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Comparison of liquid chromatography with tandem mass spectrometry and ion-exchange chromatography by post-column ninhydrin derivatization for amino acid monitoring

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ABSTRACT

Objectives: Precise quantification of amino acids (AAs) is mandatory for successful diagnosis and monitoring of patients with metabolic diseases. We compared ion-exchange chromatography (IEC) and liquid chromatography with tandem mass spectrometry (LC-MS/MS), the two methods most commonly used in clinical laboratories for the quantification of AAs in physiological samples.

Design & methods: 123 apparently healthy children were selected for the study. The plasma samples for LC-MS/MS were prepared accordingly to the aTRAQ Kit for Physiological Fluids on Sciex 3200 Qtrap, for IEC according to the protocol from Pickering laboratories on the AA analyzer Pinnacle PCX. Results were interpreted using the Pearson correlation coefficient and the percent difference Bland-Altman test.

Results: The Spearman correlation coefficients of the 14 AAs that we evaluated varied from 0.67 in Tau to 0.89 in Leu and Thr. The mean differences in measurements (IEC compared to LC-MS/MS) of 11 AAs complied with our acceptance criterion of < 15%, the differences of Ser and Tyr were higher (19.5% and −19.0%, respectively), and the measured concentrations of Cit were much lower in LC-MS/MS than IEC (31% difference).

Conclusion: The two methods are sufficiently comparable for most AAs and the reference values for individual AAs did not have to be refined, with the exception of citrulline. For the monitoring of patients on therapy (e.g. patients with phenylketonuria), it is still advisable to always use the same analytical method for the quantification of AAs.

1. Introduction

Amino acids (AAs) in biological samples of the pediatric population are important biochemical indicators of a group of inborn errors of metabolism, aminoacidopathies. Inborn errors of AA metabolism can be expressed at any time, but they are most common in the first year of the child's life and represent an important proportion of pediatric diseases. The diseases often remain clinically unrecognized, because of the low incidences of the diseases and varied accompanying clinical signs [1–3]. Quantitative analysis of AAs in biological fluids is essential for the diagnosis of aminoacidopathies and the monitoring of the diet and treatment of patients with known metabolic disorders and is, therefore, extremely important for the prevention of irreversible damages to the body. Aminoacidopathies that can be detected by quantification of AAs in biological samples are phenylketonuria, tyrosinemia, maple syrup urine disease, citrullinemia, non-ketotic hyperglycinemia, and others [4,5].

The methods for AA quantification must be sensitive, specific and accurate. Moore and Stein were the first to develop an automated AA analyzer in the 1960s based on ion-exchange chromatography [6]. Today the methods run either on ion-exchange chromatography or gas or liquid chromatography with different detectors [7]. Ion exchange chromatography with post-column ninhydrin (IEC) derivatization is considered the gold standard for the analysis of AAs in biological samples and is still the most commonly used method in clinical laboratories, because of its repeatability, wide dynamic range, excellent range of linearity and simple preparation of samples. The major downside of the method is the long analytical time and aggressive

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buffers used for separation of amino acids. In recent years, the use of the liquid chromatography with tandem mass spectrometry (LC-MS/MS) has increased, which is much quicker than IEC and is also very sensitive and selective [8,9].

For precise monitoring of patients with metabolic diseases, it is important that we compare the various methods we use with each other and determine whether the results measured by different analytical systems can affect clinical decisions. The purpose of this study was to compare the results of AA analysis in children's plasma samples with the two methods used most commonly in clinical laboratories for the quantification of AAs in physiological samples. The AAs in the plasma samples were quantified by the LC-MS/MS and IEC method to define the benefits and weaknesses of each method and evaluate the inter-method differences in measured AA concentrations and their clinical implications.

