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Enantioseparation and selective detection of D amino acids by ultra-high-performance liquid chromatography/ mass spectrometry in analysis of complex biological samples

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Abstract

The growing scientific attention in the biological function of D-amino acids leads to an increasing analytical interest for enantiomeric amino acid separation, which is still very challenging due to the lack of sufficiently sensitive, high-throughput analytical methods that can cope with often occurring matrix interferences and very low D-amino acid concentrations. Here, enantioseparation can benefit from improved resolution and chromatographic speed offered by modern UHPLC techniques and the precision of MS detection. We developed a RP-UHPLC-QqToF-MS method using pre-column OPA/IBLC derivatization for very precise discrimination of amino acids enantiomers. The method shows a superb sensitivity with limits of detection in the range of several pmol/l. It has neither shown matrix inferences in the tested very complex biological matrices (serum, plasma, urine and gut) nor stability or racemisation problems.

Keywords: chiral derivatization, chiral separation, liquid chromatography, mass spectrometry, D-amino acids, complex matrices, OPA-IBLC

1. Introduction

Amino acids (AA) are very important chiral biomolecules. They participate in gene expression, regulation of metabolism, cell signaling and immunity [1,2]. The predominantly present L-enantiomers function as building blocks of peptides and proteins or precursors for the synthesis of several important molecules. D-enantiomers are comparatively very low abundant. While even been believed to be absent in higher animals in the past [3,4], now D-AA are the focus of several studies as they are considered to be new bioactive compounds and biomarkers [5]. An accumulation of D-isoleucine, D-valine, D-leucine, and D-methionine in culture media was reported to stimulate the conversion of rod-shaped into spherical bacteria, which illustrates how D-AA can influence peptidoglycan structure and composition [6]. D-tryptophan, D-leucine, D-methionine, and D-tyrosine can inhibit biofilm formation or even disrupt existing ones [7]. Additionally, D-AAs are used in nutritional industry as markers for contamination from microorganism or for food age because D-AAs, like D-alanine or D-glutamic acid, are natural components of bacterial cell walls [8]. Moreover, the isomerization of L to less digestible D-enantiomers leads to a reduced dietary value in processed food products [9]. Main natural sources of D-AAs are next to microorganisms soils, seeds, fruits and tree leaves of several plants [10], but also meteorites have been extensively studied for D/L-AA ratio. This ratio is used to confirm an abiotic origin or possible terrestrial contamination of AAs in the analyzed material [11,12].

To further facilitate research on the presence and biological function of D-AA, sensitive and selective methods need to be developed. The aim of this work was to establish a UHPLC-QqToF-MS (ultra-high performance liquid chromatography – time of flight mass spectrometry) method for AA enantiomeric analysis, which combines the enhanced separation

possibilities of sub-2 µm particles of UHPLCs with the sensitivity and accuracy of mass spectrometric detection [12–14], and that is directly applicable to biological samples. A pre-column derivatization using o-phthalaldehyde (OPA) in combination with the chiral thiol isobuteryl-L-cysteine (IBLC) , a well-established tagging reagent (Figure 1), has been preferred and applied in UHPLC-FLD (fluorescence detection) and UHPLC-QqToF-MS. The challenges of fluorescence detection will be discussed as well as the need of detection method transfer from FLD to the more selective and therefore sensitive MS. The developed method and putative matrix interferences were tested analyzing four very complex biological matrices. Stability issues of AA derivatives were addressed as well.

2. Materials and methods

2.1. Chemicals and reagents

Methanol (Chromasolv, Sigma-Aldrich, St. Lois, USA), acetonitrile (Chromasolv, Sigma-Aldrich) and water (MilliQ) were used in LC-MS quality. OPA, IBLC, IBDC, boric acid and sodium acetate were purchased from Sigma Aldrich, as well as all used amino acid standards (enantiomeric mixtures: D/L-Ala, D/L-Asn, D/L-Asp, D/L-Cys, D/L-Glu, D/L-His, D/L-Ile, D/L-Leu, D/L-Met, D/L-Pro, D/L-Ser, D/L-Val; enantiomer pure standards: L-Arg, L-Gln, L-Lys, L-Phe, L-Thr, L-Trp, L-Tyr, D-Arg, D-Gln, D-Lys, D-Phe, D-Thr, D-Trp, D-Tyr). Compounds are indicated according to IUPAC symbolism for amino acids. Ammonium acetate buffer was bought from Biosolve (Valkerswaard, Netherlands) and an electrospray ionization (ESI) tuning mix from Agilent (Santa Clara, USA) for MS calibration.

