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Method for the quantification of underivatized amino acids on dry blood spots from newborn screening by HPLC–ESI–MS/MS

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Abstract

In our study we have developed an HPLC–ESI–MS/MS method for qualitative and quantitative analysis of underivatized amino acids on dry blood spots. The sensitive and specific instrumental performances permitted the chromatographic separation of 40 amino acids and their isomers within 10 min. The method has been set up for cases of suspected metabolic diseases revealed by newborn screening. What is new is that it is applied on the same blood spots used for newborn screening, instead of plasma, in order to avoid involvement of doctors, increased anxiety for parents, stress for patients for plasma collection, long time of waiting and further costs for analysis.
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Keywords: Newborn screening; Blood spot; Underivatized amino acids; HPLC–ESI–MS/MS

1. Introduction

Amino acids, the proteinogenic units as well as their derivatives, are very important for biological processes such as protein synthesis and metabolic pathways [1]. Enzyme deficiencies in amino acid metabolism lead to variation of their physiological concentrations causing clinical symptoms, which are usually not specific for a single disorder. Therefore, it is of great importance to simultaneously determine the levels of free amino acids in physiological samples as one or several compounds may play the role of biomarkers for one or a group of metabolic disorders [2]. For example, phenylalanine for phenylketonuria (PKU), ornithine, citrulline and argininosuccinic acid (ASA) for disorders of urea synthesis cycle, allo-isoleucine and valine for maple syrup urine disease (MSUD).

There are many chromatographic separation techniques for the analysis of amino acids, most of which require pre- or post-column derivatization in order to enhance chromatographic separation and detection. The still most widespread technique used is the automated amino acids analyser based on ion-exchange chromatography [3–5]. However, the procedure adopted for these methods is time-consuming and may cause the production of artefacts and interferences. In order to avoid this inconvenience some authors have set up methods for qualitative and quantitative analysis of amino acids without performing derivatization. First this was achieved for only a few underivatized amino acids by using HPLC or CE coupled to electrochemical detectors [6,7], but with the advent of the more sensitive and specific MS–MS techniques a wider range of amino acids, even those in trace amounts, could be detected [8–10]. So, analysis of underivatized amino acids can be done on very complex biological matrices (plasma and urine) as reported by some authors who developed a fast, simple and sensitive HPLC–ESI–MS/MS method based on the use of a volatile ion-pair reagent [11–14].

A significant number of laboratories have introduced Newborn Screening Programs for the semi quantitative analysis of amino acids on dried blood spots in order to provide an early diagnosis of metabolic diseases and thus prompting intervention [15]. However, in many of the literature descriptions of neonatal screening, the samples undergo a derivatization step and the amino acidic profile is then acquired in the form of a mass spectrum making discrimination of isomers impossible because they appear as a single peak. Such a phenomenon occurs especially for leucine that has three major isomeric forms (leucine, isoleucine and allo-isoleucine) and is also isobaric with 3-hydroxyproline and propionyl-glycine. This kind of analysis, therefore, may lead to identification of false positives if further investigations are not performed.

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We have developed an HPLC–ESI–MS/MS method making it a very rapid, simple, sensitive and specific tool for separation of 40 underivatized amino acids, including the key isomers, from a dried blood spot. The qualitative and quantitative analysis is conducted on the same blood sample previously used for newborn screening or for monitoring affected patients for diet therapy in order to closer and accurately examine the amino acidic profile excluding the risk of false positives.

