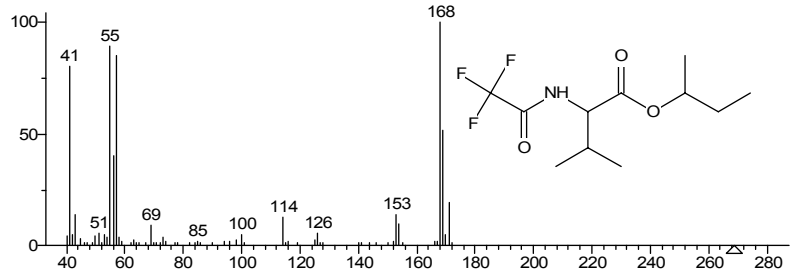
A Trace DSQ ThermoFinnigan quadruple mass spectrometer in the EI mode coupled with a Trace GC was used. The capillary column Rtx-5MS was of 30 m length × 0.25 mm, 0.25 μm film thickness, by using a temperature program from 50 ºC (1 min), then increased to 310 ºC, at 20 ºC/min, in the selected ion monitoring 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, 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 mono trifluoroacetyl butyl ester derivative of tyrosine (Fig. 1). 25 μg/ml of the internal standard were added at each sample. Each parent was informed about this study and written consents were obtained from each subject parent.

RESULTS

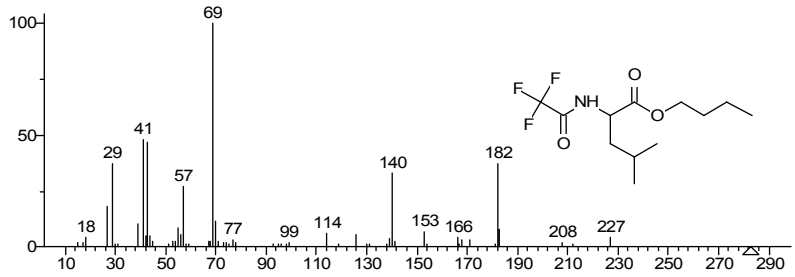
The mass spectra of the amino acids used for profiling determination are presented in Fig. 1.

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 important peaks in the mass spectra of the five amino acids tested in the two metabolic diseases are presented in the chromatogram of separation of the standard amino acids 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 $[M–COOC_4H_9]^+$.

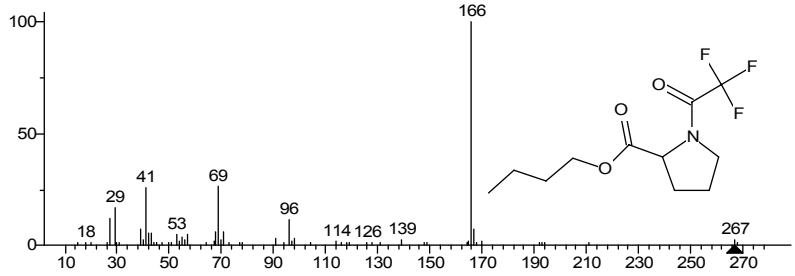
The calibration curves were obtained by injecting standard solutions containing amino acids in concentration of 5, 10, 15, 20 and 40 μg/ml with 25 μg/ml of ^{15}N-Gly added to each standard solution. Linearity of the method was calculated by representing the ratio of selected ion peak area for each amino 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: $y = 0.5117x + 0.1273$, $r = 0.98$.



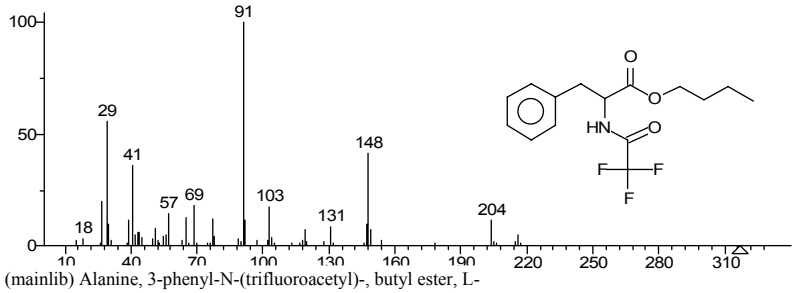
(mainlib) L-Valine, N-(trifluoroacetyl)-, 1-ethylpropyl ester



