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Review

State-of-the-art of high-performance liquid chromatographic analysis of amino acids in physiological samples

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Abstract

Nowadays, the fast and sensitive high-performance liquid chromatographic (HPLC) analysis of derivatized amino acids is a good alternative to the ion-exchange chromatography (IEC) method for plasma amino acids. However, several precautions have to be taken in order to obtain reliable data on the concentration of amino acids in physiological fluids. These include the collection, centrifugation, storage conditions and the method of deproteinization. Furthermore, the method of pre-column derivatization in connection with the protein precipitant used and the choice of the chromatographic system which determines the overall resolution are important factors. HPLC methods using pre-column derivatization with *o*-phthaldialdehyde were suitable for the accurate determination of many primary amino acids in plasma because of their high sensitivity, simplicity, speed and reliability. When the determination of secondary amino acids and cystine was also necessary, the phenylisothiocyanate method was the preferred technique. The intra-laboratory variability of the HPLC method was satisfactory while the inter-laboratory variation of this method was found to be similar to that of IEC. HPLC methods capable of separating over forty physiological amino acids seem promising for the analysis of urine samples.

Keywords: Reviews; Amino acids

Contents

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1. Introduction

Amino-acid analysis is an important technique which finds many applications in biochemical, pharmaceutical and clinical research. The analysis of physiological samples is hampered by the presence of a substantial number of amino acids which occur in widely different concentrations at the same time. Thus, the chromatographic system should exhibit high specificity, sensitivity, linearity and reproducibility.

The methods used for the determination of amino acids in physiological fluids mostly involve ion-exchange chromatography (IEC) or reversed-phase high-performance liquid chromatography (HPLC). The IEC method is based on the use of cation-exchange resins and post-column derivatization with ninhydrin [1] or *o*-phthaldialdehyde (OPA) [2] and is mostly done on a commercial amino-acid analyzer. Although the use of this instrument for amino-acid analysis has been widely advocated in the past, especially in routine applications due to its high reliability, HPLC methods are gaining more and more importance. The reasons for this are shorter analysis times, lower cost of instrumentation and maintenance, higher sensitivity and flexibility. In case of the HPLC method amino acids are separated on a reversed-phase column after being derivatized with OPA [3], phenylisothiocyanate (PITC) [4], 5-dimethylamino-1-naphthalenesulphonyl chloride (dansyl) [5] or 9-fluorenylmethyl chloroformate (FMOC) [6].

Sarwar and Botting [7] published an excellent review on the evaluation of liquid chromatographic analysis of nutritionally important amino acids in food and physiological samples. Although HPLC analysis of amino acids in hydrolysates of proteins and foods yields satisfactory results, they concluded that there is still room for improvement in the HPLC analysis of amino acids in physiological samples, especially urine. The purpose of this review was to evaluate the speed, accuracy, selectivity and reproducibility of liquid chromatographic analysis of amino acids in physiological samples using pre-column derivatization techniques.

2. Preparation of physiological samples

2.1. Collection and storage

One of the reasons that HPLC methods for the amino-acid analysis of physiological fluids have not been widely accepted is the lack of standardized conditions for sample preparation [8]. In this review we will focus on the determination of amino acids in human plasma, urine and cerebrospinal fluid (CSF) because these are the most important amino acids in clinical practice. In serum, small changes in amino-acid concentrations may occur during clotting and it is therefore used less [9]. However, before analysis of amino acids in human plasma or urine, several steps preceding the sample preparation have to be regarded. First, care is needed to ensure that the subject is medication-free or does not take medication which interferes with the analysis. Especially antibiotics and their metabolites are known to give additional peaks in the chromatogram which may interfere with the resolution of amino acids [10]. Also the use of numerous other drugs, contraceptives and differences in protein intake between subjects have to be taken into account [11].

The time of blood withdrawal and the allied problem of dietary intake may be another reason for varying results between laboratories. However, this can be easily standardized by taking blood samples between 8 and 9 a.m. before breakfast. Also the technique of blood withdrawal will not give serious problems for the quantitation of amino acids. Most laboratories collect blood by venipuncture into tubes containing heparin or potassium EDTA as the anticoagulant [9,10]. It is, however, recommended to use siliconized tubes in order to prevent blood platelets becoming activated with the resultant release of taurine and phosphoethanolamine (PEA) [9]. Haemolysis should also be prevented because this leads to false increases in the concentrations of aspartic acid, glutamic acid, PEA and taurine [9]. The delay between sample collection and preparation should be as short as possible to prevent errors in the determined concentrations caused by metabolism of blood components [12]. However, in clinical routine a delay of some hours is sometimes inevitable. It is then recommended to store the blood at 4°C before further preparation. This will reduce the otherwise markedly elevated concentrations of glutamic acid, serine and ornithine and the reduced levels of cystine and arginine to acceptable levels [12]. In order to obtain reliable plasma amino acid concentrations it is necessary to centrifuge the blood not only immediately but also at a centrifugal speed higher than 2500

g and for a sufficient time in order to remove blood platelets. Many researchers centrifuge blood samples at centrifugal speeds of 1500 g or lower for only 5–15 min and some do not even mention these details. This may lead to erroneously high concentrations of taurine and PEA. However, the speed should also not exceed values of 6000 g because damage of platelets will occur with the concomitant release of their contents [13]. Centrifugation at 2650 g for 20 min at 15–20°C, using a temperature controlled centrifuge, yields a satisfactory platelet poor plasma preparation [14].

Urine should be collected at defined times of the day or even better a 24-h collection should be used in order to make results independent of circadian rhythms [11]. It is recommended to store the collection bottle at 4°C directly after voiding and to centrifuge the urine before freezing. This is especially recommended when cells are present. CSF should be taken from the lumbar area of the subarachnoid space because most published amino-acid values refer to this area [11].

When deproteinization of the physiological samples cannot be done immediately after centrifugation, it is recommended to store the specimens at temperatures lower than −18°C to prevent further hydrolysis of proteins [9,10,12]. In particular, the amino acids aspartic acid and glutamic acid increased markedly when the plasma samples were not frozen immediately and stored at −68°C or lower [15]. Consequently, the concentration of glutamine, and curiously not that of asparagine, decreased markedly in plasma stored at −18°C for 5–6 months [12]. The concentration of cystine was also decreased under these circumstances because this amino acid is extensively bound to plasma proteins if deproteinization is not carried out within 30 min after venipunture [9–12]. More detailed information concerning preparation of biological samples was reported by Williams [10] and Deyl et al. [16].

2.2. Deproteinization

Several methods for deproteinization of physiological samples have been described in the literature [7–11,16–18]. These include precipitation with acids or organic solvents, ultrafiltration, ultracentrifugation and equilibrium dialysis. Both advantages and disadvantages of these methods have been reported in connection with the method of pre-column derivatization. Disadvantages of ultrafiltration and ultracentrifugation are that these methods do not lead to complete removal of protein [19]. Although some investigators obtained positive results with ultrafiltration of plasma [20,21], this method was found unsuitable for lipemic plasma samples. This is due to the time required to filter sufficient sample and occasionally no filtrate could be obtained, presumably because of the clogging of the membrane [8]. This does not hold true for CSF and therefore ultrafiltration, for this fluid, was found to be suitable [8]. Another problem with ultrafiltration is that the amount of free amino acids is dependent on the volume of the sample taken and it is a time-dependent procedure. Moreover, degradation of certain amino acids during the process has been reported [22]. Equilibrium dialysis against citrate buffer resulted in low losses of free amino acids and seems promising. However, this method does not appear to have been widely adopted [19].

Comparisons between methods for deproteinization of plasma with acids or organic solvents have been published by several authors [8,17,18,23]. Qureshi and Qureshi [17] found consistent results with high recoveries for most amino acids with 30% 5-sulphosalicylic acid (SSA) and 1 M $HClO_4$. The variation in recovery was large with organic solvents. Moreover, aspartic acid and glutamic acid increased and asparagine and glutamine decreased in organic solvents. Unfortunately, the amino acids tryptophan, cystine and proline were not determined.

Davey and Ersser [8] found protein precipitation with acetonitrile (ACN) most suitable in combination with a prepurification on Bond Elut SCX columns and derivatization with PITC. However, the recovery of aspartic acid was less than 90%. Although the authors concluded that SSA was also suitable as protein precipitant, a comparison with ACN was not made.

Uhe et al. [18] used the ACN method of deproteinization because this results in full recovery of total tryptophan. However, the recoveries of aspartic acid and lysine were below 90%. These authors compared the efficacy of ACN, trichloroacetic acid (TCA) and SSA as deproteinizing agents and found that the concentrations of arginine, lysine and or-

nithine and to a lesser extent those of glycine, serine and glutamic acid measured using ACN, were lower than those measured with SSA and TCA. This is also reflected by their amino-acid levels determined in plasma of healthy subjects. These data would favour the use of acid precipitation and not precipitation with ACN. The main reason these authors used the latter method was the good recovery of tryptophan. However, the low recovery of this amino acid with TCA (83%) and SSA (78%) may partly be caused by the use of rather low concentrations of these acids, viz., 2 and 1%, respectively. It has even been recommended to deproteinize plasma samples with TCA (1 part of plasma and 4 parts of 10% TCA) because of the satisfactory recovery obtained with this method [11].