92 2.2. Preparation of human serum, human plasma and urine

93

94 The developed UHPLC-FLD and UHPLC-QqToF-MS protocols were tested with human
95 serum (HuS), EDTA-plasma and urine for general suitability and putative matrix
96 interferences. Therefore, we took samples from several individual volunteers and pooled these
97 aliquots. The samples were stored at -80°C directly after sampling until analysis. The reason
98 to test the method with such a mixture of samples taken from many individuals was the
99 increased complexity of pooled samples. We wanted to capture as many possible interfering
100 compounds as possible (e.g. different diets of different persons and therefore different
101 putative interfering compounds). We do not intent to answer any biological question with
102 these experiments..
103 HuS and EDTA-plasma required furthermore protein precipitation prior analysis. After
104 careful thawing on ice, 20 µl of each sample matrix was mixed with 80 µl ice cold methanol
105 and centrifuged (15000rpm, 4°C, 15min). The supernatant was taken, evaporated and resolved
106 in water before injection. Urine was thawed on ice as well and centrifuged to avoid particle
107 injection (15000 rpm, 4°C, 15 min).

108

109 2.3. Mouse gut

110

111 About 100 mg of frozen cecal content of ten week old C57BL/6J mouse was protein-
112 precipitated by using 500 µl cold methanol (-20°C), whereas ceramic beads were used for cell
113 disruption (NucleoSpin bead Tubes, Macherey-Nagel GmbH & Co. KG). Subsequently, the
114 sample was homogenized using TissueLyser II (Qiagen) for 5 minutes at a rate of 30 Hertz.
115 This procedure was repeated three times in order to increase the extraction efficiency.
116 Afterwards, the sample was centrifuged at 14.000 rpm for 10 min at 4°C and the supernatants

were pooled for the analyses. The supernatants were kept in -20°C conditions prior to experiments.

2.4. Derivatization

The derivatization reagent OPA/IBLC was freshly prepared based on a previously described HPLC-FLD method [15]. A methanolic solution of OPA (200 mM) was mixed with 200 mM IBLC prepared in 0.1 M borate buffer corresponding to the moles ratio OPA/IBLC 1/3, which is optimal for the stability of AA derivatives [16]. The pH of the mixture was adjusted to 9 with 1M sodium hydroxide. Derivatization of amino acid in standards and samples was carefully carried out for 2 minutes under a fume hood by mixing 10µl of standards/samples with 20µl derivatization reagents prior injection. This corresponds to an excess of OPA molecules of approximately 1 x 10E8 (for the lowest tested amino acid concentration) and to 10 (for the highest tested amino acid concentration) compared to amino acids molecules in solution (0.0001 - 1.5 ppm). All glassware were heat sterilized before usage [12]. Blank samples consisting of pure solvent were included in all analysis.

2.5. UHPLC-FLD

Enantiomeric separation was initially achieved using a ACQUITY UPLC® system (Waters, Milford, USA) coupled to a fluorescence detector, which has been set to λ=300 nm for the excitation and λ= 445 nm for the emission. A BEH-C18 column with dimensions of 2.1 x 150 mm and 1.7 µm particle size (Waters) was applied with a column temperature of 30 ºC. The auto-sampler was set to 25 ºC. Mobile phase A consisted of 20 mM sodium acetate buffer that

was adjusted to pH 6.2 with acetic acid. Mobile phase B was composed of 7% acetonitrile in methanol [15]. The gradient was optimized with a flow rate of 0.35 ml/min (Table 1).

2.6. UHPLC-QqToF-MS

The ACQUITY UPLC® was coupled to a QqToF-MS (maXis, Bruker Daltonics, Bremen, Germany) to allow mass spectrometric detection. The maXis mass spectrometer is a hybrid QqToF, dual stage reflector instrument, which uses orthogonal ion acceleration. “Q” (capital letter) refers to a mass selective mass resolving quadrupole, whereas “q” indicates a quadrupole collision cell. It is therefore possible to isolated selected masses in the mass isolating quadruple and fragment these isolated masses of interest in the second quadrupole. The mass fragments can thereafter be measured according to their time of flight in the flight tube. Slight modifications of the UHPLC-FLD method were necessary to ensure compatibility with electrospray ionization. Sodium acetate was exchanged with ammonium acetate (2.5 mM). The flow rate was reduced to 0.2 ml/min for the same reason. Additional parameters were kept constant. Mass spectrometric parameters were optimized in order to achieve highest sensitivity in the mass range of the amino acid derivatives (350-500 Da) within an acquisition window of 50-1500 Da and a scan rate of 2 Hertz.