2. Experimental

2.1. Reagents

The unlabelled amino acids L-glycine (Gly), β-alanine (β-Ala), L-alanine (Ala), sarcosine (Sar), α-amino-*n*-butyric acid (α-Abu), γ-aminobutyric acid (GABA), L-serine (Ser), L-proline (Pro), L-valine (Val), L-threonine (Thr), taurine (Tau), L-leucine (Leu), L-isoleucine (Ile), δ-aminolevulinic acid (δ-ALA), L-ornithine (Orn), L-asparagine (Asn), L-aspartic acid (Asp), L-glutamine (Gln), L-glutamic acid (Glu), L-lysine (Lys), L-methionine (Met), L-histidine (His), L-phenylalanine (Phe), L-arginine (Arg), L-citrulline (Cit), L-tyrosine (Tyr), L-carnosine (Car), L-tryptophan (Trp) and L-homocystine $(Hcys)_2$ were from Fluka (Milan, Italy); ethanolamine (EA), creatinine, guanidineacetic acid (GAA), L-allo-isoleucine (a-Ile), creatine, L-α-aminoadipic acid (α-Aad), 3-methyl-L-histidine (3-Met-His), 1-methyl-L-histidine (1-Met-His), L-homoserine (Hse) and L-argininosuccinic acid (ASA) were from Sigma (Milan, Italy) and 3-hydroxyproline (3-OH-Pro) was from Merck (Milan, Italy). The internal standards L-Gly-1,2-$^{13}C_2$, DL-Ala-3,3,3-d_3, L-Glu-2,4,4-d_3, L-Val-d_8, Met-methyl-d_3, Tyr-ring-d_4, L-Leu-5,5,5-d_3, L-Orn-3,3,4,4,5,5-d_6, L-Phe-ring-d_5, L-Arg-5-^{13}C-4,4,5,5-d_4 were from Cambridge Isotope Laboratories (Rome, Italy); creatinine-methyl-d_3 was from CDN Isotopes (Rome, Italy); GAA-2,2-d_2 was from Isotec (Milan, Italy); L-Pro-d_7, L-Asp-2,3,3-d_3, L-Gln-2,3,3,4,4-d_5, L-Cit-5,5-d_2, GABA-2,2-d_2, DL-Ser-2,3,3-d_3 and $(Hcys)_2$ d_8 were a generous donation from Mayo Clinic. HPLC grade acetonitrile was from Carlo Erba (Milan, Italy) and HPLC grade methanol from Merck. Tridecafluoroheptanoic acid (TDFHA) was obtained from Sigma–Aldrich.

2.2. Standard solutions and calibration curves

The isotopically labelled internal standard mixture was prepared by diluting the stock solution containing the labelled amino acids with methanol to a concentration of 30 μmol/L for GAA and 10 μmol/L for all the others. Calibration has been achieved by applying the stable isotope dilution method in which scalar concentrations of a mixture of unlabelled amino acids were added to 150 μL of the internal standard solution. Two sets of calibration curves were determined: one using water (without matrix) and one spiked with dried blood amino acids (with matrix) for the assay of blood spots. The range of concentrations and the regression coefficients for both the curves are reported in Table 1.

Table 1
Two sets of calibration curves for the quantification of amino acids have been constructed using the range of concentrations (μmol/L) shown below. The regression coefficients reported in this table are relative to the calibration curves spiked with amino acids for the assay on blood spots

Amino acids	Range of concentrations μmol/L (no matrix)	Range of concentrations μmol/L (matrix)	R^2 (matrix)
Asp	0–300	3–303	0.998
Ser	0–600	55–655	0.997
Gin	0–500	42–542	0.998
Glu	0–600	133–733	0.990
Gly	0–900	81–981	0.994
Pro	0–200	55–255	0.993
Cit	0–200	5–205	0.999
Ala	0–900	146–1046	0.997
GAA	0–200	1–201	0.993
Tyr	0–400	18–418	0.998
Creatinine	0–300	13–313	0.993
Met	0–200	7–207	0.999
Val	0–500	72–572	0.998
a-lle	0–250	20–250	0.992
lie	0–250	23–273	0.995
Leu	0–500	54–554	0.995
Phe	0–800	30–830	0.995
Orn	0–400	66–466	0.997
$(HCys)_2$	0–100	1–101	0.999
Arg	0–400	21–421	0.999

2.3. Sample preparation

Guthrie filter papers for collection of blood spots were from Schleicher and Schuell (S&S 903). Blood spots were from newborns from hospitals adhering to the local Newborn Screening Program and from affected patients undertaking diet therapy. From every filter paper three blood spot discs (each with Ø = 3.2 mm) were punched out and 150 μL methanol containing the internal standards were added for the extraction of the amino acids. The samples were also sonicated for 20 min and the derivatization step has been omitted for the reasons described above. The sample is taken to dryness under a nitrogen flow, dissolved in 50 μL H_20 0.1% TDFHA and ready to be analysed.