(mainlib) L-Leucine, N-(trifluoroacetyl)-, butyl ester



(mainlib) L-Proline, 1-(trifluoroacetyl)-, butyl ester



(mainlib) Alanine, 3-phenyl-N-(trifluoroacetyl)-, butyl ester, L-

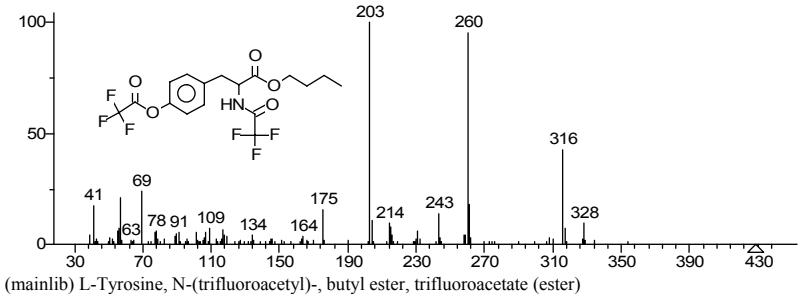


Fig. 1. The mass spectra of the five amino acids as trifluoroacetic butyl ester derivatives tested for metabolic diseases.

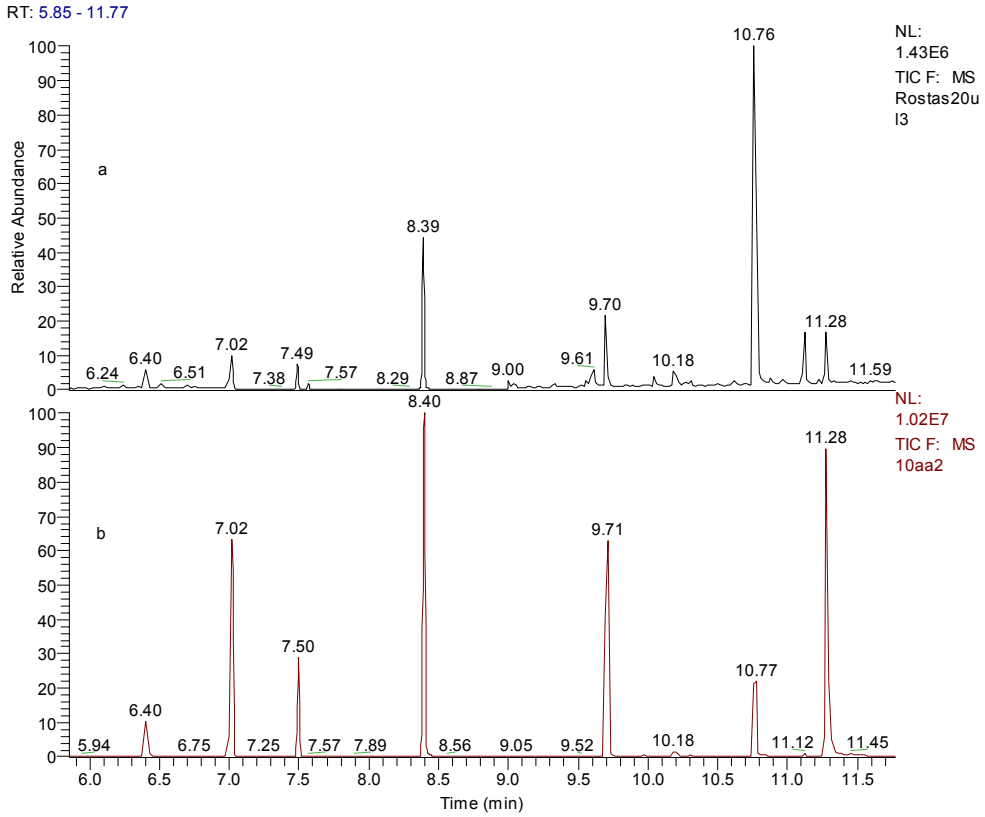


Fig. 2. Amino acid screening in the SIM-GC-MS mode for a blood sample (a) and a standard mixture of amino acids (b): ^{15}N-Gly at 6.40 min, Val at 7.02 min, Leu at 7.50 min, Pro at 8.40 min, Phe at 9.70 min and Tyr at 11.28 min.