Aristoy and Toldra [23] found amino-acid recoveries of more than 90% with ACN, TCA, $HClO_4$ and picric acid as precipitant. The use of SSA, phosphotungstic acid and ultrafiltration through a 1000 Da molecular mass cut-off membrane, resulted in important losses of some amino acids. The recoveries of aspartic acid and glutamic acid from a standard amino-acid solution were as little as 70 and 73%, respectively. These results are in contradiction with those found by some other investigators [8,14,17,18]. A possible explanation for this discrepancy is the use of different derivatization techniques (OPA vs. PITC).

Cohen and Strydom [24] reported that quantification of amino acids in biological fluids is problematic because of the reduced yield of many phenylthiocarbamyl (PTC) amino acids after deproteinization with the most common precipitants. Several authors report that a major problem with precipitation, with SSA, is the interference of this compound with aspartic acid [23–28] and glutamic acid [23,24] during chromatography. However, other investigators used SSA as deproteinizing agent without any problems because of different chromatographic techniques [14,29–31].

Irrespective of some problems encountered by several investigators, the most widely used method of deproteinization still is precipitation with SSA followed by centrifugation to remove precipitated protein [10]. This method is effective for the determination of amino acids by classical IEC, as well as by most HPLC procedures, especially those of OPA amino acids. SSA is either added to plasma or serum as a 3% solution, e.g., 4 parts of 3% SSA to 1 part of plasma or as a solid substance, 30–40 mg/ml of fluid. This mixture should be stirred immediately. After centrifugation, an aliquot of the somewhat turbid supernatant can be applied directly to the column [11]. In our experience, deproteinization of plasma samples (500 μl) with 100 μl of 24% (w/v) SSA, containing internal standard, best meets the requirements of precise profiling. Immediate vortex-mixing followed by standing for 15 min at 4°C and centrifugation results in a clear supernatant which after adjustment of pH, can be used directly for amino-acid analysis [14].

For deproteinization of urine samples containing more than 1% of urinary protein, application of 50–100 mg of solid SSA per ml of urine is recommended [16]. To remove proteins in CSF, a deproteinization with SSA (0.75 volume CSF and 0.25 volume 15% SSA) has been widely accepted [11]. For more detailed information on sample preparation, the reader is referred to the review of Deyl et al. [16] and to the more recent review of Sarwar and Botting [7].

3. Pre-column derivatization methods

In the last few years, the faster and more sensitive HPLC methods for derivatized amino acids have replaced IEC for the analysis of protein hydrolysates [8,24]. However, these methods have not yet been widely accepted for the amino-acid analysis of physiological fluids, mainly because of the increased demands of sample preparation and chromatographic separation [7,8].

Typical reagents for pre-column derivatization are OPA [3], PITC [4], dansyl [5], FMOC [6], 1-fluoro-2,4-dinitrobenzene (FDNB) [32], 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDNPAA) [33] and N,N-diethyl-2,4-dinitro-5-fluoroaniline (FDNDEA) [31]. Some of the features of these pre-column derivatization methods for amino-acid analysis are shown in Table 1. Disadvantages of the use of dansyl, FDNB, FDNPAA and FDNDEA are the formation of interfering side-products after derivatization and the necessity of removal of the reagent by drying for the latter three. The FMOC method suffers from the disadvantage that excess reagent has

Table 1
Summary of pre-column derivatization methods used for amino-acid analysis by HPLC

	OPA	PITC	Dansyl	FMOC	FDNDEA	FDNB	FDNPAA
Derivatization time (min)	<1	20	35	5	15	30	50
Sample preparation	Very simple	Complex	Simple	Simple	Complex	Complex	Complex
Automation of derivatization	Yes	No	Yes	No	No	No	No
Removal of reagent by drying	No	Yes	No	No	Yes	Yes	Yes
Solvent extraction	No	No	No	Yes	No	No	No
Quantitative yield	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Stable derivative	No	Yes	Yes	Yes	Yes	Yes	Yes
Interfering side-products	No	No	Yes	No	Yes	Yes	Yes
Interference by contaminants in eluent	No	Yes	Yes	No	No	No	No
Secondary amino acids detected	No	Yes	Yes	Yes	Yes	Yes	Yes
Detection	Fluor	254 nm	Fluor	Fluor	360 nm	365 nm	340 nm
Sensitivity	fmol	pmol	pmol	fmol	pmol	pmol	pmol
Reproducibility	Very good	Good	Good	Good	Good	Good	Good

Abbreviations: OPA = *o*-phthaldialdehyde; PITC = phenylisothiocyanate; dansyl = 5-dimethylamino-1-naphthalenesulphonyl chloride; FMOC = 9-fluorenylmethyl chloroformate; FDNDEA = N,N-Diethyl-2,4-dinitro-5-fluoroaniline; FDNB = 1-Fluoro-2,4-dinitrobenzene; FDNPAA = 1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide; Fluor = fluorescence.

to be extracted manually with pentane in order to stop the derivatization reaction and to avoid spontaneous hydrolysis of the FMOC adducts [27]. Because of these shortcomings, only OPA and PITC have been widely used as pre-column derivatization reagents.

Fürst et al. [27] have compared four pre-column derivatization methods, with OPA, PITC, FMOC and dansyl, for the HPLC determination of amino acids in biological materials. The FMOC method was only recommended if determination of secondary amino acids is desirable because this method suffers from the disadvantage that excess fluorescent reagent has to be extracted or derivatized. The use of the dansyl technique was suggested when the determination of free cystine is required because of excellent linearity for the dansylated cystine adduct. However, the interference by contaminants in the eluent and the formation of interfering side-products are major drawbacks of this technique. The simple sample preparation, the rapid derivatization, the possibility of automation and high sensitivity favour the OPA method for analyses of primary amino acids in biological fluids. Moreover, the reagent itself does not fluoresce and consequently produces no interfering peaks. The major disadvantages of this method are the inability to detect cystine and secondary amino acids and the instability of the derivatives formed. The latter disadvantage is, however, of less importance when an automated pre-column derivatization method is being used. Routine quantification of cystine is impossible with OPA due to the formation of a derivative with minimal fluorescence. This problem can be overcome by pretreating cysteine with iodoacetic acid before reaction with OPA [34], although this pretreatment may lead to incorrect values of some other amino acids [35]. When determination of secondary amino acids is desirable and if sufficient sample material is available, the PITC method is preferred. No interfering side-products are formed and the PTC derivatives are very stable, especially if stored dry in a freezer [4]. However, some interference by contaminants in the eluent has been reported with this method and the sulphur amino acids cysteine and homocysteine form mixed disulphides which may interfere with the determination of leucine [8]. Moreover, the sample preparation is lengthy and requires vacuum evaporation during the derivatization procedure which makes this method unsuitable for automation. Nevertheless, the speed of analysis, sensitivity and reliability make this technique an excellent alternative to the classic ion-exchange method for the quantitation of plasma amino acids [8,36].

McClung and Frankenberger Jr. [37] compared five pre-column derivatization techniques in terms of limit of detection, precision and stability. They concluded that the PITC method was the method of

choice as regards stability. The OPA method was found the most suitable in terms of limit of quantitation while the dansyl method was regarded as the most precise.

Fermo et al. [31] compared the OPA and the FDNDEA method for analysis of serum amino acids by HPLC. If quantitation of amino acids present at very low concentrations is the objective, OPA was found to be a suitable reagent because of the high sensitivity of this method. For the analysis of secondary amino acids, it was concluded that FDNDEA derivatization is preferable. Both methods offered acceptable reliability and reproducibility and might therefore be useful in clinical and research laboratories for routine analyses.

Differences between pre-column derivatization HPLC techniques in terms of resolution of complex samples and time of analysis not only depend on the derivatization method but also on the deproteinization technique and the reversed-phase HPLC method used. The first aspect has already been discussed (Section 2.2) and the last point will be dealt with in the next section.

4. Reversed-phase HPLC methods for analysis of amino acids in physiological samples

As has already been discussed in the previous section, only pre-column derivatization with OPA and PITC has been widely accepted for the determination of amino acids in physiological fluids. Numerous papers have been published describing various aspects of these methods. Most techniques make use of reversed-phase octyl- or octadecylsilica for the separation of the derivatized amino acids. The chromatographic run times vary between 10 [24] and 95 min [31] for standard mixtures of amino acids. Although very short analysis times may yield satisfactory results for amino-acid determination in protein hydrolysates, quantitative determination of amino acids in physiological fluids is unreliable. The chromatographic technique is important, especially when measuring aspartate and glutamate in plasma after deproteinization with SSA. When this acid is used as precipitant, many researchers encountered problems with the measurement of aspartate and sometimes glutamate because of poor resolution between SSA and the mentioned amino acids [23–28]. This poor chromatographic performance is not strictly connected with short run times because three groups use HPLC methods with chromatogram runtimes higher than 40 min [25–27]. The poor separation between SSA and aspartate is probably also not dependent on the analytical column used. Godel et al. [25] used a 4-μm Superspher CH-8 column and van Eijk et al. [26,28] and Fürst et al. [27] used a 3-μm Spherisorb ODS 2 column. Other investigators using the same columns report an excellent resolution between SSA and aspartic acid [14,29,31]. The most important difference between the studies with poor and good resolution of SSA and aspartate is the pH of the starting eluent which is 7.0 or 7.2 in the first studies [25–28] and 6.3 or 6.5 in the latter studies [14,29,31]. The use of 3-mercaptopropionic acid as sulfhydryl reagent instead of 2-mercaptoethanol which results in the more stable and slightly more hydrophilic OPA-mercaptopropionic acid derivatives, may also in part explain differences in resolution between SSA and the aspartic acid adduct. The addition of several organic modifiers may be another factor. This is especially the case with the chromatographic separation of OPA-derivatized amino acids using 3-mercaptopropionic acid as sulfhydryl reagent, as reported by Teerlink et al. [30]. These investigators included triethylamine in the starting mobile phase (pH 6.9) to allow the separation of aspartate from SSA. The analytical column they used was a 3-μm Microsphere C_{18} column and the chromatogram run time is only 12 min. Although total run time was 17 min, this is to my knowledge the only paper which describes a reliable HPLC method for the quantitation of plasma amino acids in such a short time (Fig. 1). Although secondary amino acids, cystine and some trace amino acids could not be determined, the simple sample preparation with OPA as derivatization reagent and the short analysis time, make this method very suitable for rapid routine analysis of plasma samples.