3. Results

3.1. UHPLC-FLD matrix interferences followed by method transfer

Baseline separation of enantiomeric amino acid standards could be achieved within 34 minutes using the sensitive UHPLC-FLD method (Supplementary Figure 1). Nevertheless,

separation of standard materials is so far easily achievable, but in daily routine the analyst has to deal with very complex samples. FLD detection turned out to be not well suited for such studies as e.g.strong interferences from derivatization reagents and samples themselves have been recognized (Supplementary figure 2). Since besides primary amino acids, amino esters, amino alcohols, alkyl- and aryl-amines, and heterocyclic amines react with OPA [17], a higher baseline and co-elution for some D-AA has been observed. This increases the limit of detection and might lead in miss-interpretation if identification solely relies on retention time comparison with standard compounds. Extensive sample clean-up might overcome this problem, but requires time and bargains the risk of contaminations and sample alterations. Thus, analytical methods insensitive for such interferences are on demand and consequently we transferred the method to UHPLC-QqToF-MS. The flow rate and buffer of mobile phase A were therefore adjusted. Figure 2 shows the extracted ion chromatogram (EIC) obtained for a derivatized AA standard mixture. Proline as a secondary amine does not react with OPA/IBLC reagent, glycine is achiral and was consequently not included in our study, and cysteine could not be detected. Additionally, the early elution of aspartic acid (t_R 4.1 min) resulted in poor resolution between its D and L forms showing a single peak. With these exceptions, baseline separation was achieved for all proteinogenic AA enantiomeric pairs. The high resolution ToF-MS allowed us to targeted extract m/z valuesof each AA derivative with accuracy of m/z ± 0.001 (exept Asp 0.005, Glu 0.005, Phe 0.002 and Val 0.005), which provided clean chromatograms.

3.2. LOD and linearity in UHPLC-QqToF-MS

Amino acid standards of 0.5 - 1 ppm concentration were used to determine the limits of detection, which was calculated as LOD = conc. AA [mmol/l] x 3 / SNR for pure standards

solutions and as LOD = basic AA conc + spiked conc. AA [mmol/l] x 3 / SNR at spiked concentration for biological matrices. Signal to noise ratio (SNR) was elaborated with Bruker Daltonics Data Analysis 4.2. It needs to be considered that the LOD may vary when analyzing different sample matrices. Here, we present data for pure standards (Table 2) and spiked plasma samples (Table 3). The signal-concentration response followed a linear relationship for both standard solution (data not shown) and plasma in the concentration range of 0.0001 ppm to 1.5 ppm (Table 3). Depending on the matrix and AA of interest it might be necessary to dilute the matrix before spiking the calibration curve due to the high basic concentration.

3.3. Stability of derivatization product

An additional crucial point is the stability of the derivatization product. Derivatization is completed after 2 min at room temperature and has been known to be stable for at least 18 min [15]. We tested the peak area response of the solutions of these standards after 2, 20, 40 and 60 min and observed stable signals of derivatization products for at least 40 min (97.5 - 101.8 % of original detected value). Thereafter, the signal response decreased (Figure 3). Thus, we strongly suggest the derivatization directly prior injection. A lab robot might be useful for automation of this procedure. Putative racemization of amino acids during derivatization and chromatography can be excluded because we confirmed the absence (or very low abundance as indicated by the enantiomeric purity of the vender) of the other enantiomer for several amino acids in standard mixtures and in biological samples of various concentrations (exemplarily illustrated for L-Arg, L-Asp and L-Trp in Supplementary Figure 3).

3.4. Applicability to important biological matrices

The developed UHPLC-QqToF-MS method was finally tested in four different biological matrices to evaluate possible signal interferences caused by the sample matrix itself. Due to the application of QqToF-MS, specific detection of proteinogenic amino acids could be achieved without matrix-interferences signals. Chromatograms for alanine, serine and methionine for standards and tested biological matrices are exemplarily illustrated in Figure 4 as their derivatization and ionization behavior can be used as approximation of the other amino acids [13]. The detected exact mass of AA derivatives and retention time allow certain identification. We observed very small retention time shifts when injecting different matrices (Figure 4), e.g. retention time shifts for D-AA standards in water and plasma were between 0.1 and 0.3 min, which shows a high reproducibility. Additionally, MS^2 fragmentation information can be easily obtained by various MS instrument if uncertainties in the identity of any AA exist.

Due to very presumable different matrix effects of each sample type in electrospray ionization, we relinquish to give absolute concentrations and strongly suggest determining crucial quantification parameters for each specific application. The biological matrices were used here with the purpose to test the analytical protocol for putative inferences or any other analytical problem. Consequently, we repetitively analyzed pooled samples of the selected matrices. We did not intent to answer any biological question.