2. Material and methods

2.1. Blood sample collection

123 apparently healthy children were selected for the study. They were aged 2 to 18 years, were not on any medications or other dietary supplements and had visited the hospital for gastroscopy, in which the small bowel disease was not proven (normal macroscopic and microscopic examination in gastroduodenoscopy). Venous blood samples were collected into heparinized tubes and sent to the laboratory. The samples were kept cold during transport. The plasma for the analysis was immediately separated from the cells (at the latest after 2 h after the blood was taken) and stored at −20 °C until the LC-MS/MS analysis was performed. For the IEC analysis the aliquots of plasma samples were deproteinized and frozen at −20 °C until use. Both methods were used routinely in our laboratory.

Accuracy was determined with measurement of pooled plasma, which was done by mixing plasma of random samples of healthy children and stored at −20 °C until use. To determine precision of the method a different control material Control Amino Acids (MCA Laboratory, Netherlands) was used for the two methods. Written informed consent/assent was obtained from all the participants and/or their parents. The principles of the Declaration of Helsinki were followed and the study was approved by the Slovenian National Medical Ethics Committee (#97/10/12).

2.2. Amino acid quantification by LC-MS/MS

For the analysis of AAs in plasma with the LC-MS/MS, we prepared the samples as described in the protocol of the reagent aTRAQ Kit for Physiological Fluids (Sciex, Massachusetts, USA). After precipitation of proteins with sulphosalicylic acid the AAs were derivatized with the aTRAQ reagent. After stopping the derivatization with hydroxylamine the aTRAQ internal standard is added and the prepared samples were analyzed with LC-MS/MS. The samples were analyzed in multiple reaction monitoring mode on AB Sciex 3200 Qtrap tandem mass spectrometer (Sciex, Massachusetts, USA), Perkin Elmer Series 200 HPLC system (Perkin Elmer, Massachusetts, USA). Chromatographic separation of derivatized samples was achieved at 50 °C on a C18 4.6 × 150 mm column, provided with the aTRAQ kit. The mobile phases consisted of water (LiChrosolv®, Merck, Darmstadt, Germany) as mobile phase A and methanol as mobile phase B (LC-MS Reagent, J.T. Baker, Deventer, The Netherlands), both containing 0.1% formic acid (Sciex, Massachusetts, USA) and 0.01% heptafluorobutyric acid (Sciex, Massachusetts, USA). The flow was set at 0.8 mL/min with the percentage of mobile phase B increasing from 2% to 90% after 12 min of the run, followed by a 10 min re-equilibration step.

2.3. Amino acid quantification by IEC

Quantitative analysis of AAs with IEC was done on the amino acid analyzer Pinnacle PCX (Pickering Laboratories, California, USA) with HPLC Agilent Technologies 1200 Series (Agilent Technologies, California, USA). The buffers and ninhydrin reagents were from Pickering Laboratories (California, USA). Amino acid Standard Solution, Physiological acidics and neutrals, Physiological basics were acquired from Sigma (Darmstadt, Germany). Additionally, L-glutamine was purchased from Fluka-BioChemika (Buchs, Switzerland).

Blood samples in Li-heparin were deproteinized after centrifugation with mixing equal parts of plasma and Seraprep (Pickering Laboratories, California, USA) and filtration through filters of 0.2 µm (Minisart, Sartorius, Göttingen, Germany). After injection of 100 µL of sample in the IEC the AAs were separated according to the standard manufacturer protocol using changes in ionic strength, pH of the mobile phase and the temperature of the lithium cation exchange column (4.6 × 75 mm, 5 µm) with column guard (2.0 × 20 mm). After the column separation, the samples were derivatized with ninhydrin and the formed colored complex was detected spectrophotometrically at a wavelength of 570 nm for all AAs, except for proline and hydroxyproline, which were detected at 440 nm. The intensity of the color complex was proportional to the concentration of the AAs in the sample. The identification and quantification of amino acids in the samples was based on the comparison of retention times and signal responses in standard solutions with known AA concentrations using single point calibration. Areas under the top of individual AAs were directly proportional to its concentration in the sample. The runtime of the method was 115 min.