The HPLC method described by Fekkes et al. [14] was found to be very reliable although total analysis time was much higher than that of the aforementioned method [30], viz. 49 min vs. 17 min, respectively. However, with this method over 40 physiological amino acids, including the amino acids

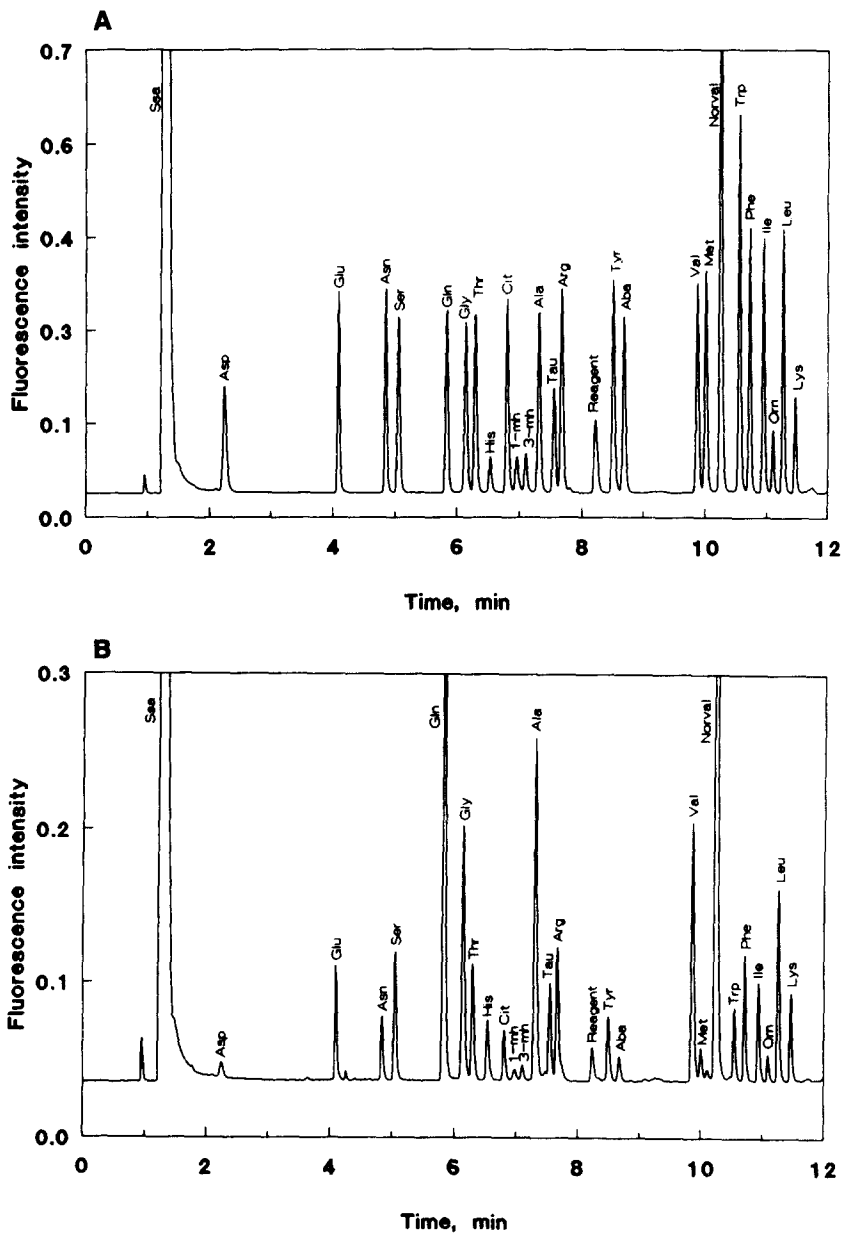

Fig. 1. Elution profile of an OPA-derivatized physiological amino acid standard (A) and a human plasma sample (B) (from Ref. [30] with permission). Column: 3-μm Microsphere C_{18} (100×4.6 mm I.D.); flow-rate, 1.5 ml/min; temperature, ambient. Ssa = 5-sulphosalicylic acid; 1-mh = 1-methylhistidine; 3-mh = 3-methylhistidine; Aba = α-aminobutyric acid; Norval = norvaline.

present in trace amounts (<10 μmol/l), can be determined in many physiological fluids (Fig. 2 and Fig. 3). The error of the method for human plasma samples, expressed as the coefficient of variation (C.V.), was below 5% for most amino acids, except for some trace amino acids. The resolution of the methylhistidines was accomplished by decreasing the pH of the eluent from 6.75 to 5.95.

Graser et al. [38] tried to reduce the analysis time of their previously published HPLC method [25] by

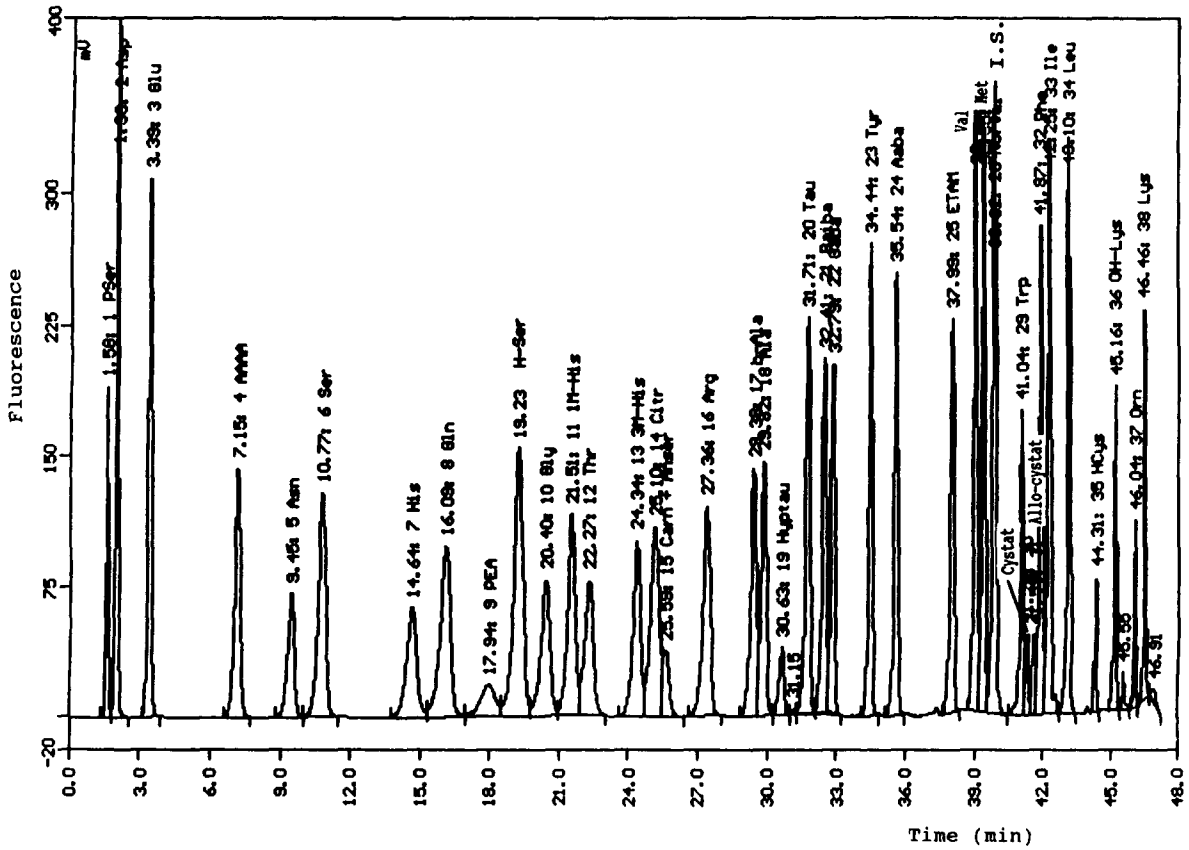

Fig. 2. Elution profile of an OPA-derivatized physiological amino-acid standard (from Ref. [14] with permission). Column: 5-μm Spherisorb ODS II (125×3 mm I.D.); flow-rate, 1.0 ml/min; temperature, 25°C. Non-standard abbreviations used: Pser=phosphoserine; AAAA=α-aminoadipic acid; PEA=phosphoethanolamine; H-Ser=homoserine; Carn=carnosine; Anser=anserine; b-Ala=β-alanine; Hyptau=hypotaurine; Baiba=β-aminoisobutyric acid; Gaba=τ-aminobutyric acid; Aaba=α-aminobutyric acid; ETAM=ethanolamine; I.S.=internal standard (=norvaline); cystat=cystathionine; allo-cystat=allo-cystathionine; HCys=homocystine; OH-Lys=hydroxylysine.

using shorter columns and a smaller particle size (3 μm), as well as by applying increased flow-rate and a steeper gradient. They were able to separate 23 major physiological amino acids in less than 13 min using a 3-μm Spherisorb ODS 2 column (125×4.6 mm I.D.). The error of this method, expressed as the C.V., ranged between 4.5 and 8.2% for the majority of amino acids. However, with this method aspartate, ornithine and some trace amino acids could not be detected, the separations between asparagine and serine, glycine and threonine, and alanine and glutamine were not optimal while phenylalanine and tryptophan were eluted together.