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 10 µg/ml and 12–20 for 20 µg/ml. Validation of the method is further studied.

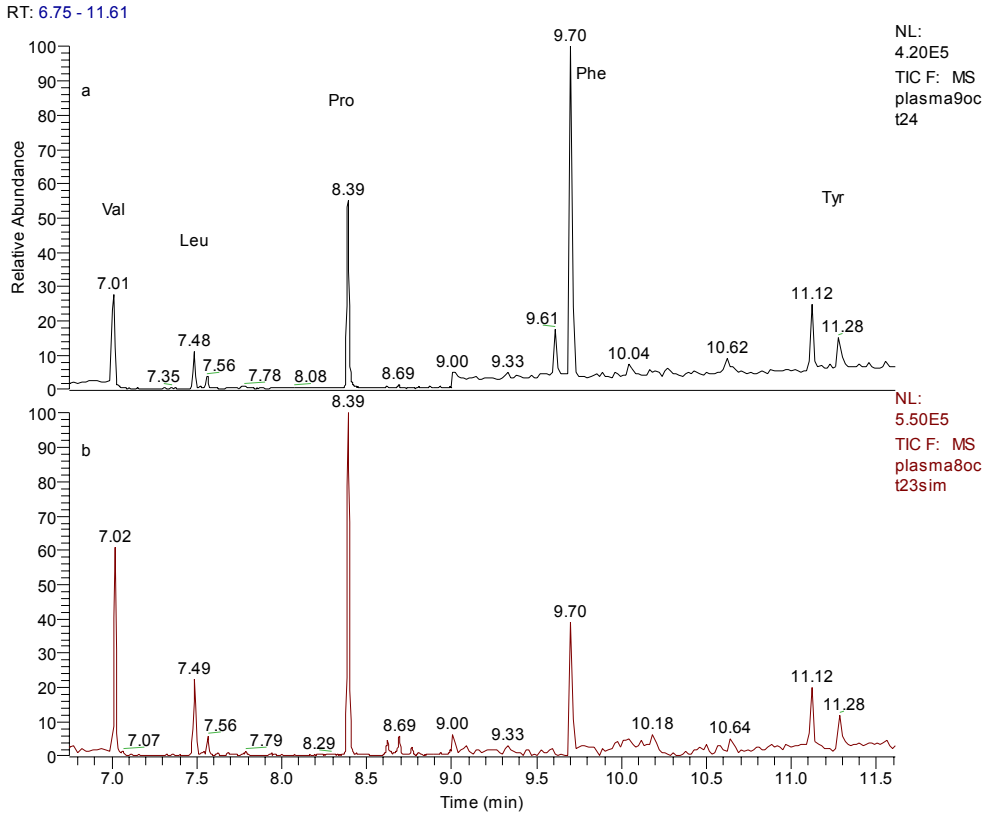


Fig. 3. Comparison of the fingerprint SIM-GC-MS chromatograms for a PKU patient between the first (a) and the third month (b) of treatment.

The amino acid quantitative values were calculated by using as internal standard ^{15}N-Gly, 25 µg 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 results obtained by using only 20 μl of blood spots showed that PKU diagnosis could be tested by calculating the ratio Phe/Tyr. In the case of a PKU patient after three months of treatment the ratio of Phe/Tyr has decreased from 2.30 to 1.02 (Fig. 3). Diagnosis of MSUD disease is obtained by the determination of Val, Leu and Pro and by calculating the ratio between aliphatic and aromatic amino acids in the blood samples.

CONCLUSIONS

The BIA method is a semi quantitative method used for PKU diagnosis but is with false positive up to 5% [1]. ID-GC-MS is rapid, sensitive, selective, simple, less expensive, measuring several amino acids at once.

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 quantitation or screening of some amino acids. The method is a minimum invasive 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.

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