Journal of Chromatography, 534 (1990) 23–35
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam

CHROMBIO. 5501

Serum amino acid analysis with pre-column derivatization: comparison of the *o*-phthaldialdehyde and N,N-diethyl-2,4-dinitro-5-fluoroaniline methods

I FERMO and E DE VECCHI

Istituto Scientifico Hosp San Raffaele, Milan (Italy)

L. DIOMEDE

Istituto di Ricerche Farmacologiche Mario Negri, Via Eritrea 62, Milan (Italy)

and

R. PARONI*

Istituto Scientifico Hosp San Raffaele, Laboratorio Centrale, Via Olgettina 60, 20132 Milan (Italy)

(First received June 12th, 1990, revised manuscript received July 18th, 1990)

ABSTRACT

We compared two pre-column derivatization methods, *o*-phthaldialdehyde (OPA) and N,N-diethyl-2,4-dinitro-5-fluoroaniline (FDNDEA), for analysis of serum amino acids by reversed-phase high-performance liquid chromatography Separations took 102 and 106 min for FDNDEA and OPA (reconditioning included), respectively, allowing a very good resolution of 30 amino acids by the former process and 38 by the latter Linearity, within- and between-day variability and advantages in terms of accuracy and speed were studied for both methods Twenty serum samples from healthy volunteers were assayed with OPA, FDNDEA and with the reference method of ion-exchange and post-column ninhydrin reaction (amino acid analyser), which took 170 min The correlation between OPA and ninhydrin was good for all the amino acids ($r = 0\ 959$) except for the last-eluting lysine Good agreement was found for FDNDEA ($r = 0.987$), which appeared in general to be a highly reproducible technique. Both pre-column methods were more sensitive than the post-column ninhydrin method

INTRODUCTION

The traditional method of amino acid analysis involves ion-exchange chromatography, followed by post-column derivatization with ninhydrin (NIN). This technique, developed by Stein *et al.* in 1958 [1], has been improved gradually over the years and there are numerous papers describing various elution strategies [2–5]. One of the most significant applications of this technique is for analysis of the pattern of free amino acids in physiological fluids [6–10]. In spite of the disadvantages of being time-consuming and requiring dedicated and expensive instrumentation, this is still regarded as the 'reference method' for amino acid analysis in biological fluids.

0378-4347/90/$03.50

A radical change of approach has been the use of pre-column derivatization procedures followed by separation of the amino acid derivatives by reversed-phase high-performance liquid chromatography (RP-HPLC). In comparison with the post-column derivatization technique, pre-column methods give shorter analysis time and higher sensitivity, although they are not optimal with respect to accuracy or precision where biological fluids are concerned.

The reagent 1-dimethylaminonaphthalene-5-sulphonyl chloride (DANS-Cl), for instance, has a long reaction time [11,12] and forms by-products that interfere in the chromatographic separation [13] The fluorenyl methylchloroformate (FMOC) derivatives show the highest sensitivity, but are not satisfactorily resolved by HPLC, because the fluorescent hydrolysis product from the excess reagent elutes in the middle of the chromatogram. The extraction procedures required to eliminate this peak reduce the reproducibility of the method [14,15].

The procedure based on formation of phenylisothiocarbamyl (PTC) derivatives, first described by Heinrikson and Meredith [16], has been satisfactorily applied to the analysis of protein hydrolysates [17,18]. However, quantification of amino acids in biological fluids is more of a problem because of the reduced yield of many PTC-amino acids after deproteinization with the most common precipitants [19]. *o*-Phthaldialdehyde (OPA) derivatization is rapid (1–3 min), but limited to primary amino acids, and the derivatives are highly unstable [20–22].

Most of these HPLC methods have been widely applied for analysis of protein hydrolysates, whose patterns are rather less complex [11,17–19,21,23,24] than those of biological fluids. Less information is available about their application to serum or urine analysis [25–28] or comparison with the reference method [29].

We assessed the validity of two pre-column techniques for amino acid analysis in serum. We chose OPA and N,N-diethyl-2,4-dinitro-5-fluroaniline (FDNDEA) as derivatizing agents. The OPA fluorophore is particularly attractive because the fluorescent products are rapidly formed in aqueous solution and can be detected with high sensitivity. Unlike OPA derivatives, DNDEA-amino acids are very stable to light and heat, and seem to be promising for this kind of analysis [30]. We analysed serum samples from twenty healthy fasting subjects by both techniques, and compared the results with those obtained with a traditional ion-exchange amino acid analyser.

