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Quantitative UPLC-MS/MS analysis of underivatised amino acids in body fluids is a reliable tool for the diagnosis and follow-up of patients with inborn errors of metabolism

W.A. Huub Waterval [1], Jean L.J.M. Scheijen [1], Marjon M.J.C. Ortmans-Ploemen, Catharina D. Habets-van der Poel, Jörgen Bierau *

Laboratory of Biochemical Genetics, Department of Clinical Genetics, Maastricht University Medical Centre, PO Box 5800, 6202 AZ, Maastricht, The Netherlands

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ABSTRACT

Background: An electro-spray ionisation ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) application for the quantitative analysis of amino acids was developed. The suitability for the detection and follow-up of patients suffering from inborn errors of metabolism (IEM) was assessed by extensive cross-validation with ion-exchange liquid chromatography (IEX-LC) with post-column ninhydrin derivatisation, participation in external quality control (ERNDIM) and analysis of samples of patients with confirmed IEM.
Methods: Prior to analysis plasma and urine samples were merely diluted 150-fold in mobile phase. Amino acids were detected in the multiple reaction monitoring mode (MRM) in the ESI-positive mode. The analytical results were compared with IEX-LC. External quality control scheme performance is presented.
Results: Comprehensive analysis of amino acids in plasma and urine was achieved with a run-to-run time of 30 min. Validation results were satisfactory and there was a very good correlation between UPLC-MS/MS and IEX-LC. Analytical results obtained in the external quality control scheme were essentially the same as those of the other participants. Patients suffering from IEM were readily identified.
Conclusion: UPLC-MS/MS analysis of amino acids in body fluids is rapid, reliable and suitable for the diagnosis and follow-up patients with IEM.

1. Introduction

Quantitative analysis of amino acids (AA) in body fluids is the basis for the diagnosis of inborn errors of metabolism affecting amino acid metabolism. The most frequently applied method for the quantitative analysis of physiological AA in body fluids is ion-exchange chromatography (IEX-LC) with post-column ninhydrin derivatisation [1]. This technique is referred to as classic amino acid analysis and is performed using dedicated equipment having excellent reproducibility. Moreover, there is a vast world wide experience with this technique. Quantitative analysis of all physiological amino acids is time-consuming, often requiring 2–3 h per sample, and is not suited for high sample throughput. Moreover, numerous artefacts hamper the method and certain compounds cannot be separated using this technique.

Tandem mass spectrometry (MS/MS) is a powerful technique for the quantitative analysis of small molecules. The technique is based on physico-chemical characteristics of analytes in an electrical field at near vacuum and subsequent collision induced fragmentation [2]. In general practice, a tandem mass spectrometer is operated in combination with high performance liquid chromatography (HPLC), which greatly increases its analytical performance [3,4]. Ions and other components from the sample matrix suppressing the signal output are removed and potential isobars are separated. HPLC-MS/MS facilitates rapid and specific analysis. Ultra-performance liquid chromatography (UPLC) has evolved from HPLC. Due to its greater analytical power, UPLC allows down scaling of sample volume and high throughput sample handling. UPLC-MS/MS supersedes HPLC-MS/MS in analytical performance [5,6].

In recent years the use of HPLC-MS/MS has become more popular in hospital laboratories and is applied to the quantitative analysis of small molecules such as metabolites and pharmaceuticals [2]. It is a powerful tool in the diagnosis of inborn errors of metabolism [7] and in newborn screening programs [8]. Most HPLC-MS/MS methods are dedicated applications for the detection or therapeutic follow-up for one specific disease or group of diseases. MS/MS applications covering

Abbreviations: AA, Amino acid(s); IEX-LC, Ion-exchange liquid chromatography; MS/MS, Tandem mass spectrometry; HPLC, High performance liquid chromatography; UPLC, Ultra-performance liquid chromatography; IS, Stable isotope-labelled internal standard(s); SSA, Sulphosalicylic acid; IEM, Inborn errors of metabolism; ERNDIM, European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism; TDHFA, Tridecafluoroheptanoic acid; MRM, Multiple reaction monitoring; LOD, Limit of detection; LOQ, Lower limit of quantification; ULQ, Upper limit of quantification; NTBC, 2-(2-Nitro-4-trifluoromethyl-benzoyl)-1,3-cyclohexanedione.

* Corresponding author. Tel.: +31 43 387 78 35; fax: +31 43 387 79 01.
E-mail address: jorgen.bierau@gen.unimaas.nl (J. Bierau).

[1] These authors contributed equally to the work presented.