2.4. Instrumentation and analysis

A PerkinElmer blood spot puncher (1296–071 Delfia) (Milan, Italy) was used for punching discs from blood spots on filter paper.

Waters 2795 Alliance HT HPLC (Milan, Italy) was coupled to a Micromass Quattro Ultima mass spectrometer (Milan, Italy) with an ESI (Electrospray Ionisation) source, together with a Discovery C_{18} column (50 mm × 2.1 mm × 5 μm) for amino acid analysis.

Analysis has been performed by using HPLC–ESI–MS/MS for a more specific and sensitive detection. Currently, two different gradients have been established, depending on the need to follow or not the whole set of amino acids. In the case of the analysis of all of the amino acids they have been divided into three groups within the same acquisition method according to

their retention times. This allowed the simultaneous monitoring of a reduced number of amino acids, and thus maximised the detection sensitivity. Chromatographic separation took 10 min with a re-equilibration time of 5 min in order to restore retention times. The mobile phase solvents were H_2O containing 0.1% TDFHA and ACN containing 0.1% TDFHA. Gradient composition started from 10% ACN and it was maintained for 1 min, then it rapidly raised to 15% at 3 min and to 20% at 5 min, at 6 min it changed to 25% increasing to 40% at 7 min, then to 75% at 8.90 min and finally to 98% at 9 min remaining till the end of the analysis.

For those amino acids which play a relevant role in detection of metabolic diseases by newborn screening such as Val, Leu/Ile/a-Ile, Met, Phe, Cit and Tyr, another gradient has been created for a shorter chromatographic run with duration time of 5 min. Elution solvents used were the same as above. Gradient composition started from 20% ACN for 1 min, it reached 30% at 3 min and then it rapidly increased up to 90% at 3.10 min remaining isocratic till 5 min. Re-equilibration time was of 1 min. For both the methods flow rate was 300 μL/min and 3 μL of sample was injected. The eluent from the LC column was split 1:10 after the column. The column temperature was set at 40 °C. MS–MS analysis was done in positive ion mode and in SRM mode following the ion transitions with appropriate instrumental parameters as described in Table 2. Source temperature was

Table 2
Ion transitions for the unlabelled and labelled amino acids and instrumental parameters for their MS–MS detection in SRM mode