EXPERIMENTAL

Chemicals

FDNDEA analysis. N,N-Diethyl-2,4-dinitro-5-fluoroaniline (FDNDEA) was purchased from Fluka (Buchs, Switzerland). Free amino acids for the preparation of DNDEA derivatives were obtained from Sigma (St. Louis, MO, U.S.A.). Analytical-reagent grade triethylamine was purchased from Fluka, and sodium hydrogencarbonate and acetic acid from Farmitalia Carlo Erba (Milan, Italy).

OPA analysis. OPA and 2-mercaptoethanol (MCE) were from Pierce (Chester,

U.K.). A standard solution of amino acids was prepared by mixing the neutral and acid amino acid solutions with the basic one (Sigma). The internal standards homocysteic acid, homoserine and norvaline were also from Sigma.

Iodoacetic acid, methyl sulphoxide and propionic acid were purchased from Janssen Chimica (Beerse, Belgium). All other chemicals, including methanol and acetonitrile, were obtained from Merck (Darmstadt, F.R.G.). Doubly distilled water was always used. All salts and reagents were analytical grade, and the buffers were filtered through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) before HPLC analysis.

NIN analysis. Lithium citrate high-performance buffer (Li-A 338 063; Li-D 338 066; Li-E 338 067; Li-F 338 068), regenerating reagent (Li-R 338 086), ninhydrin reagent (Nin-R 338 047) and the standard solution of amino acids for the amino acid analyser were obtained from Beckman Instruments (Palo Alto, CA, U.S.A.). Sodium dodecylsulphate (SDS) was purchased from Sigma and 5-sulphosalicylic acid (SSA) from Merck.

Reagents and solutions

FDNDEA. The derivatizing solution and the standard mixture were prepared as previously described [30] The internal standard (I.S.) was an aqueous solution of 6-aminocaproic acid (10 m*M*). For HPLC analysis, 3 ml of glacial acetic acid and 6 ml of triethylamine were mixed with 1 l of distilled water. The pH was adjusted to 4.3 with 5 *M* hydrochloric acid.

OPA. The OPA–MCE reagent was prepared by dissolving 5 mg of OPA in 100 μl of methanol and diluting to 1 ml with 400 m*M* sodium borate buffer (pH 9.5). Finally, 4 μl of 2-mercaptoethanol were added to this solution This reagent was freshly prepared every day and stored at 4°C. The standard amino acid mixture was prepared by mixing 100 μl of each standard physiological solution (2 5 m*M*) with 100 μl of glutamine (2.5 m*M*) and diluting 1:33 with water. Aliquots were stored at −80°C until use. The I.S. solution was 2.9 m*M* for each amino acid. The oxidizing reagent was prepared by dissolving 0.75 g of iodoacetic acid and 0.6 g of boric acid in 100 ml of water and titrating it to pH 9.5 with 2 *M* sodium hydroxide solution. The precipitating reagent was 3% SSA (w/v). The stock solutions of the HPLC buffers were made up by adjusting a solution of 250 m*M* propionic acid and 350 m*M* anhydrous disodium hydrogenphosphate to pH 6 5 with 2 *M* sodium hydroxide solution.

Apparatus

The chromatographic apparatus was the System Gold Beckman. For OPA analysis a Model 126 pump was connected through a Model 406 analogic interface with an LS-3 fluorescence detector (Perkin-Elmer, Norwalk, CT, U.S.A.). The excitation wavelength was 360 nm and the emission wavelength was 455 nm, with a fixed scale of 2.

For DNDEA-amino acid detection, we used a Model 166 spectrophotometer

(Beckman), set at 360 nm. The pump unit was equipped with a 210A valve injector (Beckman) fitted with a 20-μl loop. All modules were controlled by an IBM personal computer and the System Gold software for handling, storage and reprocessing of chromatographic data.

Amino acids were analysed by post-column ninhydrin reaction using a high-performance amino acid analyser (AA Model 6300, Beckman), set at 570 nm and interfaced with an IBM computer using the System Gold software.

Chromatographic conditions

To separate the OPA-amino acids, we used a Beckman Ultrasphere ODS column (150 mm × 4.6 mm I.D., 5 μm), protected by a guard-column (Spheri 5 ODS, 30 mm × 4.6 mm I.D., 5 μm) (Brownlee, Santa Clara, CA, U.S.A.). The mobile phase consisted of two eluents: 72% water, 20% sodium propionate buffer, 8% acetonitrile (solvent A) and 45% water, 30% acetonitrile, 25% methanol (solvent B) The flow-rate was 1.4 ml/min, and separations were carried out at room temperature; the gradient was a modification of the one reported elsewhere [27]. Isocratic conditions with 100% A were held for 30 min, then B was increased to 13% in 7 min. After 18 min, it was increased again to 25% in 2 min After 7 min, B was brought to 45% in 2 min, then after 9 min it was increased to 50% in 10 min and held for 5 min. Re-equilibration to 100% A lasted 5 min, and samples were injected after a further 10 min (see Fig. 1A).