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a wider range of disorders are the (semi-)quantitative analysis of acylcarnitines for the detection of defects in fatty acid oxidation and organic acidaemias [7,9] and the analysis of purines and pyrimidines in urine [10,11]. It has been demonstrated in non-human samples that analysis of underivatised AA by tandem mass spectrometry is possible [12,13]. Piraud and co-workers demonstrated that HPLC-MS/MS analysis of AA and related compounds for the detection of inborn errors is in theory feasible [14–16]. Extensive data on the practicality and validity of LC-MS/MS analysis of AA in comparison with the established techniques have hitherto not been reported.

In our laboratory a project was initiated to replace the classic amino acid analyser with a UPLC-MS/MS application. A reliable, robust and simplified method was developed, validated and implemented for the comprehensive quantitative analysis of underivatised AA in body fluids using UPLC-MS/MS requiring minimal sample volumes and manipulations. The analytical results were compared with those of the classic amino acid analyser and of other laboratories by means of participation in the ERNDIM external quality control scheme. The suitability of the method to detect patients suffering from inborn errors of metabolism is demonstrated.

2. Materials and methods

2.1. Chemicals

AA standards and a prefabricated AA standard solution (A9906) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Stable isotope-labelled (IS) AA were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). All solutions were prepared using LC-MS Ultra High Purity water and LC-MS-grade acetonitrile (J.T. Baker, Deventer, The Netherlands). Tridecafluoroheptanoic acid (TDFHA) 99% was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Sulphosalicylic acid (SSA) was obtained from Acros Organics (Geel, Belgium).

2.2. Patient samples

In 2008, all patient samples were routinely analysed using the method presented, in total 517 plasma samples and 365 urine samples were analysed. Samples of patients with known IEM were re-analysed. Urine samples from the patients with FIGLUria and prolidase deficiency were provided by Dr. L. Dorland, Department of Metabolic and Endocrine Diseases, University Medical Center Utrecht, The Netherlands. The urine samples from the patients with N-Aspartylglucosaminuria and Ornithine δ-amino-transferase deficiency were from the ERNDIM diagnostic proficiency testing scheme. Samples were used according to the "Code for proper use of human tissue" as formulated by the Dutch Federation of Medical Scientific Societies.

2.3. Standards

The purchased standard AA solution contained 500 µM of each AA. A stock solution containing 500 µM of L-S-Cys, PEA, L-Asn, L-Gln, L-Hci, DALA, Trp, L-Hcar, L-Sac, L-Aile, Asa was prepared in water. Prior to analysis this stock solution was mixed 1:1 (v/v%) with the purchased AA standard solution (A9906) resulting in final concentrations of 250 µM for each AA. A mixture of stable isotope-labelled AA was prepared in 0.1 M HCl and was used as internal standard for UPLC-MS/MS analysis. The concentration of the IS AA was between 70 and 800 µM. The stable isotope-labelled AA used are listed in Table 1.

2.4. Standard sample preparation for UPLC-MS/MS analysis

Patient samples were stored at −20 °C and thawed in a water bath at 37 °C prior to analysis and homogenated by vortex mixing. Ten µl of plasma or urine was mixed with 10 µl of the internal standard solution and 1500 µl of 0.5 mM TDHFA in water. The sample was then ready for analysis.

2.5. Ion-exchange liquid chromatography (IEX-LC)

IEX-LC with post-column ninhydrin-derivatisation [1] was performed using a Biochrom-20 AA analyzer (Cambridge, UK) using the separation programme for physiological fluids supplied by the manufacturer. Patient samples were stored at −20 °C and thawed in a water bath at 37 °C prior to analysis and homogenated by vortex mixing. To 150 µl of plasma or urine 150 µl of 5% (m/v) SSA containing 300 µM aminoethylcysteine as the internal standard was added. The mixture was mixed thoroughly and incubated for 15 min on ice and subsequently centrifuged for 10 min at 25,000×*g* at 4 °C. The supernatant was then filtered through a 0.2 µm nylon filter (Costar, Corning, NY) for 1 min at 25,000×*g* at 4 °C. 170 µl of the filtrate was transferred to a clean injection vial. The injected volume was 50 µl.

Table 1
Twenty isotope-labelled AA used as internal standards with the specific mass transitions (MRM), declustering potentials (DP in V), collision energies (CE in eV), dwell times (DT in ms) and retention times (RT in min).

