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APPRAISAL OF FOUR PRE-COLUMN DERIVATIZATION METHODS FOR THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FREE AMINO ACIDS IN BIOLOGICAL MATERIALS

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SUMMARY

Reversed-phase high-performance liquid chromatography (RP-HPLC) is a powerful method for assaying physiological amino acid concentrations in biological fluids. Four pre-column derivatization methods, with *o*-phthaldialdehyde (OPA), 9-fluorenylmethyl chloroformate (FMOC-Cl), phenyl isothiocyanate (PITC) and 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl-Cl), were assessed with respect to their applicability in biological research.

The methods permit the measurement of 21–26 major amino acids in 13–40 min. The superior sensitivity favours the use of OPA, FMOC-Cl and dansyl-Cl techniques. Because of instability of the OPA adducts, automated on-line derivatization is required when using this method in general practice. Application of the PITC method, although less sensitive, is useful in clinical chemistry, where sample availability is rarely a problem. Cystine determination is not feasible when using OPA or FMOC-Cl and with PITC the reproducibility and linearity are poor, whereas the dansyl-Cl method allows reliable quantitation.

The four methods are currently used to perform *ca.* 8000 OPA and 5000–6000 FMOC-Cl, PITC and dansyl-Cl analyses of biological samples per year. The results obtained with the RP-HPLC methods compare favourably with those derived from conventional ion-exchange amino acid analyses. When the guard column is regularly changed after 120 analyses, the separation remains satisfactory for at least 700 OPA, 800 FMOC-Cl, 150 PITC and 500 dansyl-Cl analyses. Careful control of factors and limitations inherent in the various methodologies is a prerequesite for proper identification and appropriate quantitation.

INTRODUCTION

For nearly 30 years, amino acid determinations have been carried out mainly by means of various automated ion-exchange post-column derivatization (mostly nin-

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hydrin) methods[1–4]. Reversed-phase high-performance liquid chromatography (RP-HPLC) is now recognized as a powerful method in biological research, and the usefulness of this technique for determining amino acids has been amply demonstrated in numerous publications[5–19]. In these reports, adequate separation and subsequent determination of free amino acids in tissues[9,11–14] and reduced analysis times and enhanced sensitivity are claimed in comparison with alternative methods[9,11,15–19]. Although this advanced technology may allow the proper application of HPLC in routine biological practice[20,21], the validity and value of this approach have not yet been adequately examined and/or verified.

In this paper we compare the applicability of four pre-column derivatization RP-HPLC methods, with *o*-phthaldialdehyde (OPA), 9-fluorenylmethyl chloroformate (FMOC-Cl), phenyl isothiocyanate (PITC) and 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl-Cl) to the analysis of physiological fluids (plasma, muscle, liver, kidney and leucocytes). Special attention was devoted to the control of factors and limitations inherent in these techniques and in the interpretations of their findings.

EXPERIMENTAL AND RESULTS

o-Phthaldialdehyde method

This derivatization method, introduced as early as 1971[22], is probably the most commonly used in RP-HPLC for the determination of free amino acids.

Manual procedure. In a previous paper[23] we reported analytical conditions allowing the precise measurement of 26 free amino acids in biological samples (plasma, muscle and liver) in the low picomole range. Derivatization was performed manually, and the adducts were detected by fluorescence. Previously reported problems of poor resolution between asparagine and serine and between valine and methionine[24] with 2-mercaptoethanol as a thiol component could be overcome by the use of 3-mercaptopropionic acid (3-MPA) and by substituting acetonitrile for methanol. Applying these optimized conditions, 23 major physiological amino acids can be separated in less than 13 min in the lower picomole range[21].

These favourable results with 3-MPA are presumably due to the introduction of an α-carboxyl group into the amino acid adduct, thereby reducing the hydrophobicity of these adducts in comparison with those formed with other mercaptans[24,25].

We use the manual OPA method commonly at the sensitivity level of 10 pmol amino acid per injection. The limit of sensitivity (0.8 pmol) was determined[21,26] at a signal-to-noise ratio of 2.5:1. Exceptions are histidine and lysine, detected at a minimum of 2.5 and 3.5 pmol, respectively.

The reproducibility was determined on the basis of 25 standard analyses and yielded values between 4.2 and 6.8% [coefficient of variation (C.V.)], respectively. The reliability of the method was assessed by twenty repeated injections of the same derivatized plasma sample, ranging between 1.8% and 6.0% (C.V.), except for tyrosine and histidine (9.8% and 8.6%, respectively)[21]. The error of the method was determined on the basis of twenty duplicate analyses of human plasma samples. The estimate of errors includes the sample preparation and all analytical procedures used. The error in a single determination, based on duplicates, ranged between 4.5 and 8.2% (C.V.) for the individual amino acids. The error in duplicate determinations ranged between 3.2 and 6.1% (C.V.)[21,26,27].

TABLE I

FREE AMINO ACID CONCENTRATIONS IN HUMAN PLASMA (nmol/ml), DETERMINED BY HPLC (PRE-COLUMN DERIVATIZATION WITH OPA, FMOC AND PITC) AND ION-EXCHANGE CHROMATOGRAPHY (NINHYDRIN POST-COLUMN DERIVATIZATION)

Results are means ± S.D. (n = 10).