DNDEA-amino acids were separated, as for OPA analysis, on a Beckman Ultrasphere ODS (150 mm × 4.6 mm I.D., 5 μm) with a Brownlee guard column. HPLC separation was obtained with a simple gradient between acetic acid buffer (A) and acetonitrile (B). The column was conditioned with 18% of solvent B. After the injection the proportion of B was increased linearly to 19.5% over 18 min. At 25 min it was increased again to 35% over 17 min. After 15 min B was brought up to 43% within 20 min, and then raised to 50% over 3 min. At 85 min it was brought down to 18% in 2 min and re-equilibrated for another 5 min before sample injection (Fig. 1B). The flow-rate was 1.3 ml/min.

A 25-cm-long column filled with a cation-exchange resin (Beckman 338 050) was used for the amino acid separation with the analyser. Complete resolution of all amino acids was achieved with a pH gradient and a temperature gradient according to the Beckman method for the analysis of physiological fluids (Beckman System 6300 Application Notes, October 1985). The flow-rate of the mobile phase was 20 ml/h and the flow-rate of Nin-R was 10 ml/h.

Biological samples

Blood samples (5 ml) were withdrawn by venipuncture in the morning from twenty healthy overnight-fasted subjects (aged 21–36 years). The blood was collected in a sterile Vacutainer®, containing a clot activator, and centrifuged at 1200 *g* for 10 min. After addition of the internal standards, the sera were immediately deproteinized and stored at −80°C until analysis

For the FDNDEA method, serum or standard mixture (100 μl) was deproteinized with 300 μl of acetonitrile after addition of the I.S. (10 μl). After centrifugation (Beckman, Microfuge 11), 100 μl of the supernatant were removed, lyophilized and derivatized.

For the OPA-amino acid analysis, 50 μl of standard amino acid solution or serum sample, added to 10 μl of the three I.S. solution, were deproteinized by the addition of 100 μl of 3% SSA. After centrifugation, 50 μl of the supernatant were derivatized.

For the analysis of amino acids by cation exchange, serum samples were diluted with 0.5% SDS (1:1, v/v) and deproteinized with 10% SSA (1:1.25, v/v). After centrifugation, 100-μl aliquots of the supernatants were injected into the AA 6300 or stored at −80°C.

Derivatization procedures

Preparation of the OPA derivatives. In a polypropylene Eppendorf tube, 100 μl of iodoacetic acid and 100 μl of OPA solutions were added to 50 μl of the SSA supernatant. The mixture was vortex-mixed and, exactly 1 min after OPA addition, 20 μl were injected into the column.

Preparation of DNDEA derivatives To each lyophilized sample, 50 μl of hydrogen carbonate (20 m*M*) and 50 μl of FDNDEA in acetonitrile (20 m*M*) were added. The mixture was heated at 100°C for 15 min and dried under vacuum. The residue was redissolved in 500 μl of acetonitrile–acetic acid buffer (1:1, v/v), and 20 μl aliquots were injected into the HPLC apparatus.

RESULTS

Serum HPLC analysis

Fig. 1 shows the chromatograms of standard mixtures analysed using the OPA and FDNDEA techniques. The analytical conditions chosen for OPA separation gave a good resolution of the typical serum amino acids, particularly of the more polar ones. Under these conditions, β-aminoisobutyric acid (BABA), 4-aminobutyric acid (GABA) and taurine (Tau) were not resolved satisfactorily. However, as the levels of BABA and GABA are not significant in normal serum, we did not try to improve this separation, which is very sensitive to the buffer pH.

In comparison with our previous conditions [30], the FDNDEA separation was considerably improved by using a Beckman Ultrasphere ODS column and an acetic acid buffer containing 0.6% of triethylamine. This counter-ion percentage results in a better resolution, particularly of the glycine–taurine, tryptophan–phenylalanine and leucine–isoleucine pairs. Altogether the new conditions allowed the resolution of 30 amino acids, as compared with 21 in our former work. The combination of a decreased flow-rate (1.3 ml/min) with a flatter gradient permitted an improvement of aspartic acid–glutamic acid–threonine separation. The separation of 1-methylhistidine, 3-methylhistidine, carnosine and anserine

was not improved because we were not interested in the determination of these amino acids.