Amino acids + internal standard	Ion transition	Dwell time (s)	Cone voltage (V)	Collision energy (eV)
EA	62.00 > 44.00	0.02	70	15
Gly	76.10 > 30.30	0.02	60	8
Gly $^{13}C_2$	79.10 > 32.30	0.02	60	8
β-Ala	90.00 > 72.00	0.02	60	10
Ala/Sar	90.40 > 44.20	0.02	60	10
Ala d_3	93.40 > 47.20	0.02	60	10
α-Abu	104.30 > 58.10	0.02	70	10
GABA	104.30 > 87.00	0.02	70	15
GABA d_2	106.00 > 89.00	0.02	70	8
Ser	106.00 > 60.00	0.02	70	15
Ser d_3	108.90 > 63.00	0.02	70	10
Creatinine	114.20 > 44.50	0.02	70	20
Creatinine d_3	117.00 > 47.30	0.02	70	15
Pro	116.00 > 70.00	0.02	70	15
Pro d_7	123.00 > 77.10	0.02	70	20
GAA	117.90 > 76.00	0.02	70	10
GAA d_2	120.00 > 78.00	0.02	70	8
Val	118.00 > 72.00	0.02	70	15
Val d_8	126.00 > 80.00	0.02	70	15
Thr/Hse	120.00 > 74.00	0.02	70	15
Tau	126.00 > 108.00	0.02	70	20
3-OH-Pro	132.00 > 86.00	0.02	70	15
Leu/lle/a-lle	132.00 > 86.00	0.02	70	15
Leu d_3	135.00 > 89.00	0.02	70	15
Creatine	132.10 > 90.20	0.02	70	10
δ-ALA	132.00 > 113.90	0.02	70	7
Orn	133.00 > 70.00	0.02	70	15
Orn d_6	139.00 > 76.10	0.02	70	30
Asn	133.00 > 74.00	0.02	70	15
Asp	134.00 > 74.00	0.02	70	15
Asp d_3	137.10 > 75.00	0.02	50	15
Gin	147.00 > 84.00	0.02	70	15
Gln d_5	151.90 > 88.00	0.02	70	15
Glu/Lys	148.00 > 84.00	0.02	70	15
Glu d_3	151.00 > 87.00	0.02	70	15
Met	150.00 > 104.00	0.02	70	15
Met d_3	153.00 > 107.00	0.02	70	15
His	156.00 > 110.00	0.02	70	15
α-Aad	162.00 > 98.00	0.02	70	15
Phe	166.00 > 120.00	0.02	70	15
Phe d_5	171.00 > 125.00	0.02	70	15
3-Met-His	170.00 > 124.00	0.02	70	15
1-Met-His	170.00 > 126.00	0.02	70	15
Arg	175.00 > 70.00	0.02	70	15
Arg ^{13}C d_4	180.00 > 75.00	0.02	70	15
Cit	176.00 > 159.00	0.02	70	15
Cit d_2	177.70 > 115.00	0.02	70	20
Tyr	182.00 > 165.00	0.02	70	15
Tyr d_4	186.00 > 169.00	0.02	70	15
Trp	205.00 > 188.00	0.02	70	15
Car	227.00 > 110.00	0.02	70	15
$(Hcys)_2$	269.00 > 136.00	0.02	70	15
$(Hcys)_2$ d_8	277.10 > 139.90	0.02	70	10
ASA	291.10 > 70.00	0.02	150	40

Table 3
Reproducibility of retention times (RT) reported as mean values (min) with their standard deviation for the underivatized amino acids detectable in SRM mode after 22 intra-day chromatographic runs of 10 min each

Amino acids	Mean RT ± S.D. (min)
3-OH-Pro	1.34 ± 0.01
Asp	1.47 ± 0.01
Asn	1.58 ± 0.01
Ser	1.65 ± 0.01
Gin	1.69 ± 0.01
Glu	1.80 ± 0.01
Gly	1.93 ± 0.01
Pro	2.08 ± 0.01
Cit	2.16 ± 0.01
α-Aad	2.21 ± 0.01
Ala	2.28 ± 0.01
α-Abu	3.02 ± 0.02
GAA	3.23 ± 0.04
EA	3.23 ± 0.02
Creatine	3.44 ± 0.01
δ-ALA	3.60 ± 0.01
Tyr	3.90 ± 0.02
Creatinine	4.25 ± 0.01
Met	4.26 ± 0.01
Val	4.33 ± 0.01
a-lle	6.05 ± 0.01
Me	6.21 ± 0.03
Leu	6.64 ± 0.05
Phe	6.78 ± 0.04
Trp	7.79 ± 0.02
1-Met-His	8.46 ± 0.02
His	8.58 ± 0.01
3-Met-His	8.67 ± 0.01
Orn	8.69 ± 0.01
ASA	8.69 ± 0.01
$(HCys)_2$	8.72 ± 0.01
Lys	8.84 ± 0.02
Arg	8.98 ± 0.01
Car	9.11 ± 0.01

100 °C, desolvation gas flow 470 L/h at 250 °C and collision gas argon kept at a pressure of 1.4×10^{-3}. The detection limits varied from 1 pmol to 40 pmol depending on the nature of the amino acids injected.

2.5. Software

Data were acquired with MassLynx 4.0 and processed for calibration and for quantification of the analytes with QuanLynx.