Georgi et al. [29] were able to determine 29 physiological amino acids in plasma. Although the chromatogram run time of their method was 62 min and they did not report on the retention time of most trace amino acids the resolution of the most important amino acids was found to be good. They used a 4-μm Superspher 100 RP-18 endcapped column (125×4 mm I.D.) and employed a ternary gradient. As many as 400–500 successive runs could be performed with this 4-μm column and only a few optimization steps were necessary when using a new column of this type. These investigators pointed to the importance of the composition and pH (6.3) of the buffer as well as the buffering ion (sodium) for both a good resolution and a good column life time. Replacing the phosphate buffer by acetate buffer resulted in a deterioration of the resolution. Increas-

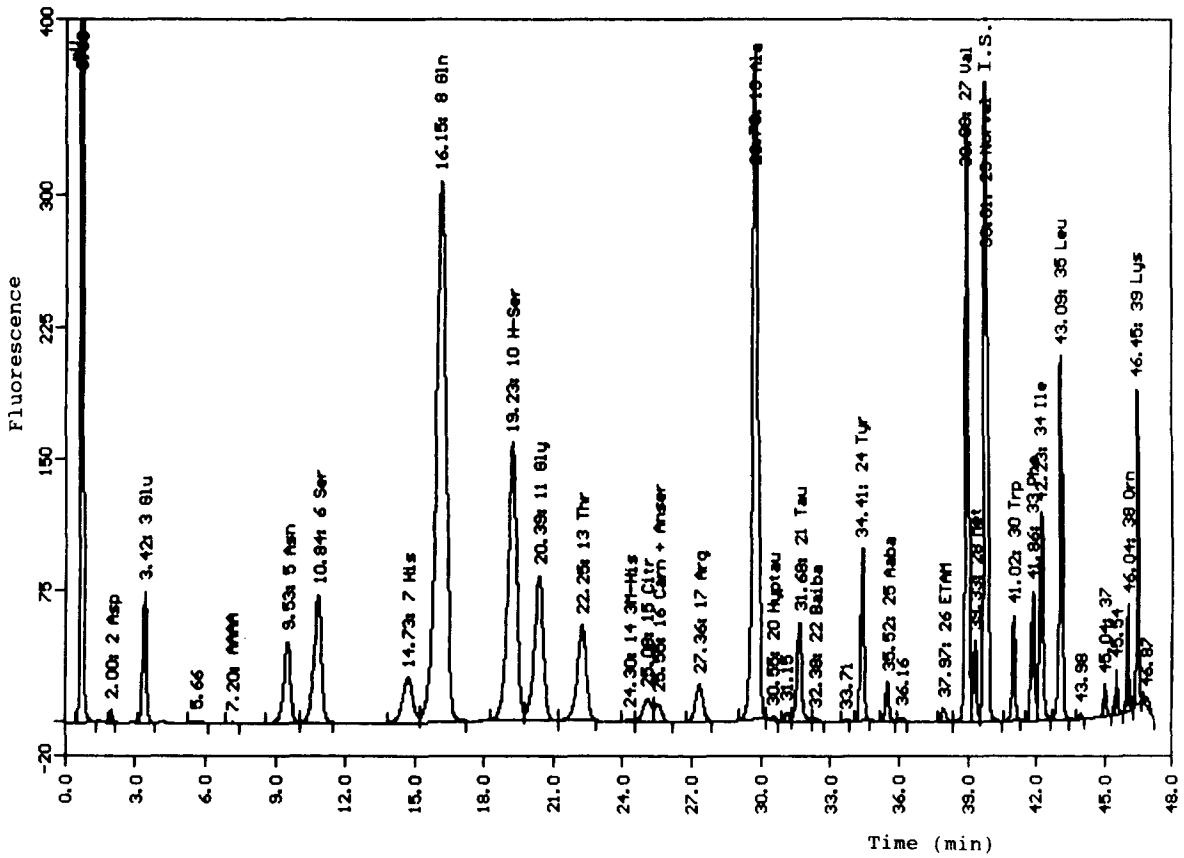

Fig. 3. Chromatogram of human plasma amino acids from a healthy volunteer under identical experimental conditions as in Fig. 2 (from Ref. [14] with permission). For abbreviations, see Fig. 2.

ing the pH of phosphate buffer from 6.2 to 7.2 also worsened the resolution. The addition of tetrahydrofuran was found disadvantageous for reproducible peak areas. This was however, not observed by other investigators [14,30]. Teerlink et al. [30] reported that it was essential to include tetrahydrofuran in the starting mobile phase in order to obtain baseline separation of glycine and threonine while Fekkes et al. [14] found a better resolution between histidine and glutamine after addition of tetrahydrofuran. A possible explanation for this discrepancy may be the concentration of tetrahydrofuran used which was only 0.2% (v/v) in the latter studies and was not given by Georgi et al. [29].

The so far described HPLC methods all concern the separation of OPA amino acids in physiological fluids deproteinized by SSA (Table 2). Several groups used ACN as precipitant [18,23,24,34,39] which has the advantage that aspartate and glutamate can be determined without being interfered with by SSA. Moreover, ACN deproteinization results in full recovery of total tryptophan whereas precipitation with SSA results in incomplete recovery of this amino acid [14,18,27,30], with the exception of a recovery of 97% found by Aristoy and Toldra [23]. On the other hand, the variation in recovery of most amino acids with ACN precipitation was reported to be higher than with SSA or $HClO_4$ [17,25]. This was however, not generally found by other investigators [18,34,39], except for the amino acids aspartate, arginine and lysine which had a lower recovery [18].

The method described by Turnell and Cooper [34] makes use of a 5-μm Ultrasphere ODS column (150×4.6 mm I.D.) and a binary gradient. Between-run precisions of amino-acid estimation in serum and urine were 2.1–12.3%. However, not all trace amino

Table 2
Summary of reversed-phase HPLC methods suitable for determination of amino acids in physiological fluids

Reference	Deproteinization method	Derivatization method	Number of compounds separated	Run time chromatogram (min)	Total (min)	Problematic amino acids
[34]	ACN	OPA	35	40	>40	Baiba-Tau
[39]	ACN	OPA	48	48	>50	–
[25]	SSA	OPA	26	41	>50	Asp,Thr,Tau-Aaba
[38]	SSA	OPA	23	13	>13	Asp,Orn,Trp,Phe, Asn-Ser,Gly-Thr
[21]	Ultrafiltration	OPA	27	45	53	Trp
[26]	SSA	OPA	30	42	55	Asp
[17]	SSA	OPA	25	45	52	Trp
[8]	ACN	PITC	37	60	>67	–
[27]	SSA	OPA	26	42	>47	Asp
[27]	SSA	PITC	26	26	>30	Orn,Trp,His,Cys
[31]	SSA	OPA	38	95	106	Baiba,Tau,1M-His
[31]	ACN	FDNDEA	30	92	102	β-Ala-Met
[40]	SSA	PITC	27	21	30	Ser-Gln-Gly
[18]	ACN	OPA	23	39	53	Glu,Arg,Lys,Orn
[41]	SSA	PITC	23	13	23	Trp-Orn,Ser-Gln Gly,Tau,Arg-Thr
[36]	Ultrafiltration	PITC	29	68	88	Gaba-Cit,Trp-Orn
[28]	SSA	OPA	30	24	28	Asp
[29]	SSA	OPA	29	62	>62	–
[30]	SSA	OPA	25	12	17	-
[14]	SSA	OPA	>40	47	49	Carn-Anserine

Abbreviations: OPA=o-phthaldialdehyde; PITC=phenylisothiocyanate; FDNDEA=N,N-Diethylamino-1-napthalenesulphonyl chloride; ACN=acetonitril; SSA=5-sulphosalicylic acid; Baiba=β-aminoisobutyric acid; Aaba=α-aminobutyric acid; 1M-His=1-methylhistidine; β-Ala=β-alanine; Gaba=τ-aminobutyric acid; Carn=carnosine.

acids were measured, the separation between β-aminoisobutyric acid (Baiba) and taurine was bad and the mean serum levels show a rather high deviation from the values reported in the literature.

Jones and Gilligan [39] employed a 3-μm Ultrasphere ODS column (75×4.6 mm I.D.) and a binary gradient. They also added Brij 35 to the OPA reagent in order to increase the fluorescence response of lysine and hydroxylysine. These investigators were able to resolve as many as 48 amino acids in less than 50 min and only a few amino acids (PEA, carnosine and homocystine) were not detected. Although the authors reported that the mean analytical recovery was greater than 95%, the concentrations of the amino acids they measured in serum were different from those found by most other investigators, especially those of taurine, hydroxylysine, glutamate and aspartate.