2.4. Statistical analysis

The accuracy and precision of both AA quantification methods were checked by the analysis of the internal quality control material (pool plasma sample) and AA control material Control Amino Acids (MCA Laboratory, Winterswijk, Netherlands). Inter-laboratory variation was assessed by participation in ERNDIM (www.erndimqa.nl) and INSTAND (www.instandev.de) external quality control schemes.

Statistical analysis was done in the GraphPad Prism software v6.0 (GraphPad Software Inc., USA). The Pearson correlation coefficient was determined to assess the association between the methods. The percent difference Bland-Altman test was calculated to determine the 95% limits of agreement between IEC and LC-MS/MS. The Bland-Altman test was interpreted from a clinical point of view, so the differences between the methods had to be small enough to not affect clinical decisions. Otherwise, the reference values had to be revised.

3. Results

Accuracy of the methods was determined by making altogether 20 measurements of pooled plasma on different days for internal analysis control while measuring children's samples (Table 1).

To determine precision two different lots of control material was used, one for LC-MS/MS and the other for IEC. All samples were measured 7 times on consecutive days and the values was compared to the ones given by the manufacturer (Table 2).

Altogether, 123 samples were analyzed by IEC and LC-MS/MS and 14 quantified AAs were used for the comparison. Summary values of all AAs quantified by both methods are listed in Table 3.

To test the normality of the distribution of differences between measurements between the two methods the Kolmogorov-Smirnov test showed that the distribution was not normal, the values of p were in all cases less than the accepted risk (α 0.05). The logarithmic values of the differences were also not normally distributed. Therefore, the Spearman coefficient of correlation was used to calculate the correlation between the measurements done by both methods. Correlation coefficients,

Table 1
Accuracy of LC-MS/MS and IEC, determined by measuring pooled samples.

Amino acid $n = 20$	Method	Average (μmol/L)	SD (μmol/L)	RSD (%)
Alanine	LC-MS/MS	498	34.0	6.8
	IEC	556	19.0	3.4
Arginine	LC-MS/MS	133	10.8	8.1
	IEC	147	6.0	4.1
Citrulline	LC-MS/MS	26.6	1.7	6.5
	IEC	32.5	4.4	13.5
Glycine	LC-MS/MS	371	29.9	8.7
	IEC	365	10.9	3.0
Isoleucine	LC-MS/MS	87.5	3.8	4.3
	IEC	77.7	3.4	4.4
Leucine	LC-MS/MS	161	7.5	4.6
	IEC	168	7.1	4.2
Lysine	LC-MS/MS	174	19.2	11.0
	IEC	209	8.9	4.3
Ornithine	LC-MS/MS	87.2	12.9	14.8
	IEC	95.4	3.1	3.3
Phenylalanine	LC-MS/MS	126	5.9	4.6
	IEC	213	8.1	3.8
Serine	LC-MS/MS	234	17.6	7.5
	IEC	199	6.8	3.4
Taurine	LC-MS/MS	148	10.6	7.1
	IEC	138	17.8	12.9
Tyrosine	LC-MS/MS	84.0	3.7	4.4
	IEC	72.0	3.9	5.4
Threonine	LC-MS/MS	127	8.8	6.9
	IEC	145	5.0	3.4
Valine	LC-MS/MS	262	15.2	5.8
	IEC	258	11.1	4.3

Table 3
Summary values of 14 amino acids in 123 samples.