3. Results and discussion

Our study started with the analysis of each amino acid in order to find the most specific and sensitive detection parameters. Every amino acid solution (400 μM) was directly injected into the mass spectrometer with the aid of a syringe pump at a rate of 10 μL/min. In MS scan we optimized the signal for detection of the *m*/*z* protonated molecules in positive ESI mode. Afterwards we studied their fragmentation pattern by using different collision energies and cone voltages in MS–MS. The most abundant product ion has been chosen in order to obtain specific ion transitions for following the amino acids in SRM under appropriate instrumental conditions as reported in Table 2. The next step was to analyse the amino acids in HPLC–ESI–MS/MS with the C_{18} column applying different gradient compositions to find the best one for both the separation and the retention times. Under the same analytical conditions the amino acids did not respond all in the same way depending on their physico-chemical characteristics: in fact some of them (Tau, β-ALA, SAR, GABA, Thr, HSE) showed a weak signal/noise response in positive ion mode. The use of HPLC was of great importance because it allowed us to differentiate between the Leu isomers (Leu, a-Ile and Ile), which are normally indistinguishable when acquired in the form of a mass spectrum (data not shown). Moreover, a-Ile and Ile are stereoisomers and fragmentation study gave an identical fragmentation pattern with the same peak intensities (Fig. 1). Detection of a-Ile is necessary as it is known to be a significantly diagnostic metabolite in the determination of MSUD (a defect of of leucine metabolism). We have been also able to separate Glu (148 *m*/*z*) and Gln (147 *m*/*z*), which have fragment 84 *m*/*z* in common, so to make their quantification possible (data not shown), important in the case of hyperammonemia. In addition to separation the absence of the derivatization step avoided contribution of Gln to Glu that occurs when butylation is conducted at 60 °C for 20 min leading to hydrolysis of a part of Gln to Glu. For quantification of the blood spot amino acids we have constructed calibration curves which showed a good linearity over the concentration ranges selected for every analyte as described in the experimental section. No matrix effect that could impair the analytical system has been observed because of the efficiency of sample preparation and the analytical methods employed.

Our HPLC–ESI–MS/MS method has been implemented from Qu's leading to some important advantages from the analytical point of view [13]. We adopted a shorter C_{18} column in order to reduce chromatographic run. To enhance sensitivity for underivatized amino acids we also used perfluorinated carboxylic acids as ion-pairing reagents volatile enough to be compatible with mass spectrometry. Hence, in our case elution solvents contained TDFHA instead of PFHA (perfluoroheptanoic acid) and TFA (trifluoroheptanoic acid) as it showed a better amino acid separation and because this way we avoided competition between the two reagents responsible of reducing detection sensitivity. Retention times were not delayed in our analyses and they were stable because of a re-equilibration time