Uhe et al. [18] added the metal chelating agent nitrilotriacetic acid to the OPA reagent to reduce the oxidation of 2-mercaptoethanol and thus improve its stability. The authors also added Brij 35 to increase the fluorescence of the lysine-OPA derivative [2]; however, they did not detect any improvement in fluorescence intensity. The separation was performed on a 5-μm Dynamax Microsorb C_{18} column (150×4.6 mm I.D.) in approximately 39 min with a total run time of 53 min. Although reproducibility was satisfactory with a mean C.V. of 5.2%, the plasma levels of glutamate, arginine and lysine were low while those for tryptophan and ornithine were high compared with the literature values. These discrepancies cannot be explained only by the procedure of sample preparation because the only amino acids which had a relatively low and variable recovery with ACN as precipitant were aspartate and lysine. Furthermore, no data on the resolution of the trace amino acids were given and the investigators did not show a chromatogram of their HPLC analysis.

Another HPLC method separating OPA derivatized plasma amino acids without acid precipitation is the one described by Blundell and Brydon [21]. These investigators obtained protein-free samples by ultrafiltration and they were able to separate 27 plasma amino acids within 53 min using a 5-μm ODS Ultrasphere column (150×4.5 mm I.D.). The between-batch precision based on normal serum determinations were 4.6–14.0% and the percentage recoveries varied from 79–121%. The reference values of plasma amino acids, including many trace amino acids and cystine, which was measured as carboxymethylcysteine after treatment of the plasma samples with 2-mercaptoethanol and iodoacetic acid, compared very good with mean literature values. However, no data on free tryptophan was given by the authors.

The aforementioned studies all concern HPLC methods with OPA-derivatized amino acids. The other widely accepted method for determination of amino acids in physiological fluids is the one using pre-column derivatization with PITC. Some investigators reported that deproteinization with SSA and other acids results in reduced yield of many PTC amino acids [23,24]. They recommended the use of ACN or ultrafiltration through a 10 000 Da molecular mass cut-off membrane as methods for deproteinization because these techniques gave amino-acid recoveries of more than 90%. On the other hand, Fürst et al. [27] found mean free amino-acid concentrations of plasma deproteinized with SSA which agreed well with values derived from conventional amino-acid analyses, with the exception of glutamic acid. These authors separated the PTC amino acids on a 3-μm Spherisorb ODS 2 column (150×4.6 mm I.D.) within 30 min. The reproducibility of their method ranged between 2.6 and 5.5% (C.V.) for all amino acids, except histidine (6.3%) and cystine (10.0%). The higher C.V. values for the latter two amino acids may be explained by the poor resolution of these compounds. A serious disadvantage of this method is that tryptophan coeluted with ornithine so these amino acids could not be quantitated in plasma.

Davey and Ersser [8] described an HPLC method for the analysis of PTC derivatives of amino acids obtained after precipitation of plasma proteins with ACN. The separation of PTC derivatives was done on a Pico-Tag free amino-acid column (300×3.9 mm I.D.) in approximately 1 h. The mean recovery for all amino acids added to plasma was 89–117%, with the exception of aspartate which was less than 90%. Although no reference values were given, the imprecision of this method compared favourably with that of the standard ion-exchange method. High intra-assay coefficients of variation were observed for hydroxyproline (14.3%), citrulline (15.7%),

methionine (20.5%) and tryptophan (6.5%) for a control plasma. An interesting point of this study is that the authors reported the relative retention times of over 90 clinically important compounds. Some drugs and their metabolites can produce substantial peaks on a chromatogram which can interfere with some of the common amino acids. These are only very occasionally present in measurable amounts in plasma but can be a significant problem in urine analysis. This was also noted by Feste [36], who separated PTC amino-acid derivatives of urinary amino acids and ultrafiltrated plasma amino acids on a C_{18} column (300×3.9 mm I.D.) in 68 min. The between-run imprecisions for urinary amino-acid concentrations were higher than those for plasma amino-acid concentrations, the amino acids present at concentrations under 10 μmol/l having the highest values. The urinary amino acids demonstrated greater errors because of the sample matrix or the fact that the concentrations of many urinary amino acids were lower than those of their plasma counterparts. Although this HPLC method has a rather long total analysis time of 88 min, there are still some problems concerning the resolution: citrulline and τ-aminobutyric acid (Gaba) eluted as a doublet peak and the separation between tryptophan and ornithine was poor.

The analysis times of the described HPLC methods using PITC derivatization for the quantitation of amino acids in plasma is quite long (66–88 min) with the exception of the method described by Fürst et al. [27]. However, the latter method did not resolve the important amino-acid tryptophan. To speed up this method, Sarwar and Botting [40] used a shorter Pico-Tag amino-acid analysis column (150×3.9 mm I.D.) than Davey and Ersser [8] and Feste [36]. They succeeded in separating 27 PTC amino acids in physiological fluids in 21 min with a total analysis time of 30 min (Fig. 4). Although the variation, expressed as C.V. of the entire method was less than 5%, some amino acids (asparagine, serine, glutamine and glycine) were not well resolved and trace amino acids were not measured (Fig. 5). Fierabracci et al. [41] reported an even more rapid

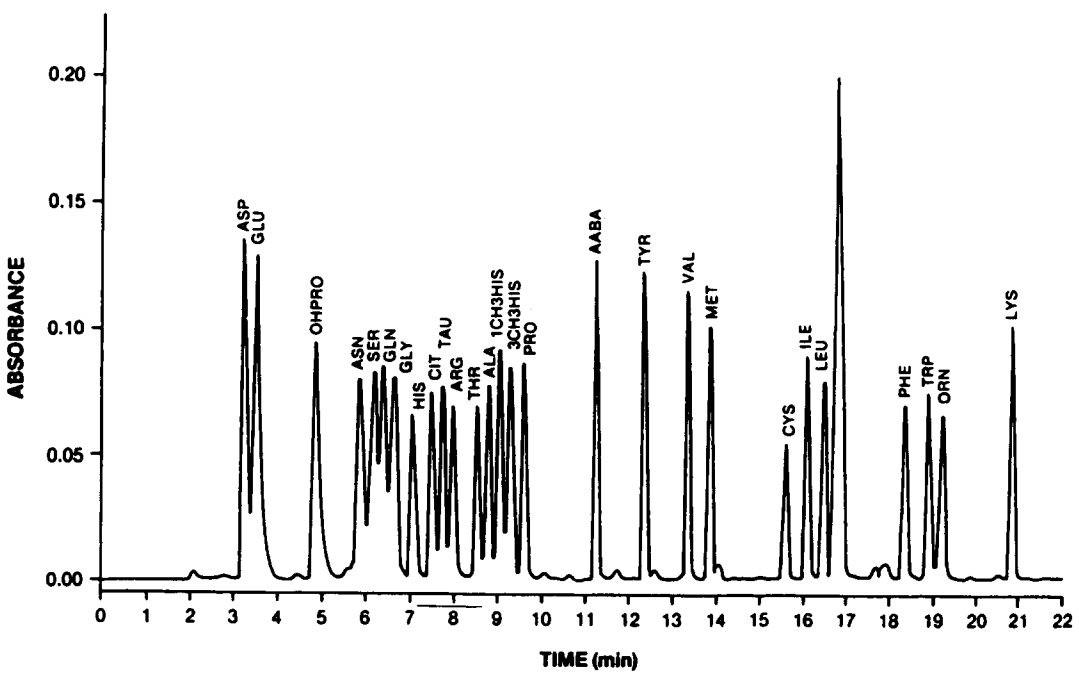

Fig. 4. Elution profile of a 27-amino-acid standard showing resolution of each PTC amino-acid by liquid chromatography (from Ref. [40] with permission). A Pico-Tag amino-acid analysis column (150×3.9 mm I.D.) was used and maintained at 47.5°C. OHPRO= Hydroxyproline; AABA=α-aminobutyric acid; 1CH3HIS and 3CH3HIS=1- and 3-methylhistidine.

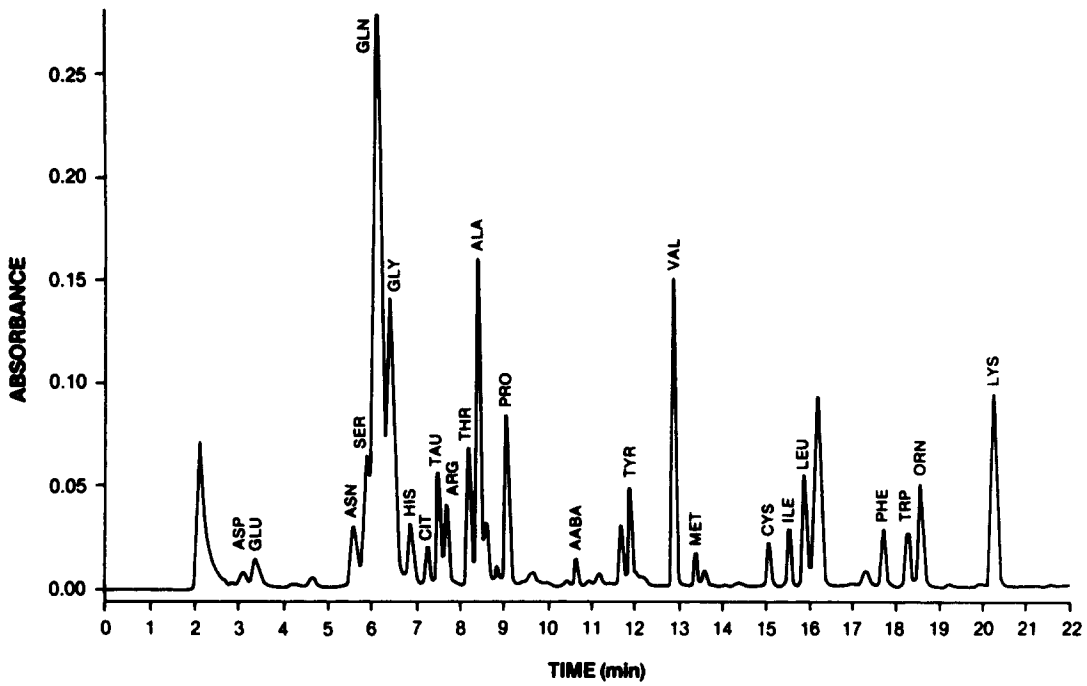

Fig. 5. Elution profile of human serum showing resolution of PTC-amino acids by liquid chromatography (from Ref. [40] with permission). The chromatographic conditions were the same as noted in the legend to Fig. 4.

method for the analysis of 23 PTC amino acids obtained after deproteinization of plasma amino acids with SSA. These investigators also made use of a C_{18} Pico-Tag column (150×3.9 mm I.D.) and separation was achieved within 13 min. The mean recovery of amino-acid standards added to plasma was 97% with the exception of aspartate (82%) and glutamate (81%). These values compare very well with those reported by Fürst et al. [27]. A disadvantage of the method is however, that tryptophan and ornithine are not resolved. Moreover, this method involves a drying step of 90–120 min in order to remove excess derivatizing reagent.