	Abbreviation	Monitored transition	DP	CE	DT	RT
S-Sulfo-DL-Cysteine-2,3,3-D_3	S-Cys*	204.8>122.8	25	12	5	0.28
Taurine-D_4	Tau*	129.7>111.8	25	10	5	0.39
L-Aspartic acid-2,3,3-D_3	Asp*	136.75>74.9	19	14	5	0.68
L-Asparagine-$^{15}N_2$	Asn*	134.8>74.9	19	15	5	0.82
DL-Serine-2,3,3-D_3	Ser*	108.7>63	18	9	5	0.88
L-Glutamine-2,3,3,4,4-D_5	Gln*	151.9>88	16	15	5	0.97
Glycine-2,2-D_2	Gly*	77.7>77.7	20	3	5	1.06
DL-Glutamic acid-2,4,4-D_3	Glu*	150.8>87	18	6	5	1.16
DL-Proline-2,3,3,4,4,5,5-D_7	Pro*	122.7>77	22	14	5	1.56
DL-Cystine-3,3,3′,3′-D_4	$(Cys)_2^*$	245.1>122.1	20	20	5	1.43
DL-Alanine-2,3,3,3-D_4	Ala*	93.7>93.7	17	3	5	1.52
DL-Valine-D_8	Val*	125.7>80	17	12	5	4.39
L-Methionine-(methyl)-D_3	Met*	152.7>106.9	16	11	5	4.59
L-Tyrosine-ring-3,5-D_2	Tyr*	183.8>137.9	19	17	5	5.45
L-Leucine-5,5,5-D_3	Leu*	134.8>89	19	11	100	7.86
L-Phenylalanine-ring-D_5	Phe*	170.8>124.9	20	15	5	8.23
DL-Homocystine-3,3,3′,3′,4,4,4′,4′-D_8	$(Hcy)_2^*$	277.1>140.1	16	10	5	8.5
L-Ornithine-5,5-D_2	Orn*	134.9>72.1	17	15	5	9.11
L-Lysine-4,4,5,5-D_4	Lys*	151>88	22	20	5	9.52
L-Arginine-guanido-$^{15}N_2$	Arg*	177>70.2	22	20	5	9.61

All standards were analysed in the ESI-positive mode.

2.6. Ultra-performance liquid chromatography conditions

Liquid chromatography was performed at 30 °C using a Acquity UPLC BEH C18, 1.7 µm, 2.1×100 mm column (Waters, Milford MA) and a gradient system with the mobile phase consisting of buffer A; 0.5 mM TDFHA and buffer B; 0.5 mM TFHA in acetonitrile (100%) at a flow rate of 650 µl/min (split less). The gradient programme used was: initial 99.5% A and 0.5% B; linear gradient to 70% A and 30% B in 14 min; hold for 3.5 min, return to initial conditions in 1 min at a flow rate of 700 µl/min, followed by equilibration for 10 min. One minute prior to the next sample injection the flow was set to 650 µl/min. Run-to-run time was 30 min. The injected volume was 5 µl.

2.7. Tandem mass spectrometry conditions

Mass spectrometry experiments and optimisation of the method were performed using a Micromass Quattro Premier XE Tandem Mass Spectrometer (Waters, Milford, MA).

The mass spectrometer was used in the multiple reaction monitoring mode (MRM) in the ESI-positive mode. The desolvatation temperature was 450 °C, and the source temperature was 130 °C. The capillary voltage was set at 0.5 kV and the cone voltage was set at 25 V. Nitrogen gas was used as desolvatation gas and as cone gas. Nitrogen gas was produced using a NM30L nitrogen generator (Peak Scientific, Renfrewshire, Scotland). The cone gas flow was 50 l/h and the desolvatation gas flow was 800 l/h. Optimal detection conditions were determined by constant infusion of standard solutions (50 µM) in solvent A using a split system. MRM and daughter-ion scans were performed using argon as the collision gas at a pressure of 3.8×10^{-3} mbar and a flow of 0.2 ml/min. Apparatus settings for detection and internal standards used are shown in Table 2.

3. Validation procedure

3.1. Linearity, accuracy and detection limits

Because of the number of analytes and complexity of the sample matrices, linearity and accuracy (recovery) were determined by spiking 3 independent plasma, and urine samples and water with AA in dissolved water. Between 0 and 1000 µmol/l per AA was added to the sample matrix. The recovery was deduced from the slope of the calibration curve and corrected for the recovery in water to minimise differences between the different standards used. The limit of detection (LOD) was determined in water at a signal-to-noise ratio of 3, lower limits of quantification (LOQ) were determined in plasma and urine at a signal-to-noise ratio of 5.

3.2. Precision (intra- and inter-assay variation)

The intra-assay variation was determined by 10 consecutive analyses of basal and spiked plasma and urine samples. In order to obtain a sample with high concentrations of AA that normally are present in trace amounts 80–150 µmol/l of these AA were added. This was also done in a