4. Discussion

The increasing scientific interest in D-AA requires more and more sensitive and selective analytical protocols. Due to several facts, like the possibility for reliable chiral separation, coverage of a very high number of analytes and suitability for full automation, liquid

chromatography (LC) has been applied for chiral separation [4,15,18]. Existing HPLC-FLD protocols are particularly challenged by matrix interferences, the very low abundances of D-AAs and huge concentration differences compared to their L-enantiomers. UHPLC, which offers a much higher separation efficiency and chromatographic speed due to the application of sub-2 µm porous particles [19, 20], in combination with the precise and sensitive detection of mass spectrometry is one way to improve enatioseparation and reach the analytical needs currently occurring.

Two strategies can be followed to resolve enatiomers in LC systems, first the application of chiral stationary phase and secondly enatioseparation based on a pre-column derivatization. Several facts led us to use pre-column derivatization for the development of our UHPLC method. The most important ones are a higher efficiency, which can be reached using chiral derivatization reagents followed by reversed phase (RP) chromatography [4], the missing availability of chiral material with sub-2µm particles for UHPLCs and a better sensitivity that can be reached with RP columns due to the high number of analytical plates. Commonly, pre-column derivatization uses fluorescence tagging for detection [15,21–23]. Unfortunately, although fluorescence detection is one trustworthy detection method, complex biological samples contain endogenous compounds that are likely to cause signal interference or may co-elute with some D-AA, which is a source of variability and inaccuracy. Mostly primary amino acids, amino esters, amino alcohols, alkyl- and aryl-amines and heterocyclic amines react with OPA [17]. Samples need consequently either an intensive clean-up or more precise detection methods are necessary. FLD application have been therefore limited to few amino acids [13]. We also observed such problems when we applied our developed UHPLC-FLD method to differently composed biological matrices and decided thus to transfer the method to UHPLC-QqToF-MS, which allows to isolate the exact masses of the derivatization products of each amino acid and is in consequence insensitive for most matrix interferences signals.

This leads eventually to a method that is much more extensive in its application. This potential of UHPLC applications in combination with pre-column derivatization for chiral separation of amino acids has been hardly described. Only three references could be found. Zhe Min developed a new florescence tagging reagent (DBD-PyPNCS) for the analysis of amino acids in human nails [14]. Visser compared NDB-PyNCS, GITC, AMBI, OPA/IBLC, S-NIFE, Marfeys- and Sangers reagent [13]. In their publication it was shown that NIFE delivered best sensitivity for alanine, serine and methionine followed by OPA/IBLC [13]. However, we decided to proceed with OPA/IBLC due to availability issues and achieved a 10 times lower LOD for OPA/IBLC compared to the published method. One further argument to proceed with OPA/IBLC is the possibility to exchange the elution order of D and L enantiomers simply by using IBDC instead of IBLC, which allows the verification of the abundance of the D-AA and might be helpful in some rare cases in which a co-eluting peak has the same mass as the AA derivative. Additionally, the exchange is very advantageous if the L-form is present in much higher concentration than the D-AA, which might be the case of bacterial cell cultures or chemically defined medium.. The development of the presented method was motivated by the fact that traditional fluorescence detection alone cannot offer the level of certainty necessary to analyze D-AA in biological samples. Although there is still some place for improvement, our MS detection allows sensitive, specific targeted and relatively fast differentiation of proteinogenic AA enantiomers in complex matrices. Analytical performance should be always evaluated for any specific application and sample matrix due possible interference during ESI.

5. Conclusions

A simple applicable, specific and sensitive UHPLC-MS method for differentiation of AA using chiral derivatization with OPA/IBLC reagent and RP chromatography has been presented. The widely implemented stationary phase C18 prevents the analyst of buying special and more expensive chiral columns. It offers moreover a high number of analytical plates and consequently very good separation efficiency. The exchangeability of the elution order of L- and D- enantiomers simply by usage of IBDC instead of IBLC might be very useful for some applications. Most importantly, the very sensitive and selective MS detection overcomes misinterpretation of detected peaks, which can result from interference signals in complex samples when methods solely rely on fluorescence detection.

6. Acknowledgment

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7. Figure caption

Figure 1: Derivatization reaction between OPA, primary amino acid and IBLC, which contains the thiol group for enantioseparation of DL-amino acids and fluorescence detection. Besides primary amino acids, their esters, amino alcohols, alkyl- and aryl-amines, heterocyclic amines react with OPA resulting in strong interferences for complex samples. The condensation between the amino group and the aromatic o-dicarboxaldehyde leads in an N-substituted isoindolin-1- one (phthalimidine) derivative [17].