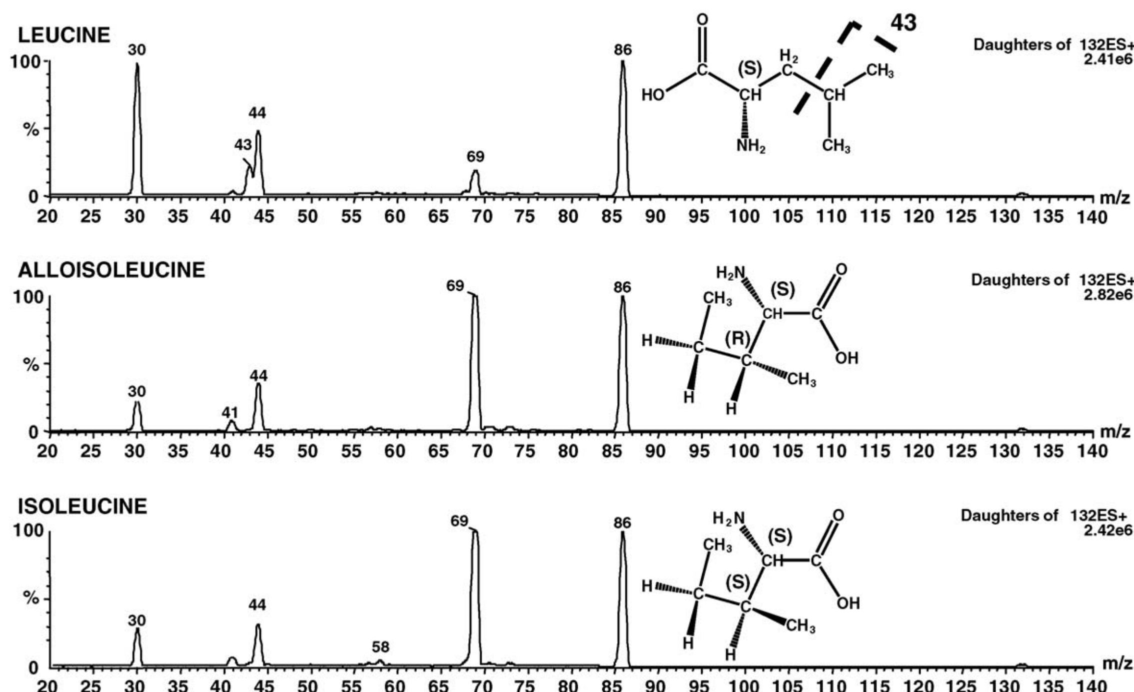

Fig. 1. Pattern fragmentation for the Leu isomers and their chemical structures.

of 5 min for the longer acquisition method and of 1 min for the more specific one. So there was no need to flush the column for 30 min after a certain number of samples as described in our reference work. Finally, we have been able to discriminate between the three Leu isomers reported previously, in particular a-Ile, which was not identified by Qu.

Within a chromatographic run of 10 min all the underivatized amino acids treated in our study could be well separated and detected, except those mentioned above because of weak signal/noise response. Their retention times are listed in Table 3 and they are reported as mean values of 22 intra-day chromatographic runs showing a very good reproducibility. This method gives a complete vision of a great number of compounds at the same time.

Our goal is to perform a closer examination of the amino acids on the same blood spot used for newborn screening whenever