Amino acid $n = 123$	Method	Lowest value (μmol/L)	Highest value (μmol/L)	Average (μmol/L)	Standard deviation (μmol/L)
Alanine	LC-MS/MS	102.2	720.6	290.2	109.5
	IEC	143.3	636.2	294.1	81.5
Arginine	LC-MS/MS	10.3	100.6	38.9	13.7
	IEC	9.5	96.5	40.5	14.3
Citrulline	LC-MS/MS	8.1	46.6	23.3	7.0
	IEC	10.6	86.2	32.2	10.7
Glycine	LC-MS/MS	110.7	485.3	245.5	62.6
	IEC	123.5	378.5	228.0	54.3
Isoleucine	LC-MS/MS	25.8	123.4	56.2	15.5
	IEC	24.3	96.5	51.3	13.7
Leucine	LC-MS/MS	41.1	248.5	102.6	31.5
	IEC	46.5	175.8	99.2	24.8
Lysine	LC-MS/MS	65.6	280.1	138.8	35.9
	IEC	71.5	249.7	144.5	29.6
Ornithine	LC-MS/MS	29.1	140.3	72.1	23.6
	IEC	32.0	146.5	74.7	21.6
Phenylalanine	LC-MS/MS	28.0	83.2	43.5	9.9
	IEC	31.6	92.6	48.6	9.1
Serine	LC-MS/MS	78.5	254.4	135.7	32.4
	IEC	68.6	165.2	110.2	20.1
Taurine	LC-MS/MS	31.2	176.7	89.9	26.7
	IEC	29.9	255.4	89.9	33.4
Tyrosine	LC-MS/MS	17.0	95.6	43.1	12.5
	IEC	21.2	90.5	51.6	11.8
Threonine	LC-MS/MS	43.0	215.0	113.9	30.7
	IEC	39.9	185.1	107.3	25.8
Valine	LC-MS/MS	88.1	382.4	199.1	51.3
	IEC	88.6	283.5	181.8	41.2

which give the power of a linear connection between comparable analytical methods, for 14 AAs varied from 0.67 in Tau to 0.89 in Leu and Thr.

The AA levels obtained by the IEC were each compared to those obtained by the LC-MS/MS using Bland-Altman analysis (Fig. 1). The acceptance criterion we have set and which does not affect the clinical decision is < 15% differences in mean values of measurements between methods. The mean difference in measurements of 11 AAs complied with our acceptance criterion, differences of Ser and Tyr were slightly above the acceptance criterion (19.5% and − 19.0%, respectively), but the measured concentrations of Cit exceeded the criterion considerably (31% difference).

Table 2
Determination of precision of LC-MS/MS and IEC for amino acids measurement.

Amino acid $n = 7$	Method	Control range (μmol/L)	Expected mean value (μmol/L)	Average (μmol/L)	SD (μmol/L)	RSD (%)	RE (%)
Alanine	LC-MS/MS	307–435	371	416	33.6	8.1	12.2
	IEC	538–778	658	702	14.9	2.1	6.7
Arginine	LC-MS/MS	245–387	316	306	24.0	7.9	−3.2
	IEC	215–329	272	290	2.2	0.7	6.7
Citrulline	LC-MS/MS	95–145	120	116	7.4	6.4	−3.5
	IEC	245–407	326	339	2.8	0.8	4.0
Glycine	LC-MS/MS	408–614	511	570	50.2	8.8	11.6
	IEC	602–886	744	798	13.7	1.7	7.3
Isoleucine	LC-MS/MS	201–309	255	245	16.2	6.6	−3.8
	IEC	136–204	170	172	1.8	1.1	1.4
Leucine	LC-MS/MS	314–478	396	378	16.5	4.4	−4.5
	IEC	334–550	442	480	40.3	8.4	8.3
Lysine	LC-MS/MS	145–197	171	170	21.6	12.7	−0.8
	IEC	284–438	361	375	4.2	1.1	3.8
Ornithine	LC-MS/MS	266–388	327	299	26.1	8.7	−8.5
	IEC	220–334	277	283	3.2	1.1	2.2
Phenylalanine	LC-MS/MS	281–419	350	333	12.3	3.7	−4.9
	IEC	403–643	523	547	6.5	1.2	4.7
Serine	LC-MS/MS	122–178	150	162	16.0	9.8	8.2
	IEC	122–174	148	130	1.8	1.4	−12.0
Taurine	LC-MS/MS	115–165	140	135	6.7	4.9	−3.6
	IEC	135–203	169	183	15.9	8.7	8.3
Tyrosine	LC-MS/MS	68–97	82.6	82.2	6.3	7.7	−0.4
	IEC	210–328	269	277	3.4	1.2	2.9
Threonine	LC-MS/MS	138–182	160	156	13.3	8.5	−2.3
	IEC	122–172	147	153	6.1	4.0	4.0
Valine	LC-MS/MS	332–446	389	409	18.3	4.5	5.2
	IEC	354–542	448	469	7.0	1.5	4.8