The chromatograms of a serum specimen, analysed by the OPA and FDNDEA techniques, are shown in Fig 2.

Fig. 1C shows the chromatogram of a standard mixture resolved by ion-exchange chromatography (amino acid analyser) and Fig. 2C shows the ion-exchange profile of the serum sample previously analysed with the pre-column techniques. The total analysis time was 170 min, and the majority of amino acids were well resolved with the exception of the asparagine–glutamic acid and tryptophan ammonia pairs.

Precision of pre-column derivatization

In order to determine the intra-day precision of the analytical procedures, 50 μl of OPA (75.5 μM) and 100 μl of FDNDEA (250 μM) amino acid standard mixtures were derivatized and injected consecutively ($n = 5$) The results are

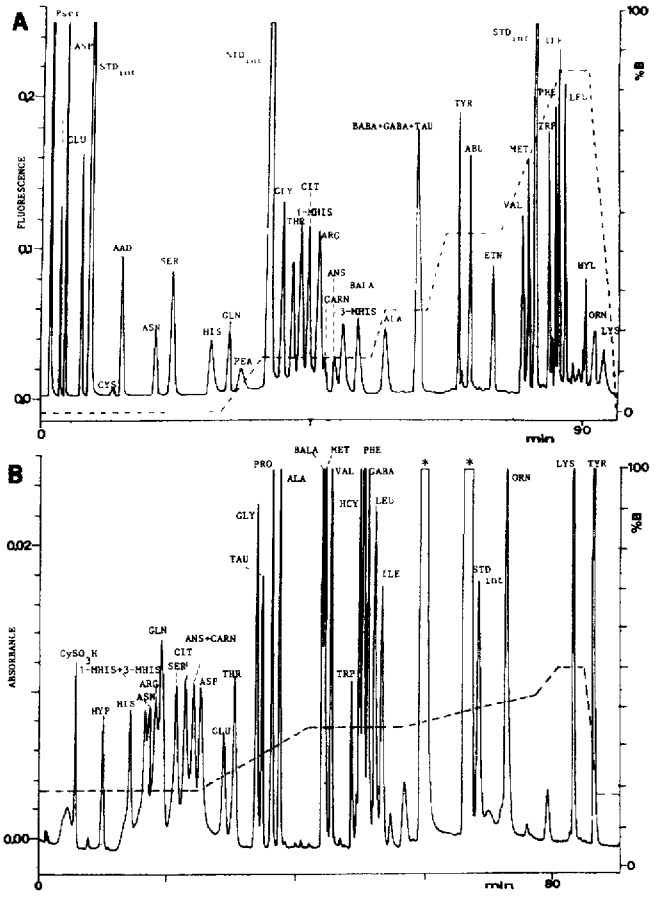

Fig 1

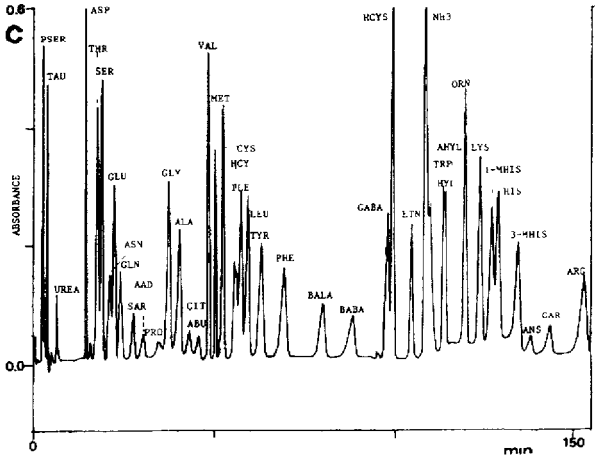

Fig 1 Analysis of standard amino acid (AA) mixtures (A) Elution profile of OPA-amino acids standard mixture (94 pmol per AA injected) Eluent A 8% acetonitrile, 20% sodium propionate buffer, 72% distilled water Eluent B. 30% acetonitrile. 25% methanol, 45% distilled water Flow-rate, 1 4 ml/min, column, Ultrasphere C_{18} Beckman (150 mm × 4 6 mm I.D , 5 μm). Detection, excitation at 360 nm and emission at 455 nm (B) Elution profile of DNDEA-amino acids standard mixture (610 pmol per AA injected). Eluent A 1 l of distilled water, 6 ml of triethylamine, 3 ml of acetic acid (pH 4 3). Eluent B: acetonitrile Flow-rate, 1 3 ml/min, column, Ultrasphere C_{18} Beckman (150 mm × 4 6 mm I D., 5 μm). Detection wavelength. 360 nm Peaks marked with an asterisk were due to an excess of FDNDEA and decomposition by-products (C) Cation-exchange separation (Beckman analyser 338 050)