From the above mentioned studies one may conclude that for the separation of OPA amino acids SSA is the preferred reagent for the deproteinization of plasma while PTC amino-acid derivatives may best be resolved after treating plasma with ACN. There is still another HPLC method described in the literature which is based on the separation of serum amino acids derivatized with FDNDEA [31]. For this method, a 5-μm Ultrasphere ODS column (150×4.6 mm I.D.) was used with a simple gradient. The total analysis time was however, rather long (102 min) and the amino acids occurring in trace amounts in serum as well as asparagine, were not well resolved or could not be detected as was the case for aspartic acid. On the other hand, the amino-acid levels measured in serum were generally in good agreement with the reference method.

5. Validation of HPLC methods for amino-acid analysis in plasma or serum

Several investigators have compared amino-acid concentrations in plasma obtained by IEC and reversed-phase HPLC methods [8,14,25,27,31,36,42]. In general, the correlations between both methods for the quantitation of plasma amino acids were good for the majority of amino acids examined, irrespective of the pre-column derivatization technique used. In each method, different amino acids gave less precise results which may result in a lower correlation for the amino acid in question, e.g., for citrulline and methionine in the study performed by Ziegler et al.

[42]. The amino acids with the highest imprecision using the PITC method include citrulline and methionine [8,36], ornithine, hydroxyproline, proline and tryptophan [8] and aspartate, histidine and 3-methylhistidine (3M-His, Fermo et al. [31] found poor precision for lysine because of stability problems of its OPA derivative, resulting in low reference values for this amino acid. Godel et al. [25] also reported a low correlation for lysine, however, the plasma concentration of this amino acid measured with their OPA method was higher than that measured with IEC. These investigators reported the lowest correlation coefficients for the amino acids occurring at a relatively low concentration; e.g. α-aminoadipic acid, 1-methylhistidine (1M-His), 3M-His and α-aminobutyric acid (Aaba). Essentially the same results regarding the so-called trace amino acids were also found in a more recent study by Fekkes et al. [14] who also used OPA as the derivatizing agent. A common finding in all studies described above is that the imprecision for plasma samples is variable, the amino acids with the lowest concentrations having the highest C.V. values.

Validation is the process of establishing that the performance characteristics of the analytical method meet the requirements for the intended applications. Method performance characteristics include precision and accuracy, limits of detection and quantitation, selectivity, linearity and ruggedness. The performance parameter precision includes retention time and peak area or height. In most studies these parameters meet the requirements, i.e., a C.V. value of less than 0.5% for retention times and less than 5% for peak area or peak height. The latter value is mostly higher for amino acids occurring at concentrations below 10 μmol/l in plasma. This parameter is in some way related to the sensitivity of the method used. This implicates that the C.V. values for the PITC method should be higher than those for the more sensitive OPA method. As can be seen in Table 3 this is true,

Table 3
Analytical parameters for the HPLC analysis of amino acids in plasma or serum

Reference	C.V. (%)		Derivatization method	Amino acids not correctly quantitated	Amino acid not detected[b]
	Intra-assay (Standard mix)	Inter-assay[a] (Control plasma)			
[34]	–	2.1–12.3	OPA	Asp, Glu, Ser, His, Gln, Phe	1MH
[21]	2.1–7.8	4.6–14.0	OPA	Tau	Trp, 1MH, β-Ala, Baiba
[26]	1.6–9.0	2.0–12.0	OPA	Trp, 3MH, ETAM	Asp, β-Ala
[17]	1.0–5.5	–	OPA	Aaba, Baiba	Cit, 1MH, β-Ala, ETAM
[8]	1.0–9.9[c]	4.0–>20.5	PITC	?	?
[27]	0.4–2.2	1.0–4.7	OPA	Trp	Asp { 1MH, 3MH, ETAM
[27]	2.6–5.5	3.6–7.0	PITC	Glu, Trp, Orn, Asp	Aaba { β-Ala, Baiba
[31]	0.9–11.0	–	OPA	Asn, Lys, Tau, 1MH, 3MH, ETAM	Baiba
[31]	0.8–6.0	–	FDNDEA	1MH, 3MH, β-Ala, Tau	Asp, ETAM, Baiba
[18]	1.2–8.0	2.0–8.2	OPA	Glu, Arg, Lys, Orn	Cit, 1MH, 3MH, β-Ala, Aaba, ETAM, Baiba
[41]	3.54±0.27	–	PITC	Asp, Cit, Trp, Orn	1MH, 3MH, β-Ala, Aaba, ETAM, Baiba
[36]	1.1–5.5	2.0–15.3	PITC	Trp, Orn, Glu, Ser, Thr, 3MH	ETAM
[30]	1.0–1.8	1.8–7.2	OPA	Tau, 1MH, 3MH	β-Ala, ETAM, Baiba
[14]	<4.0	0.3–5.6	OPA	–	–

Abbreviations: OPA=*o*-phthaldialdehyde; PITC=phenylisothiocyanate; FDNDEA=N,N-diethylamino-1-napthalenesulphonyl chloride; 1MH=1-methylhistidine; β-Ala=β-alanine; 3MH=3-methylhistidine; ETAM=ethanolamine; Aaba=α-aminobutyric acid; Baiba=β-aminoisobutyric acid.

[a] Amino acids occurring at concentrations less than 5 μmol/l were not taken into account.

[b] When OPA was used as derivatizing reagent, the amino acids proline, hydroxyproline and cystine could not be detected.

[c] High imprecision of leucine due to interference from the cysteine–homocysteine mixed disulphide.

in particular for the inter-assay C.V. values for amino acids in plasma. The accuracy of the HPLC methods using pre-column derivatization described so far in literature is excellent for most amino acids. Recoveries of amino acids added to plasma typically are 101±2.3% (mean±S.D.) [34], 89–117% [8], 96–105% [14], 85–105% [18], 79–121% [21], 96–103% [30], >95% [39] or 81–103% [41]. The recoveries of the amino acids aspartate and glutamate were consistently lower than 90% in some studies [8,41]. This was also found for tryptophan when SSA was used as precipitating agent [14,18,26,27,30,31]. The selectivity and linearity of the aforementioned HPLC methods all are satisfactory.

Concerning the quantitation, many methods report on amino-acid concentrations in plasma or serum which deviate too much from the normal values found in literature. The values reported by Turnell and Cooper [34] are too high for aspartate, glutamate, serine, histidine, glutamine and phenylalanine (Table 3). The same high values were reported for aspartate and glutamate by Jones and Gilligan [39]. However, these investigators found far too low plasma levels for asparagine, serine, histidine, glycine, threonine, arginine, taurine, methionine, valine, tryptophan and isoleucine. Both groups have in common that precipitation of serum proteins was performed with ACN and that OPA was used as the derivatizing agent. Possibly, the combination of ACN precipitation and OPA derivatization is the cause of the variable and imprecise amino-acid values.

The normal values reported by Godel et al. [25] are within the normal range, with the exception of 1M-His and taurine. However, these workers were not able to determine the important amino acids tryptophan and aspartate. The ultrafiltration method of Blundell and Brydon [21] yields fairly good amino-acid values, except for taurine which is too high. With this method free plasma tryptophan concentrations are measured, however, no data were given. The normal values of van Eijk et al. [26] were well within the normal range with the exception of a low tryptophan concentration. These authors also did not report normal values of 3M-His and ethanolamine (ETAM), amino acids normally occurring in plasma. Moreover, their method could not quantitate aspartic acid and β-alanine (β-Ala).

Davey and Ersser [8] found that the performance of their HPLC system and the ion-exchange analyzer were very similar for the majority of amino acids examined. Unfortunately, these investigators did not report normal values. The normal values presented by Qureshi and Qureshi [17] were within the normal range. A drawback of their method is that the amino acids citrulline, 1M-His, ETAM and β-Ala could not be determined. Fürst et al. [27] published several pre-column derivatization methods and did also report on the normal values found with these methods. The OPA method was fairly good, except for the low value for tryptophan. With the PITC method, the measured glutamate concentration was too low and tryptophan and ornithine coeluted. Moreover, both methods also did not measure many amino acids occurring at low concentrations in plasma (Table 3).