Amino acid	*Ion exchange (ninhydrin)*	*HPLC*		
		OPA (automated)	*FMOC-Cl*	*PITC*
Glu	23.0 ± 8.4	16.4 ± 4.7	19.1 ± 4.0	11.6 ± 3.9
Asn	38.4 ± 7.1	38.4 ± 10.9	41.2 ± 7.0	43.8 ± 9.5
Ser	119.9 ± 10.6	99.4 ± 26.6	103.3 ± 18.3	102.0 ± 13.5
Gln	574.5 ± 31.8	556.7 ± 31.5	547.3 ± 51.1	493.1 ± 56.9
Gly	262.2 ± 46.6	213.5 ± 34.7	207.1 ± 29.5	191.8 ± 23.4
Thr	114.7 ± 30.3	96.5 ± 30.1	102.1 ± 20.3	116.6 ± 20.8
His	85.7 ± 23.2	85.5 ± 16.4	69.7 ± 20.5	79.8 ± 20.3
Ala	353.5 ± 105.1	319.0 ± 99.4	303.7 ± 70.7	339.9 ± 51.5
Tau	60.7 ± 8.8	38.9 ± 9.8	38.7 ± 4.4	59.4 ± 16.8
Arg	84.8 ± 7.2	74.3 ± 29.9	74.9 ± 19.6	77.5 ± 11.6
Tyr	66.8 ± 17.2	48.1 ± 17.0	51.4 ± 11.1	54.7 ± 5.0
Val	198.7 ± 36.9	209.1 ± 40.6	185.3 ± 43.2	222.2 ± 28.3
Met	28.7 ± 12.4	35.0 ± 7.5	25.3 ± 7.0	25.9 ± 2.8
Ile	54.2 ± 12.1	51.1 ± 6.6	50.4 ± 13.8	68.7 ± 10.3
Phe	53.7 ± 12.9	52.2 ± 12.3	47.5 ± 8.2	60.3 ± 4.8
Trp	45.8 ± 17.4	34.5 ± 7.6	–	–
Leu	114.9 ± 24.7	112.7 ± 24.2	104.2 ± 29.2	141.6 ± 16.8
Orn	80.0 ± 23.2	81.4 ± 15.1	78.5 ± 10.8	–
Lys	195.9 ± 44.7	182.7 ± 39.4	178.1 ± 40.7	202.7 ± 43.6
Pro	205.3 ± 40.8	–	193.3 ± 50.6	240.8 ± 48.1

Free amino acid concentration in muscle, liver and kidney, as determined by OPA–HPLC, were in the expected physiological range and agree with those obtained by a conventional amino acid analyzer[28,29]. In plasma (Table I), the correlation between the two methods were highly significant ($r = 0.97, p < 0.001$) in 120 comparative analyses.

Automated method. The instability of the OPA–amino acid adducts seriously limits reproducibility and proper quantification. Hence it is necessary to develop an automated derivatization procedure allowing a given optimum derivatization time for the reaction to be maintained[26,27,30,31]. Another advantage of an automated on-line derivatization system is the considerably higher capacity.

As suitable refrigerated sample injectors were not commercially available at that time, we modified an LKB 2153 autoinjector to permit automated pre-column derivatization and subsequent injection[26,27,30,31]. In this system, the normal digitally pre-set functions of the autoinjector for flush cycles and the injection–fill cycles were changed into digitally pre-set functions for pump time (0–90 s) and derivatization time (0–990 s), respectively. On-line derivatization was facilitated by attaching a peristaltic pump to the autoloader. This permitted mixing of the sample with the OPA–3-MPA reagent in an external mixing T-piece (reaction vessel) in the pumping mode. The prepared samples and the reagent bottle were placed in a thermostated box on

the top of the autoinjector, which was held at ambient temperature (25°C) or cooled to 4°C. As the external reaction vessel was not thermostated, the temperature of the reactants rose to *ca.* 7°C after a 1.5-min reaction time. In the complete, automated HPLC system, the autoinjector was connected to the controller by an interface cable. Full control of the analysis time and gradient profile for each sample injection and of the number of repetitive injections from each vial was then achieved[26,27,30,31].

Compared with the manual method at similar sensitivity, considerable improvement in reproducibility could be achieved in the range 0.4–2.2%. The reliability of the method, as calculated from twenty consecutive analyses of the same plasma samples, was improved to 0.5–3.8% (C.V.). Exceptions were aspartic acid and α-aminobutyric acid (5.8% and 6.9%, respectively)[26]. The error of the method was assessed from 180 duplicate analyses of human plasma samples. The resulting variations were in the range 1.0–4.7% (C.V.) (except for aspartic acid, 10.4%), which are considerably less than observed with the manual technique[26]. As expected, the limit of sensitivity (0.8 pmol) is identical with that measured with the manual procedure.

This automated method has been used in our laboratory for analysing biological samples, such as plasma and extracts of leucocytes, muscle, liver, kidney and gut, and has now been introduced in numerous other laboratories. Typical analysis of (A) standard and (B) human plasma are shown in Fig. 1. When the guard column is regularly changed after 100–150 analysis, the separation remains satisfactory for at least 700 analyses, as indicated by unaltered relative retention time and peak width[21,26]. Indeed, the major disadvantage of the OPA method lies in the fact that only primary amines form OPA adducts[22].

Ultrasensitive HPLC applications. In certain very particular and demanding applications, it is necessary to determine trace levels of amino acids in minute amounts of samples. By using microbore HPLC columns, we achieved the resolution of all amino acids present in protein hydrolysates at a sensitivity of 25 fmol of amino acid per injection in less than 30 min (Fig. 2). The limit of sensitivity was 5 fmol at signal-to-noise ratio of 2.5. However, it must be emphasized that elution must be carried out at a flow-rate of 50–80 μl/min, and that reliable and reproducible mixing of the gradient solvents is difficult to accomplish at this flow-rate. In addition, fluorescence detection must be performed with a microcuvette of volume less than 3 μl. These crucial limitations may render microbore chromatography impractical in most applications.