Figure 2: UHPLC-MS extract ion chromatogram (EIC) obtained for the reversed phase enantioseparation of OPA/IBLC derivatized amino acid standards at concentration of 0.5 and 1 ppm (Thr, Asp, Lys, Phe, Arg, Glu, Tyr, and Trp)

Figure 3: Stability of detected peak area after t_0+x min

Figure 4: Extracted Ion Chromatograms (±0.01) of alanine, serine and methionine for standards, mouse gut, HuS, plasma and urine. Retention time windows of D-AA are marked in grey and enlarged illustrated, if the D-enantiomer has been detected.

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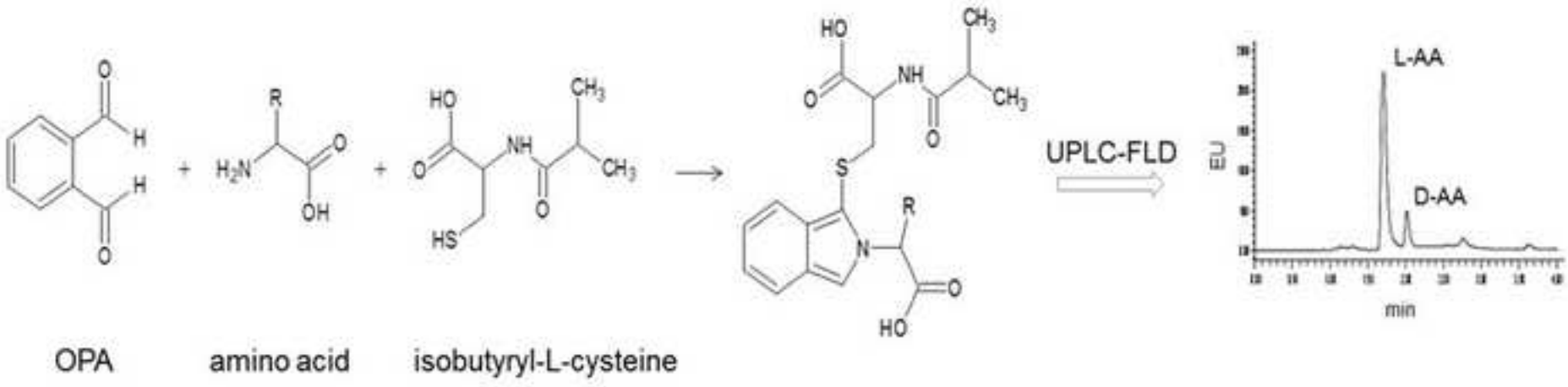
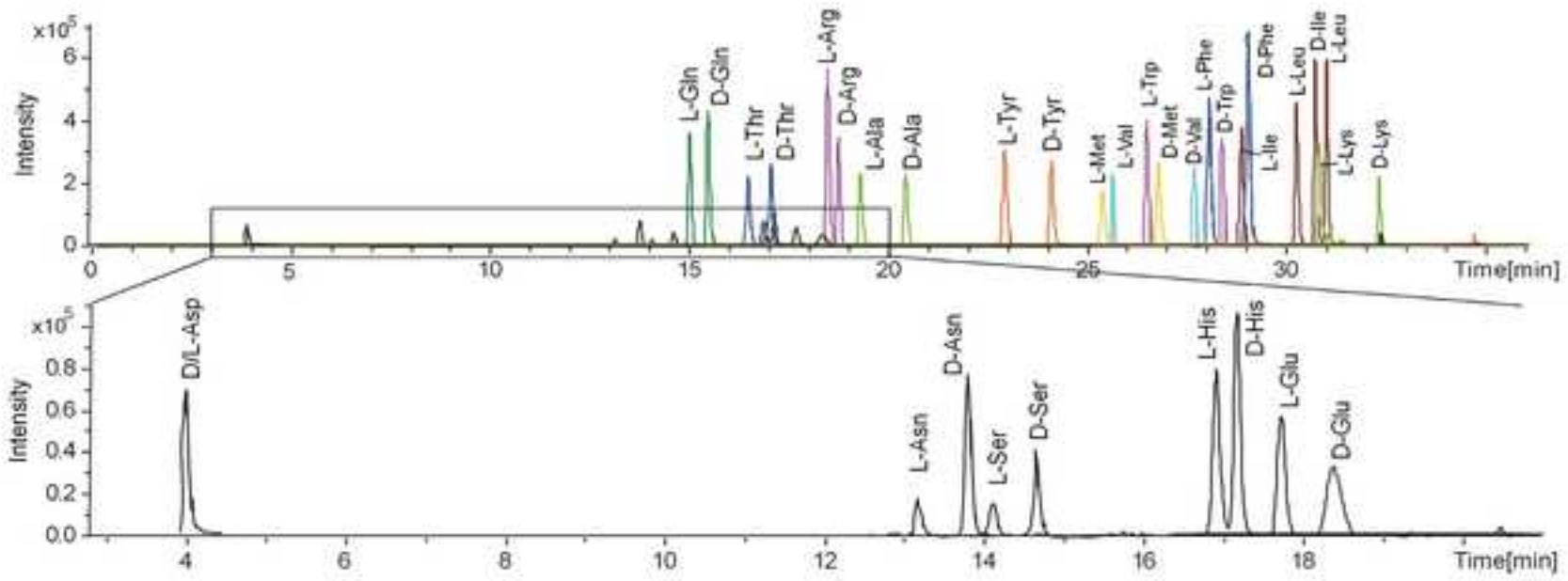
355