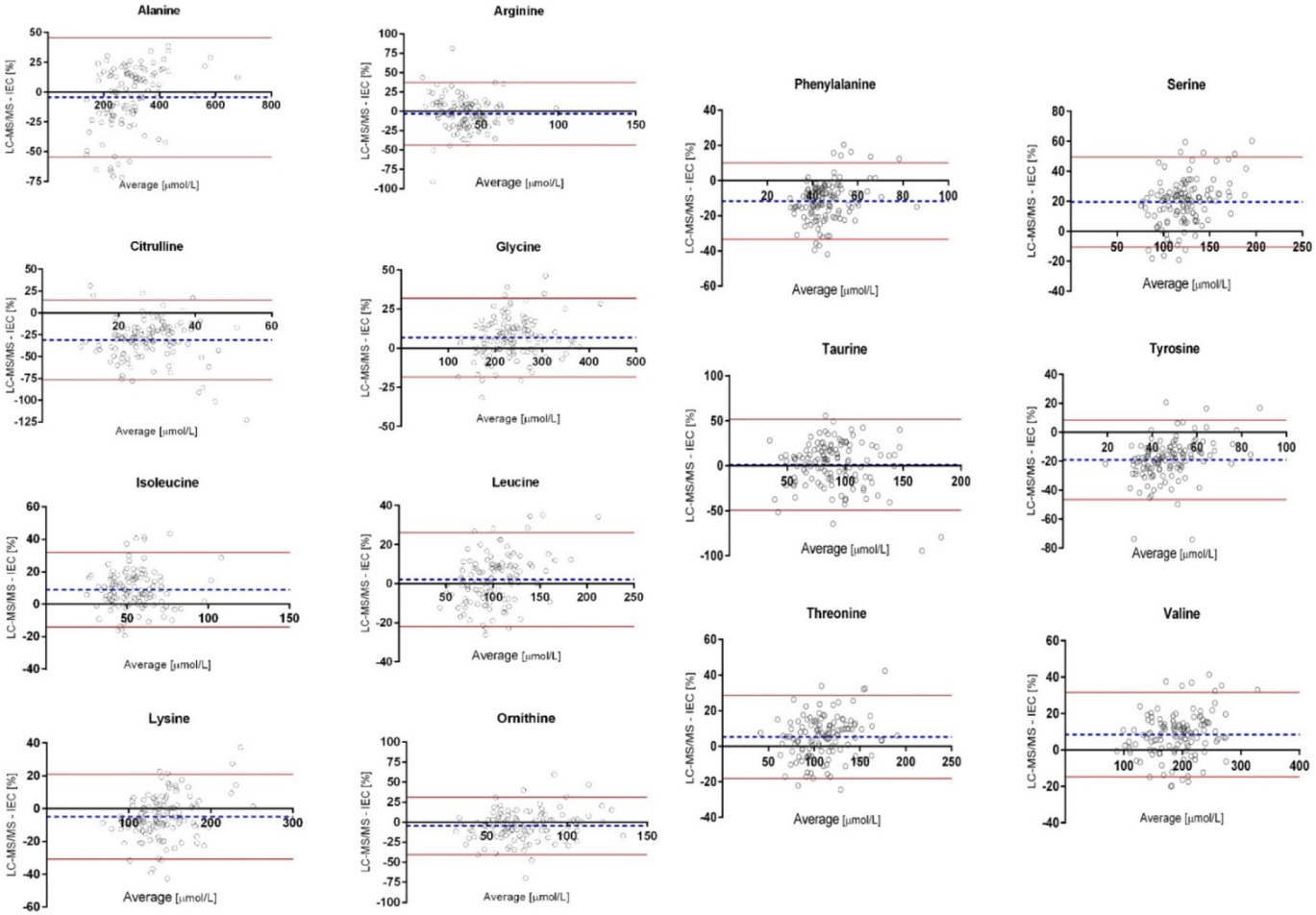

Fig. 1. The Bland-Altman analysis of the difference (in %) between liquid chromatography with tandem mass spectrometry (LC-MS/MS) and ion-exchange chromatography (IEC) amino acid analyzers for amino acid quantification.