shown in Table I. The coefficients of variation (C.V.) for FDNDEA were from 0.8% (alanine) to 6% (ornithine); for OPA analysis the range was from 0.9% (aspartic acid) to 11% (ornithine). The between-day reproducibility of the assays, evaluated by testing the two standard mixtures on different days (n = 7), is shown in Table II. The C.V ranged from 2% (alanine) to 9% (ornithine) and from 3% (serine) to 15% (ornithine) for FDNDEA and OPA, respectively. The high variability observed for the last-eluting OPA-amino acids may be explained by degradation of these unstable derivatives on the column.

Serum amino acid quantification

Serum amino acids were quantified by external calibration The ratio between the peak areas of each amino acid and the I S. was calculated daily and compared with the ratio found in biological samples.

Results expressed as mean ± S D obtained from analysis of twenty serum samples from healthy fasting subjects with OPA, FDNDEA and NIN derivatization are reported in Table III The concentrations found for each amino acid were within the normal range [9,30–32] Aspartic acid was not detectable with FDNDEA and NIN techniques because of its low physiological concentrations, usually less than 10 μM. Serum asparagine was not satisfactorily resolved by the

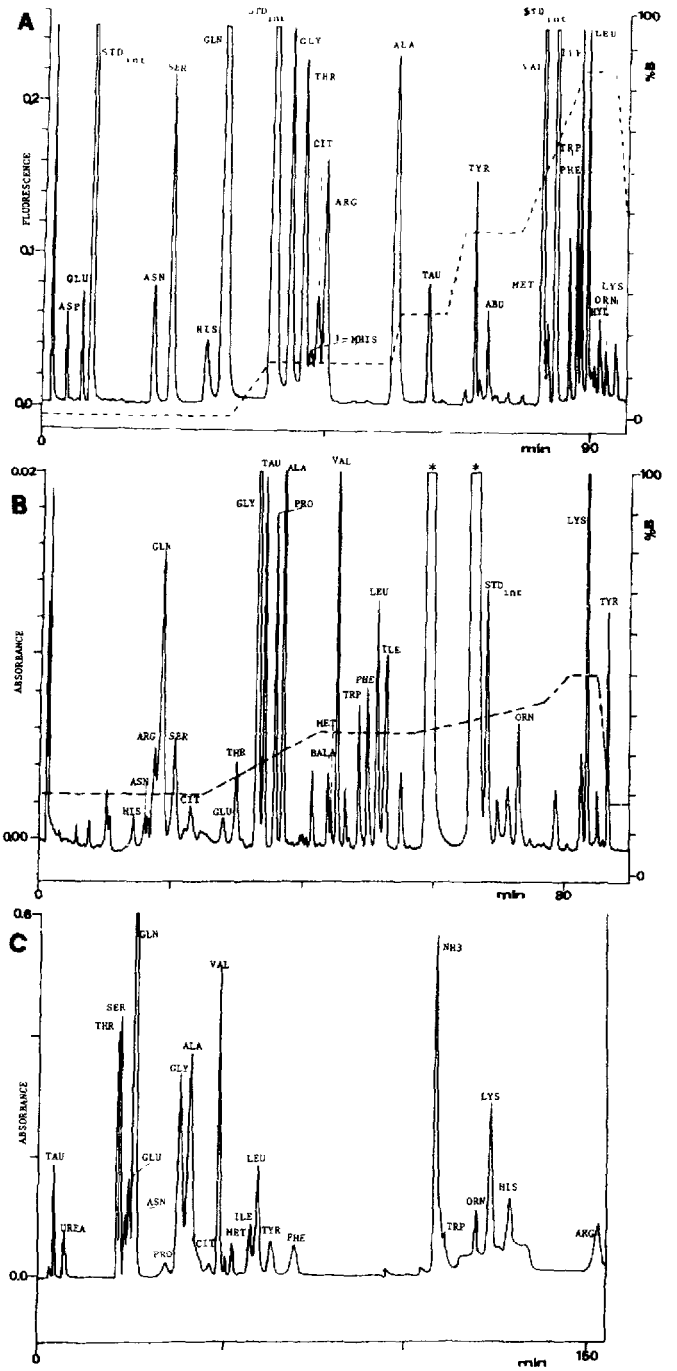

Fig 2 Amino acids separation obtained from the same serum sample with (A) OPA, (B) FDNDEA and (C) ninhydrin derivatization Chromatographic conditions as in Fig 1