- UHPLC-QqToF-MS method using pre-column derivatization for amino acid analysis
- Discussion of the advantages of the implemented MS detection over FLD
- Enantioseparation of D and L isomers with OPA/IBLC
- No detection of matrix interferences in biological samples
- Superb sensitivity for trace level analysis of D-amino acids

Figure



Figure


x10^5
Intensity
L-Gln
D-Gln
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D-Thr
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D-Arg
L-Ala
D-Ala
L-Tyr
D-Tyr
L-Met
L-Val
L-Trp
D-Met
D-Val
L-Phe
D-Trp
D-Phe
L-Ile
L-Leu
D-Ile
L-Lys
D-Lys
Time[min]
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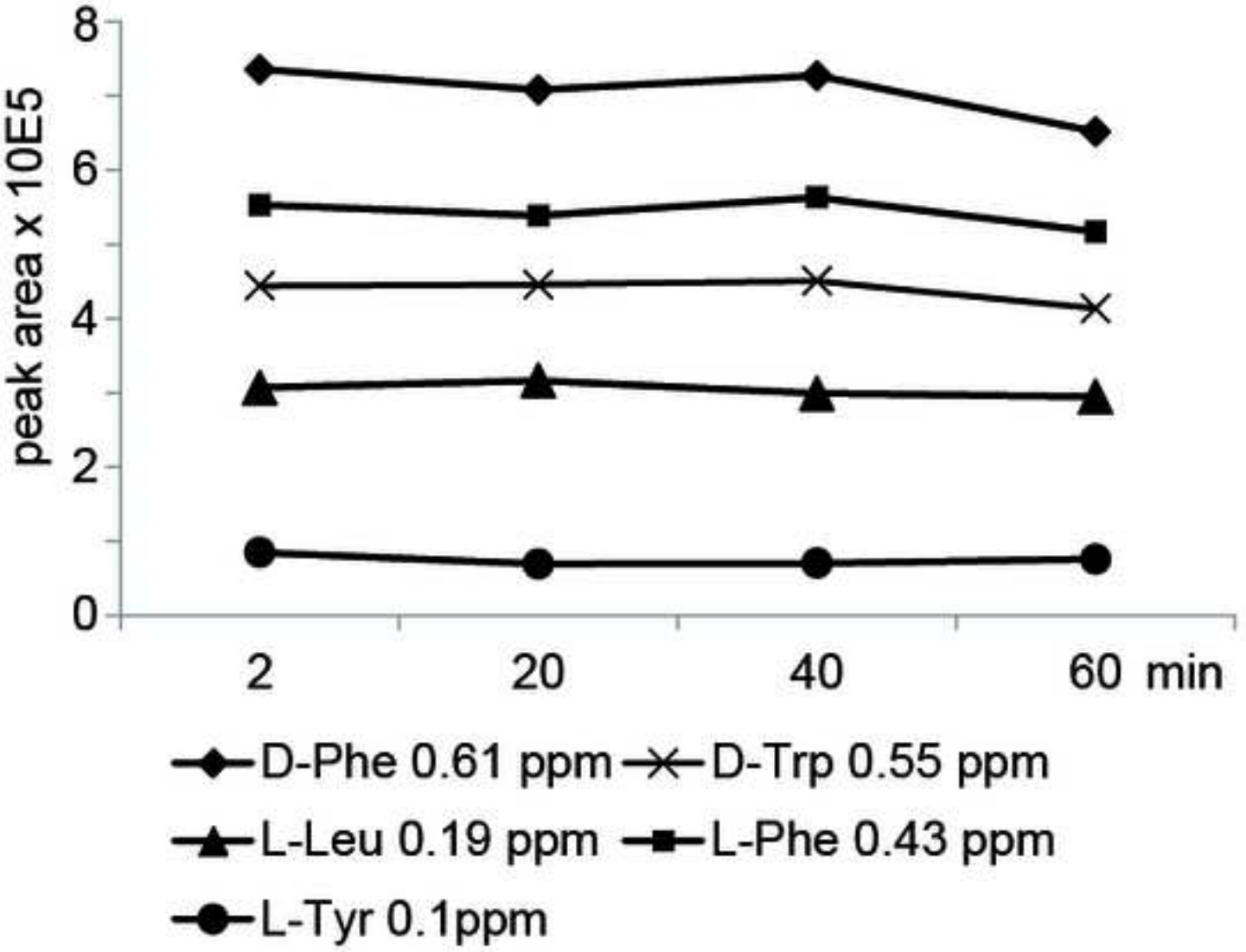
D-Ser
L-His
D-His
L-Glu
D-Glu