Fermo et al. [31] mentioned normal values, most within normal range, in serum determined with an OPA and a FDNDEA method. Values obtained by the first method were too low for lysine, too high for asparagine and could not be quantitated for taurine. With the latter method, the amino acids 1M-His and β-Ala were not quantitated while aspartic acid and ETAM could not be determined. The method described by Uhe et al. [18] yielded abnormal values for the amino acids glutamate, arginine, lysine and ornithine, while the amino acids citrulline, 1M-His, 3M-His, β-Ala, Aaba and ETAM were not measured at all. Fierabracci et al. [41] reported plasma amino-acid concentrations determined in only four healthy subjects. These values were well within the normal range with the exception of a high value for aspartic acid. A drawback of this method is that tryptophan and ornithine coeluted and many trace amino acids were not measured. The method described by Feste [36] also did not quantitate tryptophan and ornithine because of coelution of these amino acids while ETAM could not be detected. Strangely, for many amino acids which could be determined by their method, no quantitative data were given, e.g., for glutamate, serine, threonine and 3M-His. This was also the case for some other amino acids which were not reported by the authors, e.g. Aaba [17], aspartate [27], ETAM, 1M-His and 3M-His [30,31] and citrulline [41]. Ziegler et al. [42] evaluated the method derived from Fürst et al. [27] and reported normal values which were in the normal range,

except for high values of glutamic acid and taurine while tryptophan was quite low. Drawbacks of their method include coelution of SSA with aspartate and 3M-His with 1M–His and no data on many trace amino acids.

Two recent papers [14,30] reported on plasma amino-acid concentrations in healthy subjects which were in very good agreement with the literature values, except for a high value for taurine by Teerlink et al. [30]. The reason for this is probably the short centrifugation time (10 min) in combination with a somewhat low g-value (2000 g) employed by these investigators which may result in contamination of plasma by blood platelets. This is probably also the reason for the high standard deviation found by the authors, viz., 83±40 (mean±S.D.). Their method, however, did not detect the trace amino acids ETAM, β-Ala and Baiba. The method described by Fekkes et al. [14] not only yields good normal values but is also able to detect and quantitate many amino acids occurring at very low concentrations in plasma, viz. α-aminoadipic acid, PEA, β-Ala, Baiba, ETAM and hypotaurine. Moreover, a few other physiological amino acids can be determined by this method, viz. phosphoserine, cystathionine, Gaba, carnosine and anserine, homocystine and hydroxylysine (Fig. 2). The only amino acids yielding some problems are the coeluting amino acids carnosine and anserine and the unusual amino acid allo-isoleucine. This latter amino acid is present in plasma from subjects with maple syrup urine disease [8]. The high resolution of their method is favourable for the determination of some rare amino acids, thus the possibility of improper identification of amino acids will become much smaller. One of the reasons for the fairly good normal values reported by the two last mentioned groups may be the proper care of the derivatizing reagent used. Both investigators kept the OPA reagent in multiple capped vials in the autosampler which was at the same time cooled in case of the method described by Fekkes et al. [14] to minimize problems due to oxidation of the reagent. Moreover, OPA concentration in the reagent was kept rather low because otherwise some additional unidentified peaks appeared in the chromatogram [30] and excess OPA accelerates degradation of isoindole derivatives [43].

Up to now, the inter-laboratory variability of the reversed-phase HPLC method in determining the amino-acid content of human blood plasma has not been determined. In accordance with the European Quality Assurance Program, a scheme for quality control of amino-acid analysis has been set up. Participating laboratories received twelve identical vials, containing lyophilized serum and twelve different reconstituting solutions. Each month, one vial was reconstituted with the appropriate reconstituting solution and after deproteinization, the supernatant was used for two unconsecutive assays with two weeks interval. Results of amino-acid analysis of each trimester were sent to the organizers. Afterwards, each participant received a report, for the batch concerned, of the results obtained by the participating laboratories. Fekkes et al. [14] reported the results for one trimester obtained by their HPLC method using pre-column derivatization with OPA and compared these with results obtained from other laboratories using their own methods (Table 4). Their HPLC results were in excellent agreement with those of the other laboratories, most of which used a conventional amino-acid analyzer. The correlation coefficient was 0.993 when all 30 amino acids were taken into account and even reached a value of 0.999 when the amino acid with the highest C.V. (viz., Baiba) was excluded.

To estimate the inter-laboratory variation of the reversed-phase HPLC method for amino-acid analysis, we analyzed the results of the first two trimesters of 1995 of the European Quality Assurance Program. In these trimesters 94 laboratories participated in the study, of which 6% used an HPLC method. My own laboratory also participated in this quality study and the inter-assay (or within-laboratory) variability of our HPLC method of OPA-derivatized amino acids was compared with the inter-laboratory variability of both the HPLC methods and of all methods together (Table 5). The within-laboratory variability of our HPLC method was less than 4% for the amino acids shown, except 8.0% for threonine, 9.2% for Aaba, 12.1% for α-aminoadipic acid and 25.0% for glutamine. The last three amino acids were present at concentrations less than 10 μmol/l. These amino acids show, as expected, the highest variability. The inter-laboratory variability of the HPLC method was much higher than the intra-laboratory variability with a range from 8.6 to 40.4% for most amino acids with

Table 4
HPLC analyses of amino acids in spiked plasma: comparison with other laboratories

Amino acid	HPLC			Other laboratories[a]			
	Mean[b] (μmol/l)	S.D.	*n*	Mean[b] (μmol/l)	Median[b] (μmol/l)	S.D.	*n*
Aspartic acid	24.3	0.9	11	24.8	25.0	7.7	511
Glutamic acid	220.0	0.5	11	233.0	227.0	62.1	521
α-Aminoadipic acid	3.3	0.5	8	6.3	4.0	5.4	57
Asparagine	101.0	1.6	11	111.0	100.0	53.0	460
Serine	66.2	1.4	11	63.9	63.0	11.9	526
Histidine	47.2	0.9	11	45.6	45.0	9.6	503
Glutamine	334.0	11.8	11	322.0	308.0	112.0	505
Glycine	234.0	6.2	11	229.0	230.0	33.4	526
Threonine	249.0	2.2	11	235.0	239.0	34.7	536
1-Methylhistidine	5.8	0.8	6	5.0	5.0	3.0	98
Citrulline	33.3	1.2	3	38.7	37.0	12.3	498
3-Methylhistidine	23.2	–	1	21.0	20.0	5.2	388
Arginine	120.0	2.2	11	119.0	117.0	24.7	521
β-Alanine	2.0	0.0	2	5.0	4.0	3.2	26
Alanine	565.0	6.9	11	551.0	565.0	82.5	538
Taurine	46.5	2.0	11	49.1	48.0	8.4	481
β-Aminoisobutyric acid	2.9	2.4	7	89.7	61.5	93.4	22
Tyrosine	37.4	0.8	11	38.8	36.0	11.2	531
α-Aminobutyric acid	7.4	0.5	11	6.4	5.5	4.4	236
Ethanolamine	10.7	0.6	3	40.1	29.0	33.1	34
Valine	261.0	1.8	11	240.0	245.0	40.6	533
Methionine	44.6	1.4	11	52.4	52.0	12.3	536
Tryptophan	24.6	0.5	11	25.4	25.0	8.6	227
Cystathionine	11.8	0.5	4	27.3	12.0	34.5	171
Phenylalanine	427.0	7.4	11	404.0	405.0	59.2	538
Isoleucine	64.7	0.5	7	63.7	61.0	17.3	527
Leucine	226.0	3.1	11	220.0	224.0	39.3	544
Homocystine	45.0	2.8	3	43.5	45.0	24.6	172
Ornithine	52.4	1.2	11	53.0	51.0	14.7	517
Lysine	445.0	11.3	11	427.0	428.0	68.1	531

Reproduced from Ref. [14] with permission
[a] 89 Laboratories participating in the European Quality Assurance Program.
[b] Results are not corrected for recovery.

the exception of Aaba (95.8%) and glutamine (84.4%; Table 5). The inter-laboratory variations of the HPLC plus ion-exchange methods for the amino acids in Table 5 showed essentially the same range as those of the HPLC method alone.

From the data shown in Table 4 and Table 5, it can be concluded that the precision and quantitation of amino acids in plasma or serum using the HPLC method are comparable to those of the ion-exchange method. Whether peak height or peak area measurement is used for the quantitation of amino acids depends on the separation efficiency of the column used. What is more important for obtaining reliable data is the use of an internal standard which should be added before or during the precipitation step. Apart from the confounding factors already discussed in Section 2, several other factors may influence the quantitation of amino acids in physiological fluids. Among these are age, sex, pregnancy, protein intake and circadian rhythm.

6. Conclusions

(1) The delay between the collection and preparation of a physiological sample should be as short as possible to prevent errors in the quantitation of the amino acids due to metabolism.

Table 5
Coefficients of variation for serum amino acids determined by HPLC and ion-exchange plus HPLC methods[a]

Amino acid	C.V. (%)		
	HPLC		Ion-exchange + HPLC
	Within-laboratory (*n*)	Inter-laboratory (*n*)	Inter-laboratory (*n*)
Alanine	1.5 (12)	8.6 (60)	9.4 (1106)
Arginine	1.5 (12)	17.2 (52)	11.9 (1099)
Glutamic acid	1.6 (12)	22.8 (66)	12.5 (1067)
Glycine	3.3 (12)	12.6 (47)	9.7 (1087)
Aspartic acid	0.1 (12)	28.8 (54)	53.9 (1042)
Lysine	3.0 (12)	25.8 (66)	15.7 (1110)
Ornithine	2.8 (12)	32.0 (60)	17.5 (1090)
Phenylalanine	2.0 (12)	18.4 (64)	17.7 (1096)
Serine	2.8 (12)	38.2 (66)	17.1 (1110)
Taurine	1.3 (12)	40.4 (60)	20.4 (1076)
Threonine	8.0 (12)	18.9 (56)	15.4 (1101)
Tryptophan	2.1 (12)	25.3 (48)	33.0 (504)
α-Aminoadipic acid	12.1 (12)	27.3 (23)	55.5 (247)
α-Aminobutyric acid	9.2 (12)	95.8 (41)	84.0 (460)
Glutamine	25.0 (12)	84.4 (48)	136.5 (925)

[a] Abstracted from the report of the European Quality Assurance Program for amino-acid analysis, trimesters 1 and 2 of 1995, with permission.