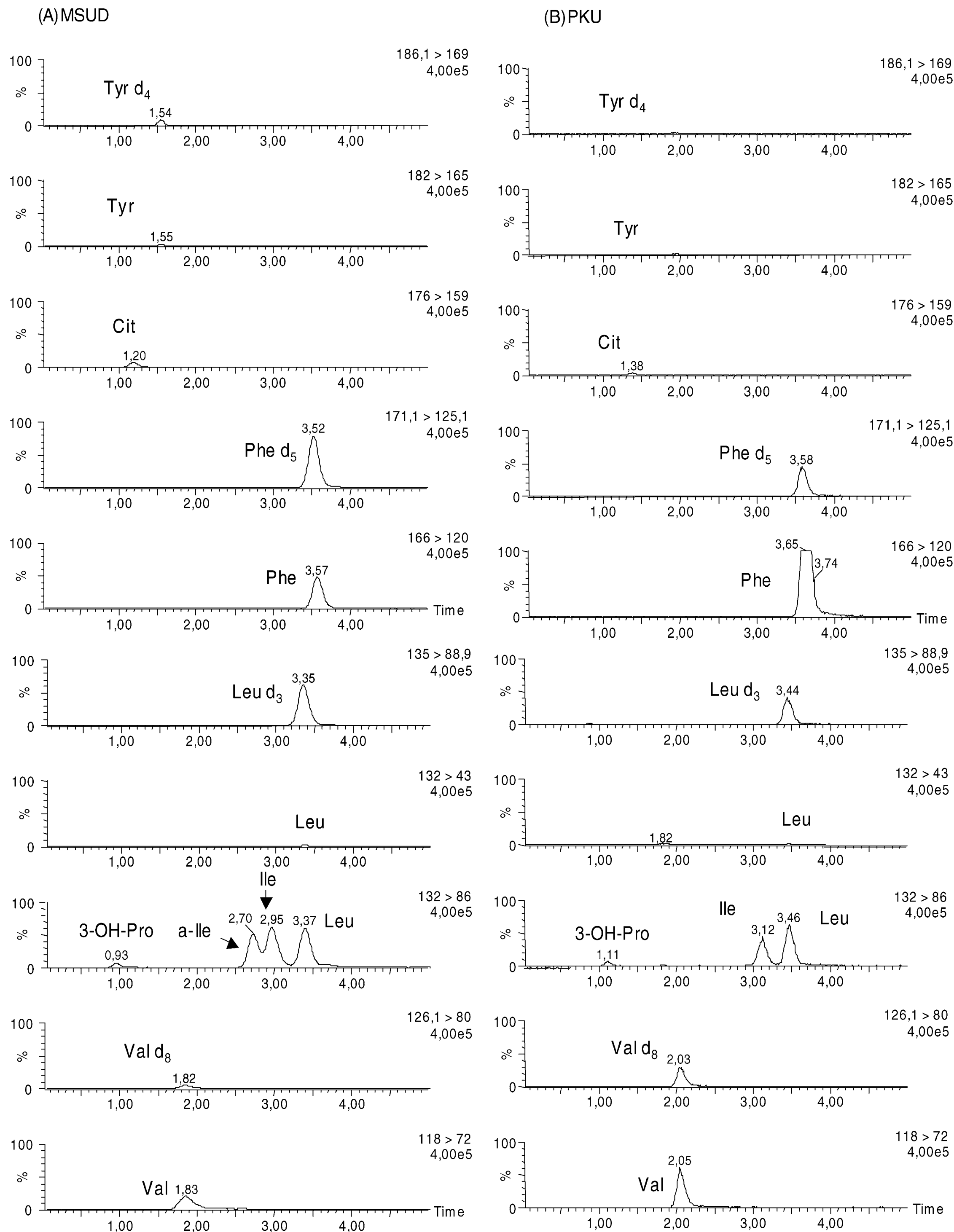

Fig. 2. Chromatographic profiles of a MSUD patient (A) vs. a PKU patient (B).