Another ultrasensitive application is the narrow-bore method, employing RP-HPLC columns of I.D. 1.8 mm[19]. Representative separations from (A) a standard and (B) a plasma sample are shown in Fig. 3. The sensitivity of the analysis is about 1 pmol of amino acid per injection and the reproducibility and reliability range between 4 and 8% (C.V.). The separation of 23 major free amino acids can be accomplished in 22 min. The limit of sensitivity is about 150 fmol at a signal-to-noise ratio of 2.5.

The great advantage of narrow-bore chromatography compared with conventional column technology is the considerably reduced consumption of expensive and polluting organic solvents, the actual use of such reagents with the narrow-bore method being only 15–20% of that with conventional RP-HPLC.

FMOC-Cl-method

Pre-column derivatization with FMOC-Cl permits the fluorimetric detection of primary and secondary amino acids as stable FMOC adducts[32–34].

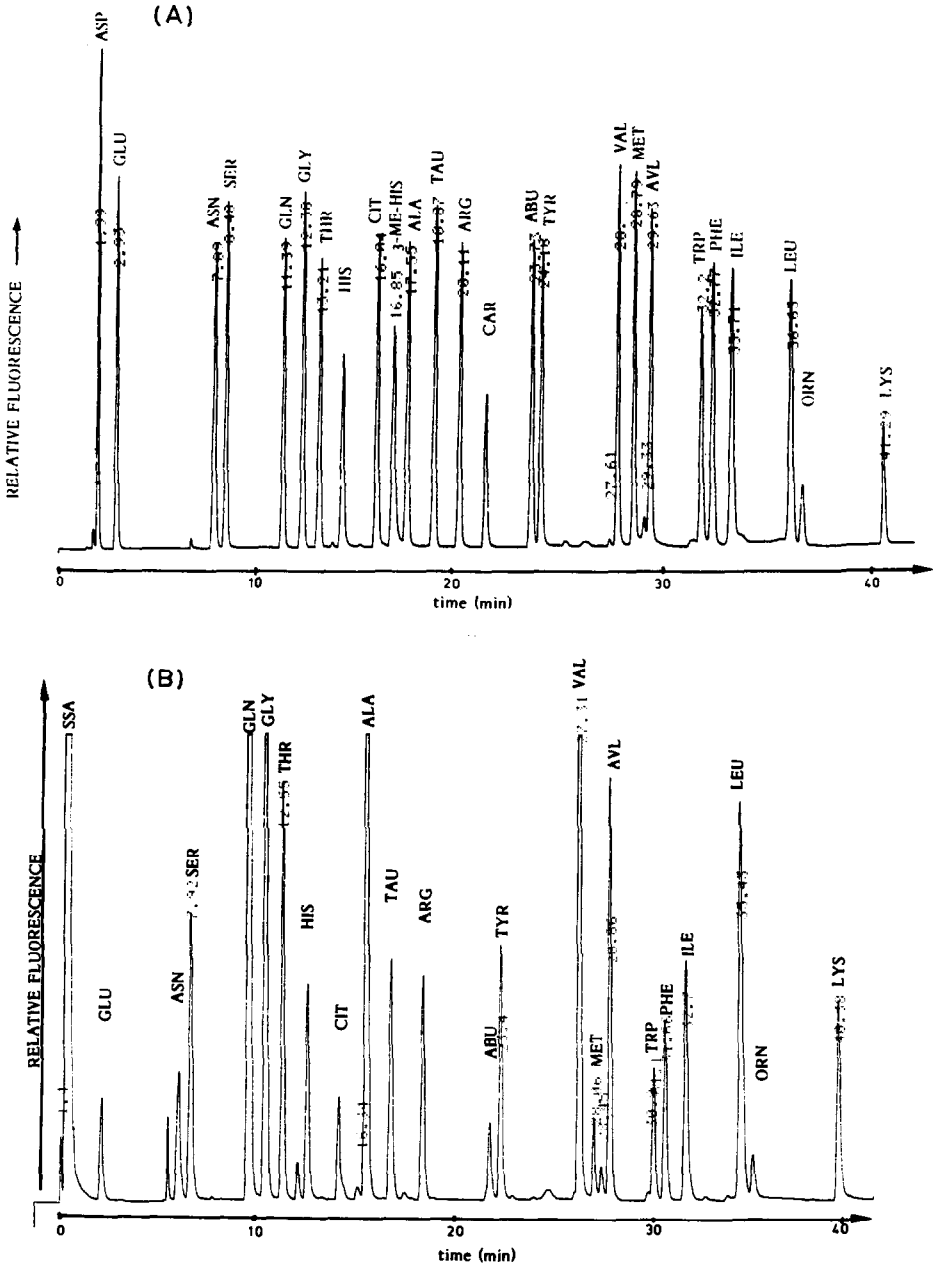

Fig. 1. Typical chromatograms of an (automated) OPA–3-MPA derivatized (A) amino acid standard and (B) human plasma. SSA = sulphosalicylic acid (deproteinization agent); CYA = cysteic acid; CIT = citrullin; TAU = taurine; CAR = carnosine; SAR = sarcosine; ABU = α-aminobutyric acid; AAD = α-aminoadipic acid; AVL = norvaline (internal standard); ORN = ornithine. Chromatographic conditions: column, Spherisorb ODS II (3 μm) (150 × 4.6 mm I.D.); guard column, 10 × 4.0 mm I.D.; eluent A, 0.5% tetrahydrofuran in 12.5 m*M* sodium phosphate buffer (pH 7.2); eluent B, 35% methanol and 15% acetonitrile in 12.5 m*M* sodium phosphate buffer (pH 7.2); flow-rate, 1.0 ml/min; gradient, 0 min 0% B, 3 min 0% B, 20 min 35% B, 36 min 60% B, 40 min 70% B, 43 min, 100% B, 45 min, 100% B, 47 min, 0% B; temperature, 25°C; fluorescence detection, λ_{ex} 330 nm, λ_{em} 450 nm; injection volume, 20 μl; standard, 10 pmol per amino acid.

