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o-PHTHALDIALDEHYDE PRECOLUMN DERIVATIZATION AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF POLYPEPTIDE HYDROLYSATES AND PHYSIOLOGICAL FLUIDS

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SUMMARY

A rapid and ultrasensitive fluorescence amino acid analysis system has been developed which utilizes *o*-phthaldialdehyde as a precolumn derivatizing agent. *o*-Phthaldialdehyde in the presence of mercaptan reacts rapidly with primary amino acids to form intensely fluorescent derivatives. These derivatives are analyzed with good selectivity by high-performance liquid chromatography employing 3-μm particle size reversed-phase columns. Resolution of the amino acid derivatives is accomplished with a methanol gradient in 0.1 *M* aqueous sodium acetate, pH 7.2. The quantitation of the individual amino acid derivatives is reproducible within an average relative deviation of $\pm 1.5\%$ and has a detection limit of less than 100 fmoles. Amino acid mixtures obtained by either enzymatic or acid hydrolysis of polypeptides are efficiently resolved with an analysis time of less than 18 min. Methods for the amino acid analysis of physiological fluids such as serum, urine and cerebrospinal fluid were also developed which employ the above separation procedure for the identification and quantitation of amino acids and other biological amines. Mixtures which contained as many as 48 components were resolved with an analysis time of less than 50 min.

INTRODUCTION

The ability to separate and quantitate amino acids has many applications in the areas of protein chemistry and clinical chemistry. Analyses are usually accomplished by separating amino acid mixtures using classical ion-exchange chromatography[1] followed by post-column derivatization with ninhydrin[2,3] or a fluorogenic reagent[4–7]. This technique resolves most amino acids with good detection limits, especially if either fluorescamine or *o*-phthaldialdehyde (OPA) is used as the post-column derivatizing reagent. However, this procedure generally requires specialized equipment and an analysis time greater than 1 h for peptide hydrolysates and up to 3–4 h for the analysis of physiological fluids.

Recent studies have demonstrated that amino acid analyses can also be per-

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formed successfully using OPA as a precolumn derivatizing reagent. OPA in the presence of either 2-mercaptoethanol or ethanethiol reacts rapidly with primary amino acids to form highly fluorescent, thio-substituted isoindoles[8,9]. These derivatives are analyzed with good selectivity and sensitivity by reversed-phase high-performance liquid chromatography (HPLC). Using this method, peptide hydrolysates can be analyzed on 5-μm particle size ODS columns (250 × 4.6 mm) with an analysis time of 30–45 min[10–17]. In our laboratory, two approaches were investigated to determine if the above analysis time could be further reduced. One approach was to decrease the column length of the 5-μm particle size ODS columns used in the analysis while the other approach was to investigate the use of 3-μm particle size ODS columns. In addition, the feasibility of employing the 3-μm particle size column for the analysis of polypeptide hydrolysates and physiological fluids was examined.

EXPERIMENTAL

Materials

Methanol and acetonitrile were distilled-in-glass grade (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and used without further treatment. High purity water was obtained with a system from Hydro Service and Supplies (Durham, NC, U.S.A.). Solutions of amino acid standards (2.5 μmol/ml), constant boiling HCl (6 *N*), fluoraldehyde OPA reagent solution (OPA in borate buffer containing 0.8 mg/ml Brij 35* and 2-mercaptoethanol), iodoacetic acid and sodium acetate (pHix buffer grade) were obtained from Pierce (Rockford, IL, U.S.A.). Individual amino acid and biological amine standards, synthetic human calcitonin, aminopeptidase M and lyophilized reference serum were purchased from Sigma (St. Louis, MO, U.S.A.). Carboxymethylated human calcitonin was prepared by the method of Jones *et al.*[18]. All other chemicals were reagent grade. Enzymatic digestion buffers contained 0.01% pentachlorophenol (Pierce) as a preservative. Rat cerebrospinal fluid (CSF) was generously supplied by Drs. E. H. Cantor and S. Spector of the Roche Institute, Nutley, NJ, U.S.A.

Preparation of amino acid standards

Stock solutions of individual amino acids and biological amines were prepared in water at a concentration of 10 nmol/μl. Standard mixtures used for calibration during quantitative analysis were prepared by combining the appropriate stock solutions followed by dilution with water to yield a final concentration of either 25 or 50 pmol/μl for each individual component. The working standard mixtures were stable for approximately 1 month of continual use if stored at −20°C. After this time, appreciable amounts of methionine were lost with a concomitant increase in the methionine sulfoxide content of the standard. The glutamine peak in the calibration experiments also decreased with time, presumably due to the conversion of glutamine to pyroglutamic acid. For the ammonia standard, a 50 nmol/μl solution in water was prepared from ammonium acetate.