(2) In order to obtain reliable data on amino-acid concentrations in plasma, blood should be centrifuged immediately after withdrawal and under conditions, concerning centrifugal speed, time and temperature which prevent contamination of the plasma with blood platelets.

(3) It is recommended to deproteinize physiological samples immediately. If this is impossible, the samples should be frozen at temperatures of −68°C or lower to prevent further hydrolysis of proteins.

(4) SSA, ACN and ultrafiltration have been successfully used as deproteinizing agents for the analysis of free amino acids in plasma or serum.

(5) Automated pre-column derivatization with OPA was found to be a very sensitive and simple technique for the HPLC analysis of primary amino acids in physiological samples. When determination of secondary amino acids is desirable or when stable derivatives are needed, the PITC method is the preferred technique.

(6) SSA appeared to be a very suitable agent for deproteinization of plasma in combination with the OPA method, while PTC-amino acid derivatives were best resolved after treating plasma with ACN.

(7) The faster and more sensitive HPLC methods with pre-column derivatization are a very good alternative to the IEC method for the amino-acid analysis of plasma or serum. The correlations between both methods for the quantitation of plasma or serum amino acids were satisfactory for the majority of amino acids examined.

(8) The HPLC methods for the analysis of urinary amino acids still demonstrated greater errors mainly because of the more complex sample matrix and the lower amounts of amino acids occurring in urine than in plasma.

(9) With a PITC method it was possible to quantitate twenty-seven nutritionally important amino acids in serum within 21 min.

(10) The use of an OPA method enabled the accurate determination of the 24 major primary amino acids in plasma within 12 min.

(11) The within-laboratory variabilities of the HPLC methods for plasma amino-acid analysis using pre-column derivatization with PITC were somewhat higher than those employing pre-column derivatization with OPA.

(12) The determination of over forty primary physiological amino acids was possible within 47 min using an OPA method. The high resolution of

this method not only favoured the quantitation of rare amino acids but also the chance of proper identification of the major amino acids.

(13) This OPA method exhibited very good intra-laboratory variability for most amino acids and seems promising for the analysis of primary amino acids in urine.

(14) Preliminary data showed that the inter-laboratory variability of the HPLC method was similar to that of IEC.

(15) HPLC methods using automated pre-column derivatization with OPA were the best choice for the determination of primary amino acids in physiological fluids because of their high sensitivity, simplicity and reliability and the relatively short time in which the analysis of a clinically urgent sample can be done.

7. List of abbreviations

Aaba (Aba)	α-Aminobutyric acid
ACN	Acetonitrile
Baiba	β-Aminoisobutyric acid
β-Ala	β-Alanine
1M-His	1-Methylhistidine
3M-His	3-Methylhistidine
CSF	Cerebrospinal fluid
C.V.	Coefficient of variation
Dansyl	5-Dimethylamino-1-naphthalenesulphonyl chloride
ETAM	Ethanolamine
FDNB	1-Fluoro-2,4-dinitrobenzene
FDNDEA	N,N-Diethyl-2,4-dinitro-5-fluoroaniline
FDNPAA	1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide
FMOC	9-Fluorenylmethyl chloroformate
Gaba	γ-Aminobutyric acid
HPLC	High-performance liquid chromatography
IEC	Ion-exchange chromatography
OPA	o-Phthaldialdehyde
PEA	Phosphoethanolamine
PITC	Phenylisothiocyanate
PTC	Phenylthiocarbamyl
SSA (Ssa)	5-Sulphosalicylic acid
S.D.	Standard deviation
TCA	Trichloroacetic acid

References

[1] P.B. Hamilton and R.A. Anderson, Anal. Chem., 31 (1959) 1504.
[2] J.R. Benson and P.E. Hare, Proc. Natl. Acad. Sci. USA, 72 (1975) 619.
[3] B.N. Jones, S. Paäbo and S. Stein, J. Liq. Chromatogr., 4 (1981) 565.
[4] R.L. Heinrikson and S.C. Meredith, Anal. Biochem., 136 (1984) 65.
[5] V.T. Wiedmeijer, S.P. Porterfield and C.E. Hendrich, J. Chromatogr., 231 (1980) 410.
[6] S. Einarsson, B. Josefsson and S. Lagerkvist, J. Chromatogr., 282 (1983) 609.
[7] G. Sarwar and H.G. Botting, J. Chromatogr., 615 (1993) 1.
[8] J.P. Davey and R.S. Ersser, J. Chromatogr., 528 (1990) 9.
[9] T.L. Perry and S. Hansen, Clin. Chim. Acta, 25 (1969) 53.
[10] A.P. Williams, J. Chromatogr., 373 (1986) 175.
[11] H.J. Bremer, M. Duran, J.P. Kamerling, H. Przyrembel and S.K. Wadman (Editors), Disturbances of Amino Acid Metabolism: Clinical Chemistry and Diagnosis, Urban and Schwarzenberg, Munich, Germany, 1981, p. 181/505.
[12] A. Schaefer, F. Piquard and P. Haberey, Clin. Chim. Acta, 164 (1987) 163.
[13] M. Picard, D. Olichon and J. Gombert, J. Chromatogr., 341 (1985) 445.
[14] D. Fekkes, A. van Dalen, M. Edelman and A. Voskuilen, J. Chromatogr. B, 669 (1995) 177.
[15] J.C. Dickinson, H. Rosenblum and P.B. Hamilton, Pediatrics, 45 (1970) 606.
[16] Z. Deyl, J. Hyanek and M. Horakova, J. Chromatogr., 379 (1986) 177.
[17] G.A. Qureshi and A.R. Qureshi, J. Chromatogr., 491 (1989) 281.
[18] A.M. Uhe, G.R. Collier, E.A. McLennan, D.J. Tucker and K. O'Dea, J. Chromatogr., 564 (1991) 81.
[19] M.J. Thornber, N. Buchanan and K.L. Manchester, Biochem. Med., 19 (1978) 71.
[20] T. Gerritsen, M.L. Rehberg and H.A. Waisman, Anal. Biochem., 11 (1965) 460.
[21] G. Blundell and W.G. Brydon, Clin. Chim. Acta, 170 (1987) 79.
[22] I.J. Lima, J.J. MacKinchan and J. Sabino, J. Pharmacokin. Biopharm., 11 (1983) 483.
[23] M.C. Aristoy and F. Toldra, J. Agric. Food Chem., 39 (1991) 1792.
[24] S.A. Cohen and D.J. Strydom, Anal. Biochem. 174 (1988) 1.
[25] H.G. Godel, T.A. Graser, P. Foldi, P. Pfaender and P. Fürst, J. Chromatogr., 297 (1984) 49.
[26] H.M.H. van Eijk, M.A.H. van der Heijden, C.L.H. van Berlo and P.B. Soeters, Clin. Chem., 34 (1988) 2510.
[27] P. Fürst, L. Pollack, T.A. Graser, H.G. Godel and P. Stehle, J. Chromatogr., 499 (1990) 557.
[28] H.M.H. van Eijk, D.R. Rooyakkers and N.E.P. Deutz, J. Chromatogr., 620 (1993) 143.
[29] G. Georgi, C. Pietsch and G. Sawatzki, J. Chromatogr., 613 (1993) 35.

[30] T. Teerlink, P.A. van Leeuwen and A. Houdijk, Clin. Chem., 40 (1994) 245.
[31] I. Fermo, E. De Vecchi, L. Diomede and R. Paroni, J. Chromatogr., 534 (1990) 23.
[32] R.C. Morton and G.E. Gerber, Anal. Biochem., 170 (1988) 220.
[33] S. Kochbar and P. Christen, Anal. Biochem., 178 (1989) 17.
[34] D.C. Turnell and J.D.H. Cooper, Clin. Chem., 28 (1982) 527.
[35] J.D.H. Cooper and D.C. Turnell, J. Chromatogr., 227 (1982) 158.
[36] A.S. Feste, J. Chromatogr., 574 (1992) 23.
[37] G. McClung and W.T. Frankenberger, Jr., J. Liq. Chromatogr., 11 (1988) 613.
[38] T.A. Graser, H.G. Godel, S. Albers, P. Foldi and P. Fürst, Anal. Biochem., 151 (1985) 142.
[39] B.N. Jones and J.P. Gilligan, J. Chromatogr., 266 (1983) 471.
[40] G. Sarwar and H.G. Botting, J. Assoc. Off. Anal. Chem., 73 (1990) 470.
[41] V. Fierabracci, P. Masiello, M. Novelli and E. Bergamini, J. Chromatogr., 570 (1991) 285.
[42] F. Ziegler, J. Le Boucher, C. Coudray-Lucas and L. Cynober, J. Autom. Chem., 14 (1992) 145.
[43] M.C.G. Alvarez-Coque, M.J.M. Hernández, R.M.V. Camañas and C.M. Fernández, Anal. Biochem., 178 (1989) 1.