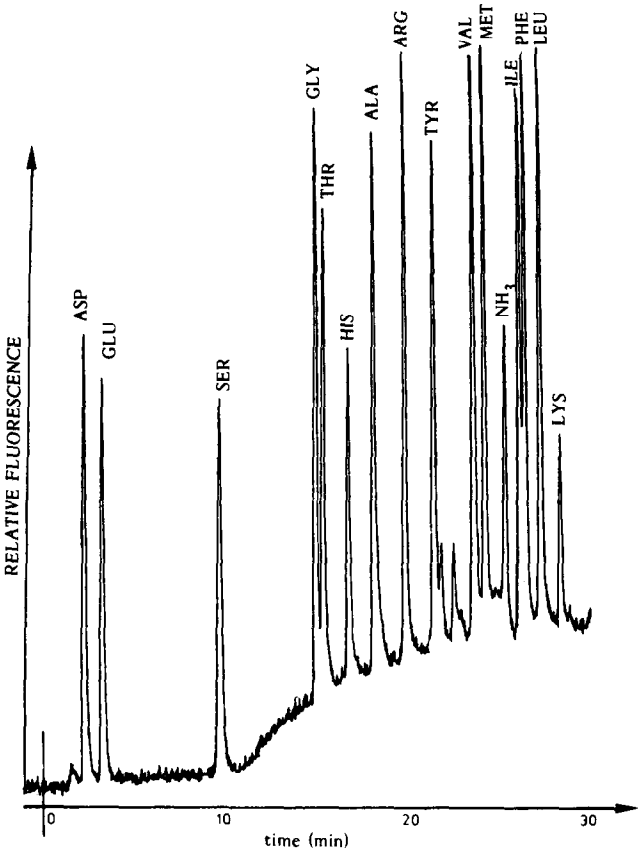

Fig. 2. Typical microbore HPLC of a hydrolysate standard mixture (OPA–3-MPA derivatization). Chromatographic conditions: column, Spherisorb ODS II (3 μm) (250 × 1.0 mm I.D.); eluent A, 0.5% acetonitrile + 2.5% methanol in 12.5 m*M* sodium phosphate buffer (pH 7.2), eluent B, 25% acetonitrile + 5% methanol in 12.5 m*M* sodium phosphate buffer (pH 7.2); flow-rate, 80 μl/min; gradient, 0 min 0% B, 20 min 100% B, room temperature; fluorescence detection, λ_{ex} 330 nm, λ_{em} 450 nm; injection volume, 1 μl; 25 fmol per amino acid.

A typical chromatogram of a physiological standard mixture (Fig. 4A) demonstrates the separation of 22 amino acids in 23 min. An application of this method to biological material (human plasma) is shown in Fig. 4B. Elongation of the column and extension of the running time permit the satisfactory separation of additional amino acids, such as citrulline and hydroxyproline[34].

The mean free amino acid concentrations in human plasma determined by employing HPLC with FMOC-Cl, as compared with conventional ion-exchange analysis of the same plasma samples from ten healthy subjects, are included in Table I. As shown, the results of these two methods are in good agreement. Exceptions are histidine and taurine, yielding considerably lower concentrations (20 and 30%, respectively) with FMOC-Cl than those found by ion-exchange chromatography. Measurements of free amino acids in muscle, liver or kidney tissues are troublesome with the FMOC-Cl method owing to the low buffering capacity of the reaction mixture.