4. Discussion

The most widespread analytical method in clinical laboratories for the quantification of AAs is still IEC with post-column ninhydrin derivatization, which has been used for many years as a gold standard [2,7]. Many laboratories are implementing a new LC-MS/MS method, using it also for other analyses, while simultaneously running an IEC. The key factors for moving the AA quantification to an LC-MS/MS are its specificity and the shorter analysis time, and the small volume of the sample needed, which is especially important in pediatric patients. IEC is also specifically tailored for AA analysis, while with LC-MS/MS numerous analytes besides AAs can be quantified. A comparison of the methods was carried out with the intent to determine whether the differences between the methods influenced clinical decisions. The AAs can be quantified in plasma, urine, and liquor with both methods. However, since disorders in AA metabolism are usually first detected by an increase in their blood concentrations, we compared the measurements of plasma samples.

Accuracy and precision of the methods was determined to test the suitability of methods for measurements. With the LC-MS/MS the RSD values were considerably higher in determination of ornithine (14.8%) and lysine (11%), however measurements of all amino acids satisfied the conditions we set for use in clinical laboratory diagnostics (RSD < 15%). Phenylalanine, isoleucine, leucine and tyrosine, which are crucial for following-up of phenylketonuria and tyrosinemia patients and with all patients on dietary supplements, had good reproducibility with RSD lower than 5%. IEC was more accurate for most amino acids as most had RSDs lower than 5%, higher RSDs were calculated for citrulline (13.5%) and taurine (12%). The deviation in citrulline was probably due to the poor separation with IEC as it elutes just next to alanine, which is normally present at much higher concentrations. Precision was determined with purchased amino acid control material and calculated from 7 measurements on consecutive days. Results with LC-MS/MS were for most amino acids up to 8.5% lower than the expected mean values, for IEC values of most amino acids were up to 8.6% higher than the expected mean values. All measurements on both LC-MS/MS and IEC were lower than 15% and also within the expected control range and are suited for clinical laboratory use.

The quantified AAs did not have a normal distribution. The reason for this abnormalities are most likely the systemic factors present in both methods, which cause a non-normal the distribution of the differences between the two groups of measurements. The quantification principle between the methods is also another factor: IEC uses single point calibration, while LC-MS/MS uses isotopically labeled internal standards with known concentrations of AAs. Spearman correlation coefficients for most AAs had values ranging from 0.70 (alanine) to 0.89 (leucine, threonine), indicating a high degree of linear correlation between the methods. A weaker coefficient of correlation was calculated for taurine (0.67), indicating a moderate linear association of the methods. Kaspar and colleagues also determined similar correlation and a high degree of correlation between the LC-MS / MS and the IEC methods, with the weakest correlation in arginine (Pearson correlation coefficient 0.56), but they used a different reagent kit (iTRAQ) and urine samples using the AB Sciex API 3200 tandem mass spectrometer and the IEC Biochrom 20 AA analyzer [10].

The agreement between the results of measurements was evaluated using the Bland-Altman test and diagram, comparing LC-MS/MS to IEC. The differences in the mean of measurements of the AAs were < 15%, with the exception of citrulline (− 31%), serine (19.5%) and tyrosine (−19%). A worse match was in serine, whereby the IEC method yielded 19.5% lower results. Reduced serine values are clinically relevant, as they may indicate disorders due to its shortage, which must be taken into account when interpreting [3]. The cause of the observed significant differences between the determined serine concentrations could be overlapping or the bad peak separation with the IEC method [11].