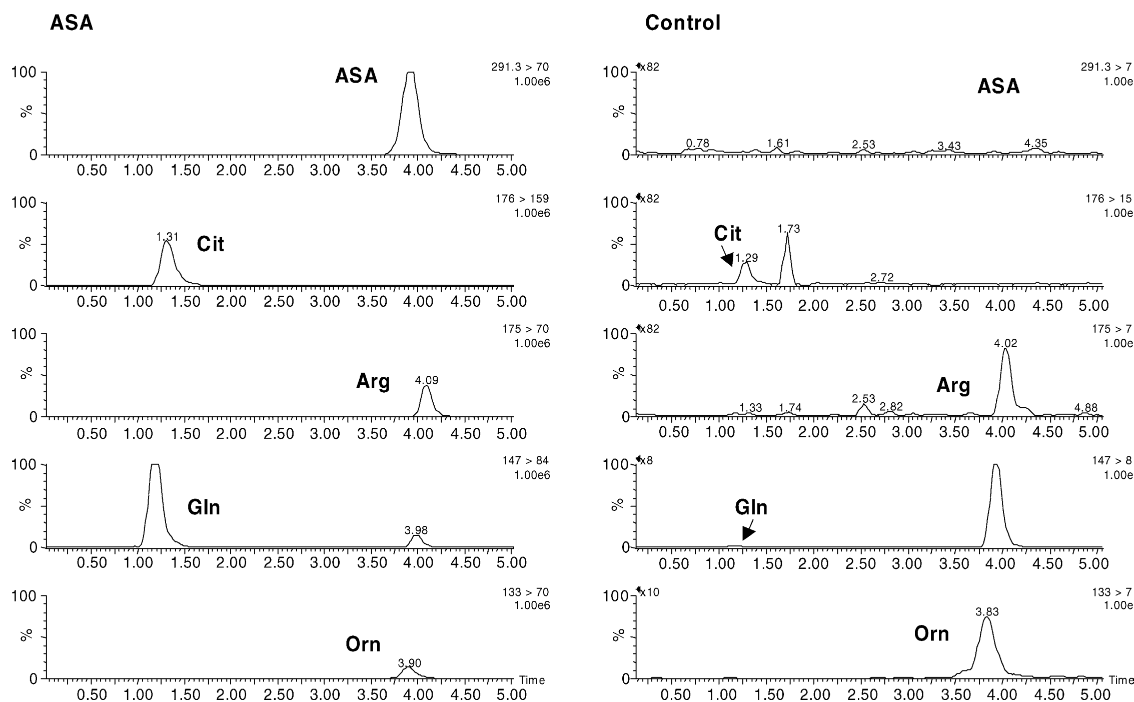

Fig. 3. Chromatographic profiles of an ASA patient vs. a normal control.

there is a case of suspected pathology, so to exclude the risk of false positives. The benefits deriving from the application of such a kind of method are: no involvement of doctors, no increased anxiety for parents, no stress for patients for plasma collection, as it usually occurs for the classical method for amino acids, and no further costs for analysis. For example, when elevated Leu concentration (>312 μM) is found by newborn screening the same blood sample is analysed by HPLC–ESI–MS/MS so to separate Leu isomers, and if the peak corresponding to a-Ile appears together with elevation of Ile and Leu, classical MSUD is diagnosed.

In our study we applied the method on blood spots deriving from a healthy subject, a patient with suspected MSUD, a patient with PKU and a patient with a defect in urea cycle resulting from analysis by newborn screening used as a routine procedure where the amino acidic profile was acquired in the form of a mass spectrum. Newborn screening showed elevated concentrations of Leu for the suspected MSUD and of Phe for PKU, and relatively high levels of Cit and Arg in the case of the defect in urea cycle. In order to exclude any risk of mistake in diagnostic evaluation and on the basis of the metabolites involved, a closer examination has been performed on the same blood spots by applying the more specific HPLC–ESI–MS/MS method with the 5 min gradient. We have set up this method in order to follow the amino acids which show elevation in diseases like those considered above within a shorter time of analysis. The sample with elevation of Leu in newborn screening showed the presence of a-Ile in HPLC–ESI–MS/MS leading to the diagnosis of classical MSUD (Fig. 2A). The patient for whom a high concentration of Phe was detected by newborn screening was confirmed suffering from PKU by our method (Fig. 2B). Finally, the blood sample which showed relatively high levels of Cit and Arg by newborn screening indicating a defect in urea cycle presented the appearance of elevated ASA and Gln in HPLC–ESI–MS/MS in comparison with a normal control (Fig. 3). This method permitted a more detailed glance into the urea cycle and diagnosis of ASA synthetase deficiency could be established. Moreover, the absence of the derivatization step could make detection of ASA and quantification of Gln possible. A-Ile, Phe and ASA may be considered biomarkers for the metabolic diseases identified in our patients. Our examples confirm the importance to simultaneously monitor a wider number of amino acids as in some pathologies such as ASA synthetase deficiency elevation of more than one amino acid may occur.

We aim to develop other specific and sensitive HPLC–ESI–MS/MS methods, which permit to follow only some amino acids and in particular those which are involved in the metabolic disease suspected with newborn screening analysis. Chromatographic runs may become shorter and so analysis may be performed more rapidly.

4. Conclusions

In this work we have developed and optimized an HPLC–ESI–MS/MS method for the qualitative analysis of 40 underivatized amino acids and for the quantification of 20 of them. We were able to simultaneously separate the different compounds including both the proteinogenic amino acids and their derivatives. Sample preparation was very simple, fast and free of interferences without the derivatization step. Analysis was rapid, robust, sensitive and specific permitting a more accurate vision of the wide spectrum of amino acids present in the blood spots previously examined for newborn screening or for monitoring patients in diet therapy. Our future purpose is to

increase the number of amino acids to be monitored, especially those methylated and acetylated, because they are not detectable by traditional methods. Furthermore, we aim to enhance the analytical method to make it more specific and sensitive for the amino acids to be monitored.

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