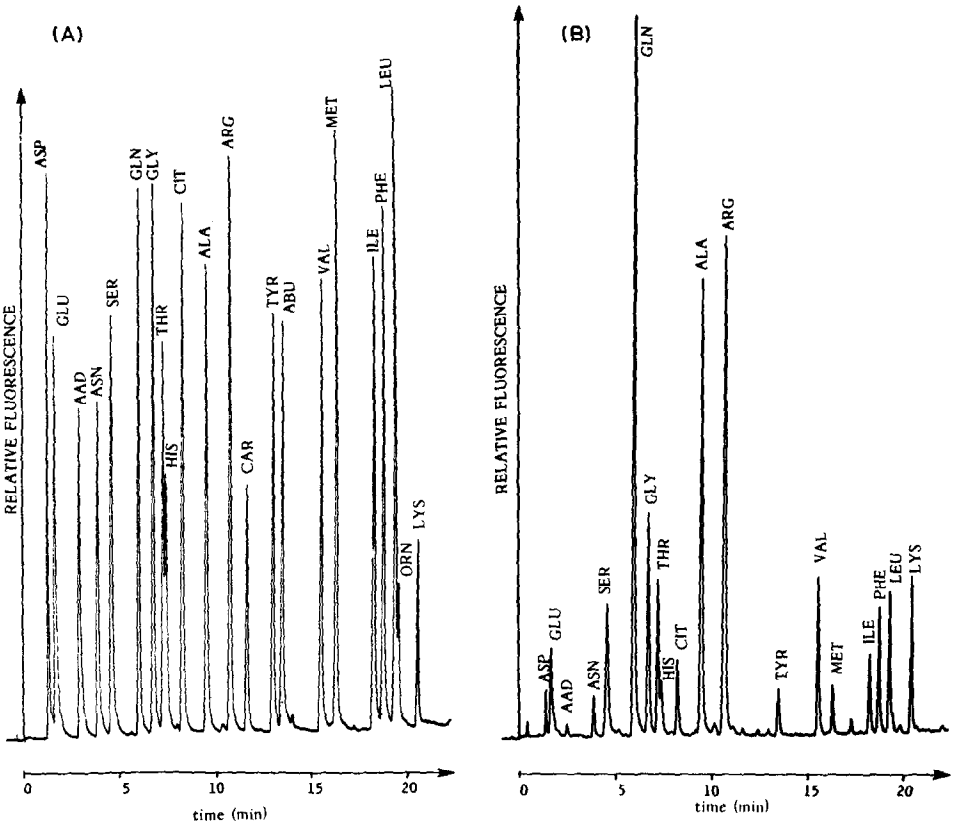

Fig. 3. Narrow-bore HPLC of (A) a standard mixture consisting of 22 amino acids and (B) TCA-precipitated rat plasma after derivatization with OPA–3-MPA. Abbreviations as in Fig. 1. Chromatographic conditions: column, Spherisorb ODS II (3 μm) (200 × 1.8 mm I.D.); eluent A, 0.5% tetrahydrofuran in 12.5 m*M* sodium phosphate buffer (pH 7.2); eluent B, 25% acetonitrile in 12.5 m*M* sodium phosphate buffer (pH 7.2); flow-rate, 200 μl/min; gradient, 0 min 0% B, 20 min 100% B; room temperature; fluorescence detection, λ_{ex} 330 mm, λ_{em} 450 nm (Merck F-1000); injection volume, 1 μl; standard, 1 pmol per amino acid.

The reproducibility of the FMOC-Cl derivatization procedure was calculated from twenty standard analyses. The C.V. ranged between 1.9 and 3.6%, except for histidine (4.6%). This can be explained by the low relative fluorescence response of FMOC–histidine which is only about 20% of that of the other FMOC–amino acids. This difference is probably caused by intramolecular quenching[35]. It is worth noting that determination of free tryptophan and cystine is not feasible with the FMOC-Cl method because the fluorescence of the adducts is also quenched[35,36]. The error of the method, based on 30 duplicate analyses of human plasma samples, ranged between 1.1 and 5.9% (C.V.), with the exception of histidine (9.2%). The detection limit at a signal–to-noise ratio of 2.5 was 1.0 pmol for all amino acids, except histidine (4.5 pmol).

The FMOC-Cl method suffers from the disadvantage that excess of strong fluorescent reagent has to be extracted manually with pentane in order to stop the

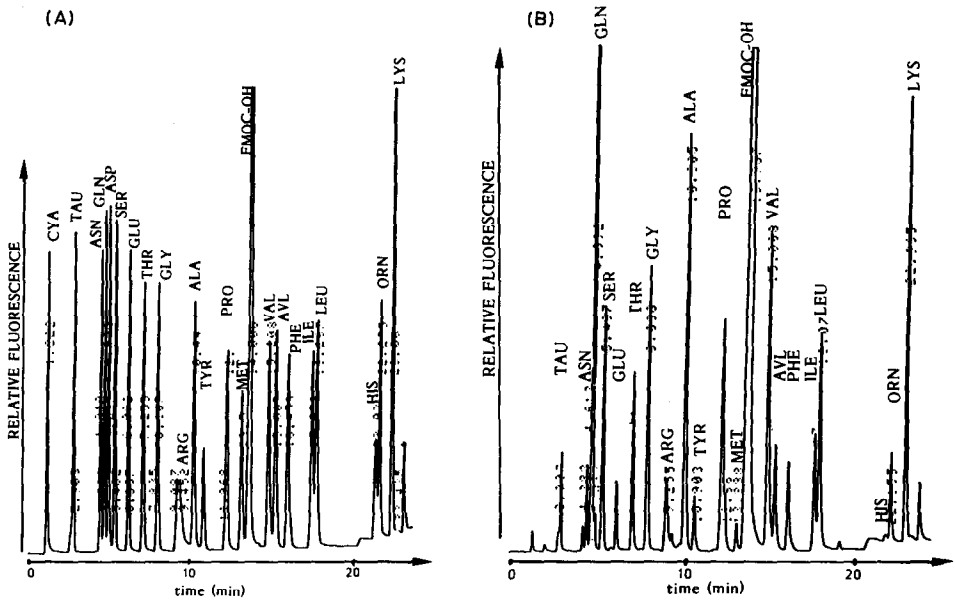

Fig. 4. Elution profiles of (A) an amino acid standard and (B) human plasma after derivatization with FMOC-Cl. Abbreviations as in Fig. 1. Chromatographic conditions: column, Superspher CH-8 (125 × 4.0 mm I.D.); eluent A, 20% acetonitrile in 50 m*M* sodium acetate buffer (pH 4.2); eluent B, 70% acetonitrile in 50 m*M* sodium acetate buffer (pH 4.2); flow-rate, 1.5 ml/min; gradient, 0 min 10% B, 3 min 10% B, 9 min 30% B, 17 min 60% B, 18 min 100% B; temperature, 35°C; fluorescence detection, λ_{ex} 265 nm, λ_{em} 310 nm (Merck F-1000); injection volume, 20 μl; standard, 5.2 pmol per amino acid, except His (10.4 pmol) and Orn (2.6 pmol).

derivatization reaction and to avoid spontaneous hydrolysis of the FMOC adducts[34]. This laborious manual extraction procedure prevents the wide acceptance of this method. This shortcoming, however, might be overcome by using specially designed autosamplers and/or a combination of the FMOC-Cl and OPA methods[37].