TABLE I

INTRA-DAY PRECISION OF PRE-COLUMN DERIVATIZATION TECHNIQUES

Values are the mean ± S D area/pmol for each amino acid, five aliquots of the OPA and FDNDEA standard mixtures were injected consecutively

Amino acid	OPA		FDNDEA	
	Area/pmol	C V (%)	Area/pmol	C V (%)
Asp	0 54 ± 0 005	0 9	6 5 ± 0 22	3 4
Glu	0 41 ± 0 006	1 5	9 1 ± 0 27	3 0
Asn	0 21 ± 0.002	0 9	5 5 ± 0 30	5 0
Ser	0 41 ± 0.009	2 2	9 4 ± 0 09	0 9
His	0 30 ± 0 010	3 0	4 2 ± 0 25	6 0
Gln	0 40 ± 0.010	2 5	10 1 ± 0 35	3 0
Gly	0 60 ± 0 010	1 7	11.2 ± 0 10	0 9
Thr	0 63 ± 0 015	2 0	13 2 ± 0 39	3 0
Ala	0 32 ± 0 020	6 2	10 8 ± 0 09	0 8
Tyr	0 37 ± 0 020	5 4	21 1 ± 0 60	3 0
Val	0 47 ± 0 020	4.2	10 0 ± 0 17	2 0
Met	0 42 ± 0 020	4.8	9 5 ± 0 27	3 0
Trp	0 28 ± 0 015	5.3	7 4 ± 0 12	2 0
Phe	0 36 ± 0 020	6.6	11 6 ± 0 12	1 0
Ile	0 52 ± 0 020	3 8	10 5 ± 0 10	0 9
Leu	0 41 ± 0 020	4 9	10 1 ± 0 50	5 0
Orn	0 18 ± 0 020	11 0	15 8 ± 1 00	6.0
Lys	0 11 ± 0 010	9 0	16 3 ± 0.40	2 0
Mean ± S D		4.2 ± 2 7		2 8 ± 1 7

ninhydrin or the FDNDEA method. Using these techniques a partial separation of the asparagine peak from the surrounding amino acids was observed, whereas in the OPA chromatogram it was well resolved from serine. This observation and a more accurate integration method may justify the higher values found in the OPA analysis The greatest differences (−48%) were observed between results for OPA-lysine and the corresponding ninhydrin derivatives. Since proline is a secondary amino acid, it was not quantified by the OPA assay.

The amino acid levels for each individual subject were generally in good agreement with the reference method (Fig. 3). Equations calculated by pooling the total amino acids values from twenty patients were $y = 0.921x - 2\ 74$ for OPA and $y = 0.983x - 2.73$ for FDNDEA. Correlation coefficients were $r = 0.959$ and $r = 0.987$, respectively Because of the stability problems of the lysine derivatives, the reference method was less well correlated with OPA than with FDNDEA. When this particular amino acid was excluded, the correlation coefficient improved considerably ($r = 0.977$).

TABLE II

BETWEEN-DAY PRECISION OF PRE-COLUMN DERIVATIZATION TECHNIQUES

Values are the mean ± S D area/pmol for each amino acid, the OPA and FDNDEA standard mixtures were injected on different days (n = 7)

Amino acid	OPA		FDNDEA	
	Area/pmol	C V (%)	Area/pmol	C V. (%)
Asp	0 56 ± 0 020	3 5	4 7 ± 0 28	6 0
Glu	0 49 ± 0.040	8 0	10 7 ± 0 35	3 0
Asn	0 28 ± 0 015	5 0	5 7 ± 0 35	6 0
Ser	0 58 ± 0.020	3 0	8 8 ± 0 45	5 0
His	0 32 ± 0.020	6 0	4 4 ± 0 39	9 0
Gln	0 45 ± 0.030	6 0	10 7 ± 0 41	4 0
Gly	0 61 ± 0.060	10 0	10.4 ± 0 31	3 0
Ala	0 40 ± 0.040	10 0	10.5 ± 0 22	2 0
Thr	0 69 ± 0 030	4 0	10.6 ± 0 40	4 0
Tyr	0.50 ± 0.060	12 0	18 0 ± 0 91	5 0
Val	0.60 ± 0.040	6.0	11 2 ± 0 38	3 0
Met	0.50 ± 0 040	8 0	10 0 ± 0 41	4 0
Trp	0.40 ± 0.040	10.0	6 8 ± 0.48	7 0
Phe	0.45 ± 0.050	11 0	9 1 ± 0 41	4 0
Ile	0 60 ± 0 030	5 0	9 0 ± 0 39	4 0
Leu	0.51 ± 0 060	10.0	10 9 ± 0 75	7 0
Orn	0 13 + 0 020	15 0	16 3 ± 1 50	9 0
Lys	0 14 ± 0 020	14.0	20 1 ± 0 65	3 0
Mean ± S D		8 0 ± 3 0		4 0 ± 2 0