Figure


peak area x 10E5
8
6
4
2
0
2
20
40
60 min
D-Phe 0.61 ppm
D-Trp 0.55 ppm
L-Leu 0.19 ppm
L-Phe 0.43 ppm
L-Tyr 0.1ppm

Figure

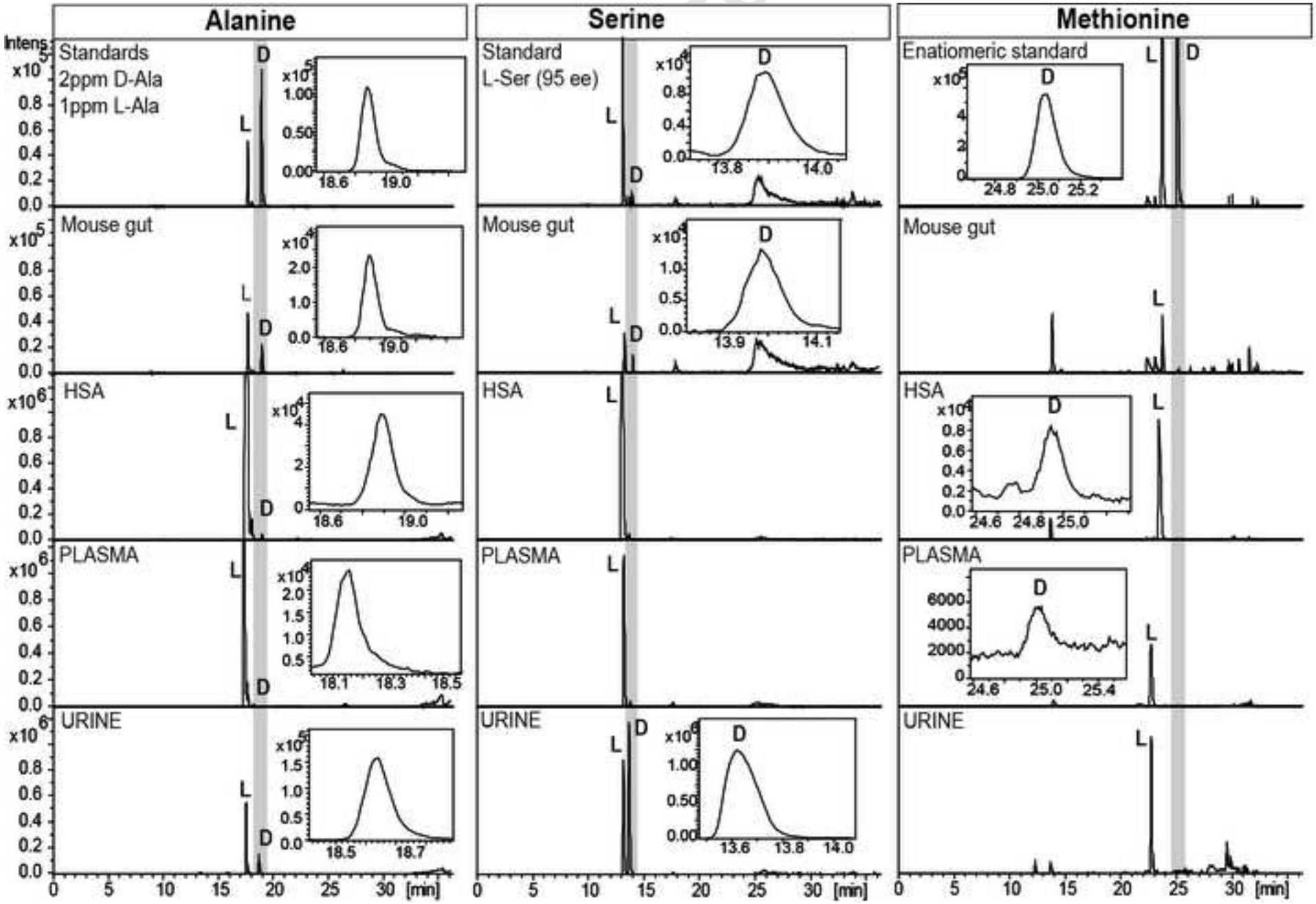

Alanine
Serine
Methionine
Intens.
Standards
2ppm D-Ala
1ppm L-Ala
Standard
L-Ser (95 ee)
Enatiomeric standard
Mouse gut
HSA
PLASMA
URINE
L
D
[min]

Table 1: Optimized gradient for enantiomeric separation of AA

time (min)	**Flow (mL/min)**	**%A**	**%B**
0	0.350	100.0	0.0
15.00	0.350	70.0	30.0
28.00	0.350	50.0	50.0
31.00	0.350	20.0	80.0
32.00	0.350	20.0	80.0
33.00	0.350	10.0	90.0
34.00	0.350	10.0	90.0
34.50	0.350	100.0	0.0

Table 2: Masses, retention time, and limits of detection for D-amino acids standards measured by UHPLC-MS using OPA/IBLC derivatization

D - AA	**$[M+H]^{+}$ [a] (m/z)**	**RT (min)**	**LOD (pmol/L)**
Ala	379.1322	20.5	11.46
Arg	464.1962	18.8	8.27
Asp	422.1380	13.8	27.09
Asn [b]	423.1221	4.1	76.43
Gln	436.1537	15.4	8.93
Glu [b]	437.1377	15.3	29.33
His	445.1540	17.1	19.22
Ile	421.1792	30.7	2.79
Leu	421.1792	31.0	2.83
Lys	436.1901	32.4	24.75
Met	439.1356	26.6	12.25
Phe	455.1635	28.8	7.31
Ser	395.1271	14.6	62.52
Thr	409.1428	17.1	40.17
Trp	494.1744	28.2	10.47
Tyr	471.1584	22.2	3.82
Val	407.1635	27.7	6.87

[a] = calculated masses for protonated molecules of AA derivatives, [b] = limited soluble in water under used conditions

Table 3: Masses, LODs and calibration parameters for D-amino acids measured in plasma sample matrix by UHPLC-MS using OPA/IBLC derivatization. 4 calibration points were used except for aspartic acid, glutamic acid and lysine.