PITC method

Derivatization with Edman's reagent (PITC) results in the formation of phenylthiocarbamyl (PTC) adducts of primary and secondary amino acids, which can be separated by RP-HPLC[38,39]. After phenylthiocarbamylation (within 5 min at room temperature), excess of coupling reagent must be thoroughly evaporated by vacuum centrifugation (Speed Vac; Savant, Farmingdale, NY, U.S.A.) to furnish acceptable separation conditions and to protect the column. The PTC–amino derivatives are stable for several days and can be automatically injected with an autosampler[27].

The elution profile of a PITC-derivatized standard mixture containing 21 physiological amino acids, including proline and cystine, is depicted in Fig. 5A and that of a human plasma in Fig. 5B. It is important to note that under the conditions used, tryptophan coelutes with ornithine. The mean free amino acid concentrations of plasma acquired from ten healthy subjects agreed well with values derived from conventional amino acid analyses, with the exception of glutamic acid (Table I).

The reproducibility of the method ranged between 2.6% and 5.5% (C.V.) for all

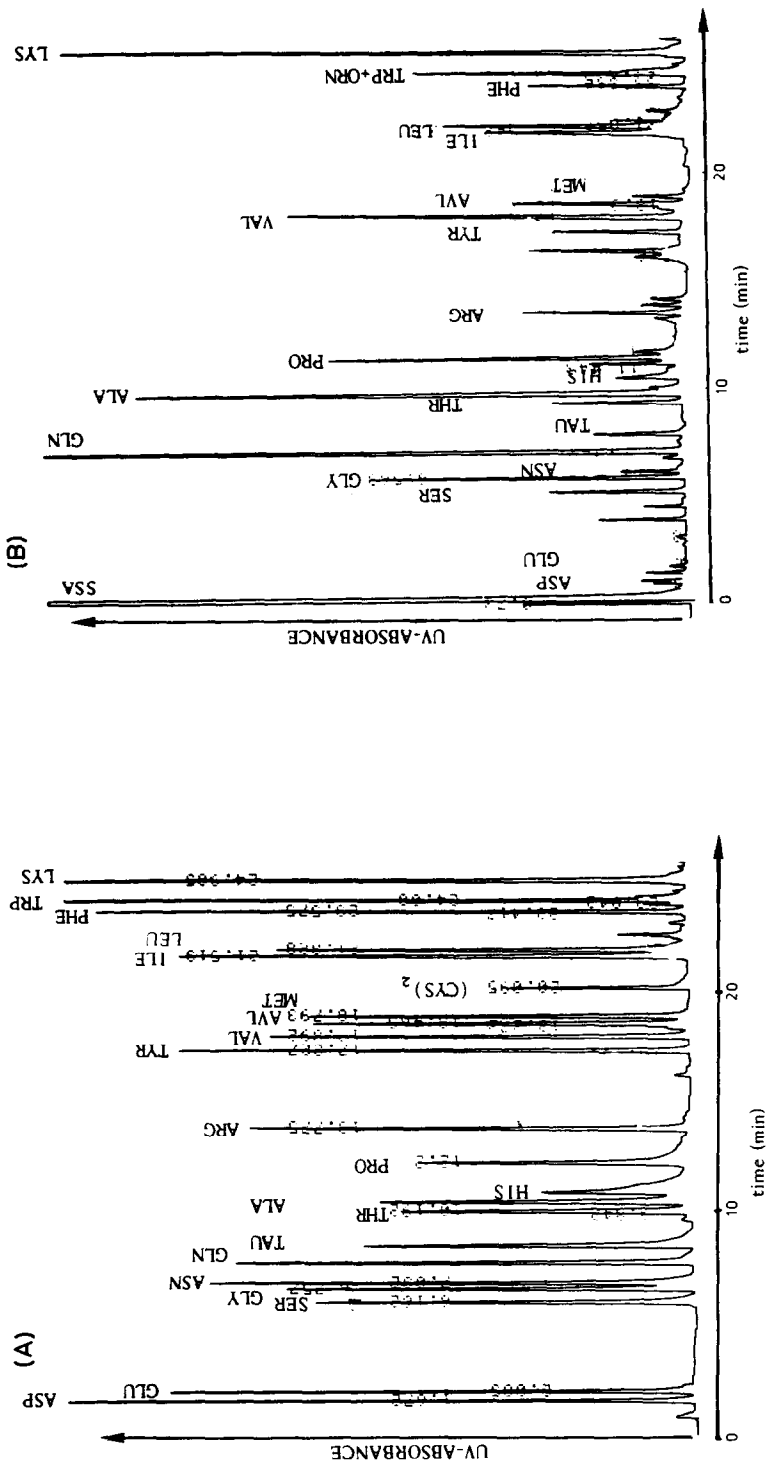

Fig. 5. Elution profiles of (A) an amino acid standard and (B) human plasma after derivatization with PITC. Abbreviations as in Fig. 1. Chromatographic conditions: column, Spherisorb ODS II (3 μm) 125 × 4.6 mm I.D.); guard column, 10 × 4.0 mm I.D.; eluent A, 12.5 m*M* sodium phosphate buffer (pH 6.8); eluent B, 60% acetonitrile in 12.5 m*M* sodium phosphate buffer (pH 6.8); flow-rate 1.2 ml/min; gradient, 0 min 0% B, 5 min 6% B, 19 min 48% B, 25 min 100% B; temperature, 37°C; UV detection, 254 nm (LKB 2151 variable-wavelength monitor); injection volume, 20 μl; standard, 500 pmol per amino acid.