DISCUSSION

A large number of papers have been published about the analysis of protein hydrolysates by HPLC and pre-column derivatization reactions [11,17–19,21,23,24]. Much less information is available about the application of these methods to the separation and quantification of amino acids in physiological fluids [25,29]. We studied two pre-column derivatizing agents (OPA and FDNDEA) for the quantification of serum amino acids. The good correlation found between the results obtained with the amino acid analyser and the two HPLC methods suggests that these alternative procedures might be useful in clinical and research laboratories for routine analyses.

If the objective is the quantification of amino acids present at very low concentrations, such as aspartic acid, then OPA may be a suitable agent, because of the very high sensitivity of the method [27–30] For the analysis of secondary amino acids (proline and hydroxyproline), FDNDEA derivatization is preferable, as it has the advantages of high stability, insensitivity to light and easy adaptation to large-scale automated analysis

TABLE III

AMINO ACIDS ANALYSED BY DIFFERENT TECHNIQUES IN SERUM FROM TWENTY HEALTHY VOLUNTEERS

Values are the mean ± S.D of the levels in all twenty subjects. N D = not determined.

Amino acid	OPA (μM)	FDNDEA (μM)	Ninhydrin (μM)
Asp	6 ± 12	N D	N D
Glu	69 ± 41	64 ± 38	74 ± 14
Asn	90 ± 14	70 ± 45	68 ± 32
Ser	135 ± 32	135 ± 33	135 ± 28
His	64 ± 14	68 ± 14	74 ± 18
Gln	550 ± 108	522 ± 115	577 ± 87
Gly	266 ± 74	264 ± 39	303 ± 53
Pro	–	249 ± 71	236 ± 50
Ala	330 ± 71	338 ± 77	354 ± 46
Thr	127 ± 31	127 ± 30	132 ± 23
Tyr	58 ± 18	62 ± 18	65 ± 10
Val	241 ± 60	246 ± 53	251 ± 42
Met	25 ± 5	24 ± 5	26 ± 5
Trp	47 ± 9	54 ± 12	45 ± 17
Phe	60 ± 17	62 ± 18	60 ± 7
Ile	64 ± 15	66 ± 15	73 ± 25
Leu	118 ± 24	129 ± 25	139 ± 17
Orn	56 ± 28	55 ± 29	62 ± 29
Lys	70 ± 24	116 ± 20	145 ± 42

Both pre-column techniques offer acceptable reliability and reproducibility and the advantage of shorter analysis times than the amino acid analyser (102 and 106 min against 180 min). In addition, the pre-column separations can be carried out at room temperature on a standard HPLC apparatus, without the need for an expensive dedicated ion-exchange chromatograph.

Although the use of an amino acid analyser remains the reference method for the quantification of amino acids in biological fluids, we have demonstrated the validity and advantages of the OPA and FDNDEA methods for the analysis of amino acids in serum These methods could also be used for the separation and quantification of other biogenic amines.

ACKNOWLEDGEMENTS

This research was partially supported by CNR (Consiglio Nazionale delle Ricerche) We thank Marilena Lomartire for her valuable technical assistance. Luisa Diomede is a fellow of the Inner Wheel Club (Monza, Italy).