Tyrosine and phenylalanine are AAs important for the detection and monitoring of patients with phenylketonuria. On average, tyrosine was measured 19% higher with IEC than with LC-MS/MS and phenylalanine was measured 11.7% higher with IEC. According to the literature data, the methods differ more at higher concentrations of phenylalanine and cautiousness is needed especially in the monitoring of patients with phenylketonuria [11,12]. Clinically relevant AAs for the detection and monitoring of aminoacidopathies are also branched-chain AAs, leucine, valine, isoleucine, and in addition glycine and threonine. For the above-mentioned AAs, the LC-MS/MS method measured from 2.1% (leucine) to 8.9% (isoleucine) higher results compared to IEC. Even with alanine, the IEC method measured 4.3% higher results compared to LC-MS/MS. In the Bland-Altman diagram of alanine, the distribution of points in the form of a trumpet was observed, which means that the average error changes with the concentration, and therefore, at higher concentrations of the analyte, larger differences are obtained. The best match in all quantified AAs was observed in taurine, where only 1.1% higher results were obtained with the LC-MS/MS method. The consistency of the measurements by both methods was also evaluated for arginine (−3.1%), lysine (−4.9%) and ornithine (−4.5%), and found that the IEC method measured < 5% higher results compared to the LC-MS / MS.

That the analytical methods compared well was shown in a study carried out by Le and colleagues [11], where they used an underivatized method on LC-MS/MS and compared it with IEC AA analyzer. For most of the AAs that we have also quantified, < 15% of the difference in the mean of the measurements was calculated except for glycine (21%), alanine (20%) and serine (18%), where the LC-MS/MS method measured higher values. As was also observed in our study, tyrosine was 19% higher when quantified by the IEC method. Waterval and colleagues [13] also compared an underivatized LC-MS/MS method with IEC and got good comparison except for citrulline and lysine where the calculated differences of averages were > 15%. The cause was probably the inferior chromatographic separation using the IEC method.

The advantages of IEC are good repeatability, high resolution, and short and undemanding preparation of samples. The high cost of maintaining the instrument, the use of expensive buffers and reagents (low pH buffer, ninhydrin), longer analysis time (2.5 h) and the relatively large required volume of samples, represent its weaknesses. The problem may also be the co-elution of AAs and interference due to the possible presence of compounds that react with ninhydrin. The advantage of the method is, on the other hand, that additional metabolites [8,9,11,13,14] can be detected if they appear in the chromatogram, which is not possible in an LC-MS/MS method when using MRM. The advantages of the LC-MS/MS method are specificity, shorter analysis time (25 min) and a smaller quantity of sample required for the analysis. In addition, the use of the labeled IS for each individual AA contributes to a more reliable quantification of the results. The weakness of the method is the longer preparation of the sample, as it involves the derivatization process, and finally the processing of data [8,13,14]. Lastly, the LC-MS/MS reagents are significantly cheaper and as the maintenance of the apparatus is comparable to the maintenance of the IEC analyzer the LC-MS/MS method is more cost-effective.

5. Conclusion

The presented research was focused on the comparison of LC-MS/MS and IEC for the quantification of the most important AAs in human plasma. When monitoring AAs levels in patients with a metabolic disease it is of utmost importance that the clinicians are informed about the method used. The inter-method differences have implications for diagnosis and clinical management of patients as the differences can be clinically important. For this reason, different reference intervals must be used according to the method. This is especially important in infants and toddlers and in pregnant patients where stringent blood monitoring is paramount. The measurements on LC-MS/MS and IEC are sufficiently comparable for most AAs and we did not have to redefine the reference values for individual AAs, with the exception of citrulline. For the monitoring of patients on therapy (e.g. patients with phenylketonuria), it is still advisable to always use the same analytical method for the quantification of AAs.

Conflict of interest

Authors declare no conflict of interest.

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