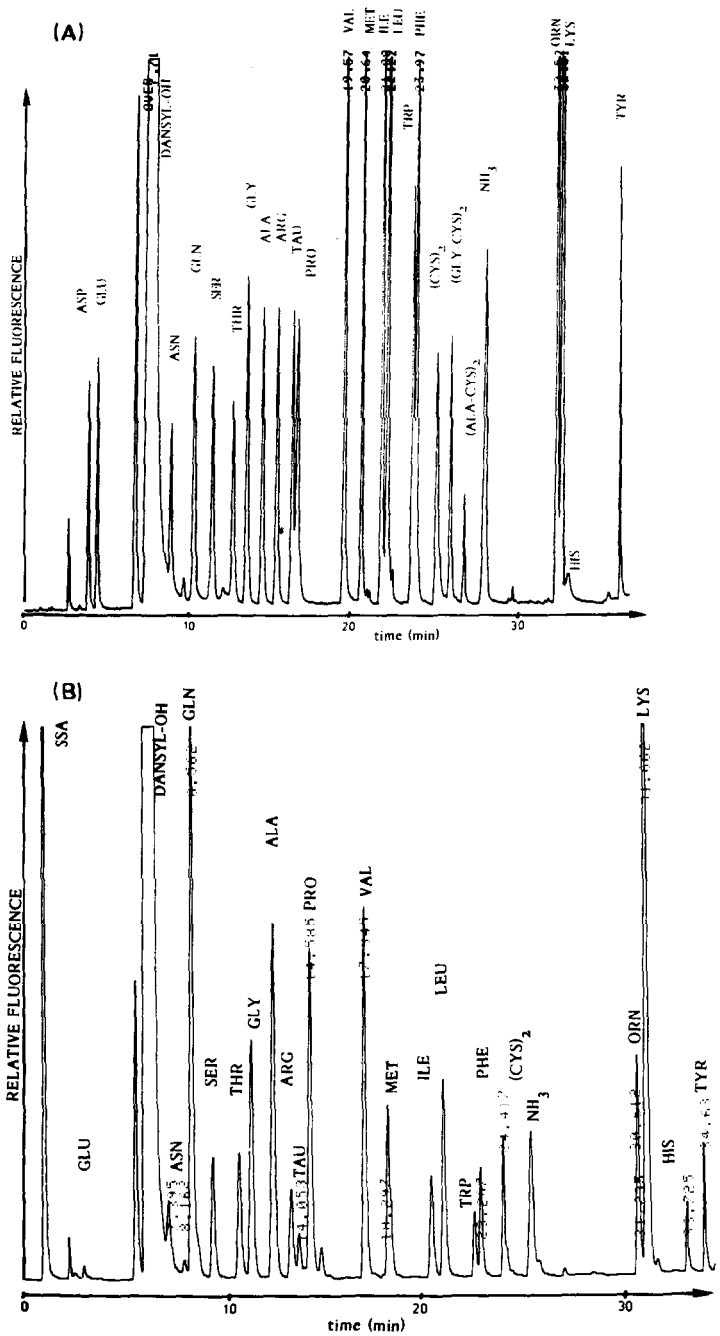

Fig. 6. Elution profile of (A) a standard mixture containing 22 amino acids and the synthetic peptides $(Gly\text{–}Cys)_2$ and $(Ala\text{–}Cys)_2$ and (B) a human plasma after derivatization with dansyl-Cl. Abbreviations as in Fig. 1. Chromatographic conditions: column, Spherisorb ODS II (3 μm) (125 × 4.6 mm I.D.); guard column, 10 × 4.0 mm I.D.; eluent A, 3% tetrahydrofuran in 15 m*M* sodium phosphate buffer (pH 6.3); eluent B, 60% acetonitrile in 15 m*M* sodium phosphate buffer (pH 6.3); flow-rate, 1.2 ml/min; gradient, 0 min 15% B, 15 min 30% B, 18 min 40% B, 25 min 45% B, 30 min 60% B, 35 min 60% B, 40 min 100% B; temperature, 25°C; fluorescence detection, λ_{ex} 330 nm, λ_{em} 550 nm (Merck F-1000); injection volume, 20 μl; standard, 40 pmol per amino acid and peptide except cystine (20 pmol).

amino acids except histidine (6.3%) and cystine (10.0%). Apart from the poor reproducibility, PTC–cystine also revealed an inferior linearity, and therefore the determination of free cystine is not practicable with this method.

As UV detection has to be employed, the sensitivity of the PITC method is low compared with that of the other methods. The limit of sensitivity is 50 pmol at a signal-to-noise ratio of 2.5, which is *ca.* 50 times less sensitive than detection of OPA or FMOC adducts. This is in excellent agreement with most previous studies[38,40], but is in contrast to Bidlingmeyer *et al.*[39], who reported a 10-fold lower detection limit (4 pmol at a signal-to-noise ratio of 5) when analysing a standard amino acid mixture. However, such a level is of little practicable value for routine analysis of biological materials.