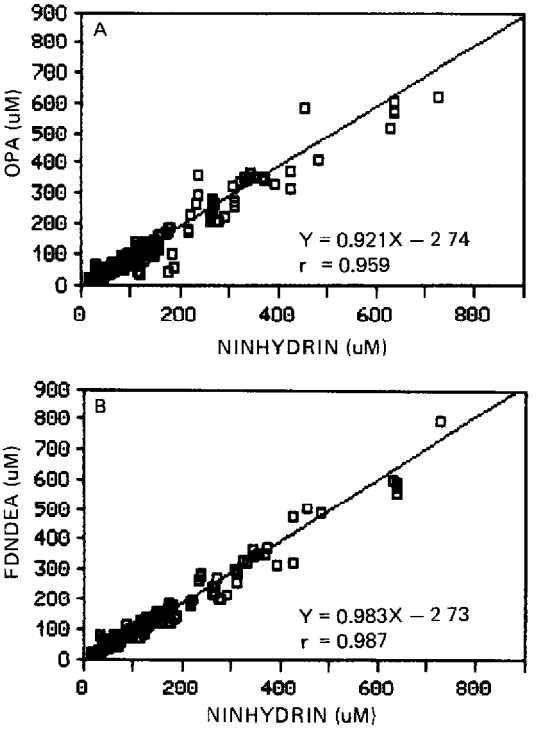

Fig 3 Correlation between HPLC pre-column derivatization and ion-exchange chromatography with ninhydrin postcolumn reaction (A) Pre-column derivatization with OPA (B) Pre-column derivatization with FDNDEA Linear regressions were calculated from the results for single amino acids from samples of twenty subjects

REFERENCES

1 S Moore, D H Spackman and W H Stein, *Anal Chem*, 30 (1958) 1185
2 P B Hamilton, *Anal Chem*, 35 (1963) 2055.
3 T L Perry, D Stedman and S Hansen, *J Chromatogr*, 38 (1968) 460
4 A Smith, P J Peterson and L. Fowden *J Chromatogr*, 62 (1971) 144
5 P Adriens, B Meesschaert, W Wuyts, H Vanderhaeghe and H Eyssen, *J Chromatogr*, 140 (1977) 103
6 M D Armstrong and U Stave, *Metabolism*, 22 (1973) 561.
7 K Murayama and T Sugawara, *J Chromatogr*, 224 (1981) 315
8 J H Peters, S C Lin, B J Berridge, Jr., J G Cumming and W R Chao, *Proc Soc Exp Biol Med.*, 131 (1981) 281
9 J Wuu, L Wen, T Chuang and G Chang, *Clin. Chem*, 34 (1988) 1610
10 P L Y Lee and R. H Slocum, *Clin Chem*, 34 (1988) 719
11 K T Hsu and B L Currie, *J Chromatogr*, 166 (1978) 555
12 B Oray, H S Lu and W Gracy, *J Chromatogr*., 270 (1983) 253
13 N Seiler, *Methods Biochem Anal*, 18 (1970) 259
14 S Einarsson, B Josefsson and S Lagerkvist, *J Chromatogr*, 282 (1983) 609
15 I Betner and P Foldi, *Chromatographia*, 22 (1986) 7.
16 R L Heinrikson and S C Meredith, *Anal Biochem*, 136 (1984) 65

17 B A Bidlingmeyer, S A Cohen and T L Tarvin, *J Chromatogr*, 336 (1984) 93.
18 H Scholze, *J Chromatogr*, 350 (1985) 453
19 S A Cohen and D J Strydom, *Anal Biochem*, 174 (1988) 1
20 J D H Cooper, G Ogden, J McIntosh and D C Turnell, *Anal Biochem*, 142 (1984) 98
21 H S Sista, *J Chromatogr*, 359 (1986) 231
22 A Fiorino, G Frigo and E Cucchetti, *J Chromatogr*, 476 (1989) 83
23 H Humagat and P Kucera, *J Chromatogr*, 239 (1982) 463
24 C R Krishnamurti, A M Heindze and G Galzi, *J Chromatogr*, 315 (1984) 321
25 F J Marquez, A R Quesada, F Sanchez and I Nunez de Castro, *J Chromatogr*, 380 (1986) 275
26 H G Biggs and L J Gentilcore, *Clin. Chem.*, 30 (1984) 851
27 D C Turnell and J D H Cooper, *Clin Chem*, 28 (1982) 527
28 T A Graser, H G Godel, S Albers, P. Foldi and P Furst, *Anal Biochem*, 151 (1985) 142
29 G Blundell and W. K Brydon, *Clin Chim Acta*, 170 (1987) 79
30 I Fermo, F M. Rubino, E Bolzacchini, C Arcelloni, R Paroni and P. A Bonini, *J Chromatogr.*, 433 (1988) 53
31 P Felig, E Marliss and G F Cahill, *N Engl J Med.*, 281 (1969) 811
32 M D Armstrong and U Stave, *Metabolism*, 22 (1973) 549
33 R Scriver, D M Gregory, D Sovetts and G Tissenbaum, *Metabolism*, 34 (1985) 868