D - AA	$[M+H]^{+a}$ (m/z)	calibration range (mg/L)	calibration curve y = aC + b	R^2	LOD (pmol/L)
Ala	379.132	0.01 - 0.75	y = 2 003 274.81 C + 294 580.47	0.9432	21.75
Arg	464.196	0.0002 - 1.0	y = 740 377.10 C + 127 178.72	0.9807	19.6
Asn	422.138	0.01 - 0.75	y = 641 037.87 C + 66 622.62	0.9577	28.37
Asp [b]	423.122	-	-	-	-
Gln	436.154	0.0002 - 1.0	y = 1 532 437.43 C + 239 481.50	0.9808	16.83
Glu [b]	437.138	-	-	-	-
His	445.154	0.0001 - 0.5	y = 577 640.12 C + 41 467.93	0.9865	61.23
Ile	421.179	0.0001 - 0.75	y = 3 519 703.40 C + 326 974.14	0.9951	7.78
Leu	421.179	0.0001 - 0.75	y = 2 880 663.20 C + 163 095.19	0.9965	7.08
Lys [c]	436.190	-	-	-	-
Met	439.136	0.0001 - 0.75	y = 1 856 244.56 C + 53 060.88	0.9903	22.11
Phe	455.164	0.0002 - 1.0	y = 2 859 517 C + 216 411.03	0.9988	20.63
Ser	395.127	0.0001 - 0.75	y = 794 772.77 C + 51 842.50	0.9973	33.92
Thr	409.143	0.0002 - 1.5	y = 1 158 402.21 C + 65 359.65	0.9969	79.43
Trp	494.174	0.0002 - 1.0	y = 1 237 427.67 C + 37 510.10	0.9975	20.39
Tyr	471.158	0.0002 - 1.5	y = 248 853 C + 54 497	0.9780	64.35
Val	407.164	0.01 - 0.75	y = 2 278 485.89 C + 224 692.79	0.9830	159.18

[a] = calculated values for protonated molecules of AA derivatives, [b] = limited soluble in water under used conditions, [c] = basic concentration too high, LOD calculation possible with 10 fold dilution of the matrix