In addition to poor sensitivity, inferior linearity with cystine and the lengthy sample preparation, we observed rapid deterioration of the columns when analysing biological fluids. This is a serious shortcoming of the PITC method. According to our experience, a maximum of 150 physiological analyses could be performed in spite of the use of rigorous sample preparation and suitable guard columns. The reduced column lifetime is probably caused by traces of the PITC reagent obviously present in the samples, despite careful vacuum evaporation.

Dansyl-Cl method

Dansyl-Cl is a well known fluorogenic reagent for the determination of primary and secondary amines[41,42]. According to Tapuhi *et al.*[43], dansyl–amino acid adducts are formed under optimal conditions within 35–50 min at room temperature in the

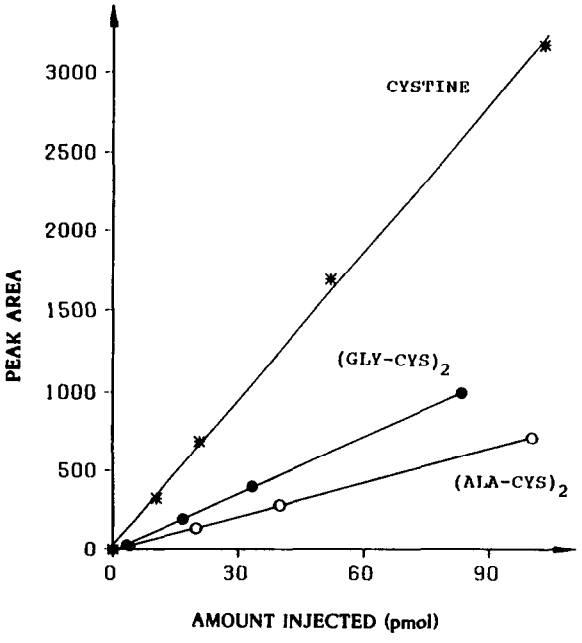

Fig. 7. Linearity obtained with dansyl derivatives of cystine [$(Cys)_2$], bis glycyl-L-cystine [$(Gly–Cys)_2$] and bis-L-alanyl-L-cystine [$(Ala–Cys)_2$] in the range 5–150 pmol.

dark[42]. After separation by RP-HPLC, the dansylated derivatives are detected fluorimetrically. Under these conditions, histidine formed multiple peaks whereas for cystine, ornithine, lysine and tyrosine only the didansyl derivatives were detectable. The minor histidine peak (corresponding to the monoderivatized adduct) eluted between asparagine and glutamine and therefore did not interfere with the determination of the other amino acids.

Fig. 6A shows a typical chromatogram of a dansylated standard mixture containing 22 primary and secondary amino acids, including cystine, and two cystine-containing short-chain peptides. The elution profile of a dansylated human plasma is depicted in Fig. 6B. All the major physiological amino acids can be separated in 35 min.

The reproducibility of the method was calculated from ten standard analyses; the C.V. ranged between 1.5% and 4.1% for amino acids and peptides, except histidine (6.4%). The poor reproducibility for histidine can be explained by the low relative fluorescence response of the didansylated adduct (only about 10% of the other amino acids), which is caused by intramolecular quenching[35] and by the formation of two dansyl derivatives. The error of the method was calculated from 30 duplicate analyses of rat plasma and ranged between 1.7% and 4.5% (C.V.).

In contrast to the PITC method, the dansyl-Cl technique shows excellent linearity for cystine and also for cystine-containing short-chain peptides (Fig. 7). Hence, the dansyl-Cl method appears to offer the only quantitative approach for measuring free cystine in biological material by HPLC techniques[44].

CONCLUSION

Each of the four methods investigated permits the measurement of the concentrations of the 21–26 major free amino acids in 13–40 min[21,23,26,27,34,44]. The sensitivity, errors of the methods, advantages and disadvantages and problems with certain "difficult" amino acids are summarized in Table II.

Automated derivatization and the wide range of applicability clearly favour the

TABLE II

HPLC ANALYSES OF FREE AMINO ACIDS: COMPARISON OF FOUR DERIVATIZATION METHODS

Parameter	*OPA (automated method)*	*FMOC-Cl*	*PITC*	*Dansyl-Cl*
Limit of sensitivity, pmol (Signal-to-noise ratio = 2.5)	0.8	1.0	5.0	1.5
Error of the method (C.V.,%) (based on duplicate determinations)	1.0–4.7	1.1–5.9	3.6–7.0	1.7–4.5
Reproducibility (C.V.,%)	0.4–2.2	1.9–4.6	2.6–5.5	1.5–4.1
Stable adducts[a]	N	Y	Y	Y
Detection of secondary amines/cystine[a]	N/N	Y/N	Y/Y	Y/Y
Laborious	–	+ + +	+ +	+
Problematic amino acids	Asp, Trp	His, Trp	Orn, Trp, His, $(Cys)_2$	His, Asn

[a] Y = yes; N = no.

OPA method for routine analyses of primary free amino acids (except cystine) in biological fluids. If determination of secondary amino acids is desirable, the use of the FMOC-Cl method or a combination of the OPA and FMOC-Cl techniques[37] is recommended. The use of the dansyl-Cl technique is suggested when the determination of free cystine or cystine-containing short-chain peptides is required. Application of the PITC method might be beneficial in frame of clinical and/or protein chemistry, if sufficient sample material is available.

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