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* Polyoxyethylene lauryl ether.

Derivatization procedure

The general procedure for derivatization was as follows: 5–10 μl aliquots of standards or unknown samples were mixed with 5 μl of the fluoraldehyde reagent. After 1 min, 20–100 μl of 0.1 *M* sodium acetate (pH 7.0) were added, the solution mixed and a 20-μl sample was subjected to analysis.

The commercial fluoraldehyde reagent can be replaced by a solution prepared by dissolving 50 mg of OPA in 1.25 ml of absolute methanol followed by the addition of 50 μl of 2-mercaptoethanol and 11.2 ml of 0.4 *M* sodium borate (pH 9.5)[15]. Whereas this solution is stable for only 1–2 weeks, the commercial fluoraldehyde reagent is stable for at least 6 months if stored at 4°C, can be used directly without modification and also contains Brij 35, which was found to increase the fluorescence response of lysine and hydroxylysine[14,15].

Hydrolysis of peptides

The general procedure for the hydrolysis of peptides was as follows: peptides (50–500 pmoles) were dried in a hydrolysis tube, the tube sealed under vacuum after the addition of 200 μl of constant boiling hydrochloric acid (6 *N*) and the tube then heated at 110°C for 22 h. After hydrolysis, the hydrochloric acid was removed by lyophilization and the resulting residue dissolved in 25–50 μl of water. The derivatization with OPA was performed as above, usually on 10-μl aliquots.

For total enzymatic hydrolysis, peptides (100–500 pmoles) were dissolved in 49 μl of 0.2 *M* sodium phosphate (pH 7.0). To this solution, 1 μg of aminopeptidase M was added and the resulting mixture incubated at 37°C for 24 h. Aliquots (5 μl) were derivatized and then analyzed by HPLC. A digest blank containing 49 μl buffer and 1 μg aminopeptidase M was also prepared and analyzed.

Preparation of biological samples

Serum, urine and CSF samples (25 μl) were mixed vigorously with acetonitrile (75 μl). Following centrifugation at 10,000 *g* for 2 min, 10-μl aliquots were subjected to OPA derivatization and analyzed.

Chromatography system

The HPLC system consisted of two Beckman Model 100A pumps (Fullerton, CA, U.S.A.), a Beckman Model 421 microprocessor for generation of elution gradients and a Gilson Model 121 filter fluorometer (Middleton, WI, U.S.A.). The fluorometer was equipped with a 9-μl cuvette and filters for excitation at 305–395 nm and emission at 420–650 nm. All sample injections were performed with a Beckman Model 210 injection valve, equipped with a 20-μl sample loop. The following reversed-phase HPLC columns were used for chromatographic separations: Ultrasphere ODS columns (4.6 mm I.D.; particle size, 5 μm) of three different lengths, 250 mm, 150 mm and 45 mm; Ultrasphere ODS column (75 × 4.6 mm I.D.; particle size, 3 μm) and Microsorb C-18 column (100 × 4.6 mm I.D.; particle size, 3 μm). All HPLC columns were obtained from Rainin Instruments (Woburn, MA, U.S.A.). The above columns were fitted with a guard column (45 × 2.1 mm I.D.) packed with CO:PELL ODS sorbent (particle size, 40 μm) (Rainin Instruments) when physiological fluids and enzymatic digests were being analyzed. To minimize extracolumn dilution of peak volumes, the capillary tubing (0.18 mm I.D.) used to connect the HPLC column

to the injector and detector was made as short as possible[19]. Chromatographic peaks were recorded and integrated by a Beckman C-R1A integrator system. Gradients were formed between two degassed solvents. Solvent A was tetrahydrofuran–methanol–0.1 *M* sodium acetate (pH 7.2) (5:95:900) and solvent B was methanol. Further details of the chromatographic procedure are given in the figure legends.

RESULTS AND DISCUSSION

Recent studies have demonstrated that amino acid mixtures derivatized with OPA can be successfully resolved by HPLC using gradient elution with methanol and 5-μm particle size ODS columns. In these studies, the reversed-phase columns were either 250 or 300 mm in length and each analysis required 30–45 min[11,14,15,17]. This analysis time constitutes a definite improvement when compared with the 1–3 h required for analysis by classical ion-exchange methods. However, if a further decrease of the analysis time could be obtained, a significant reduction in the total time required for multiple analyses would be achieved. This is especially important when numerous samples such as tryptic peptides derived from large proteins must be analyzed[18,20,21]. The first attempt at decreasing the analysis time was by increasing

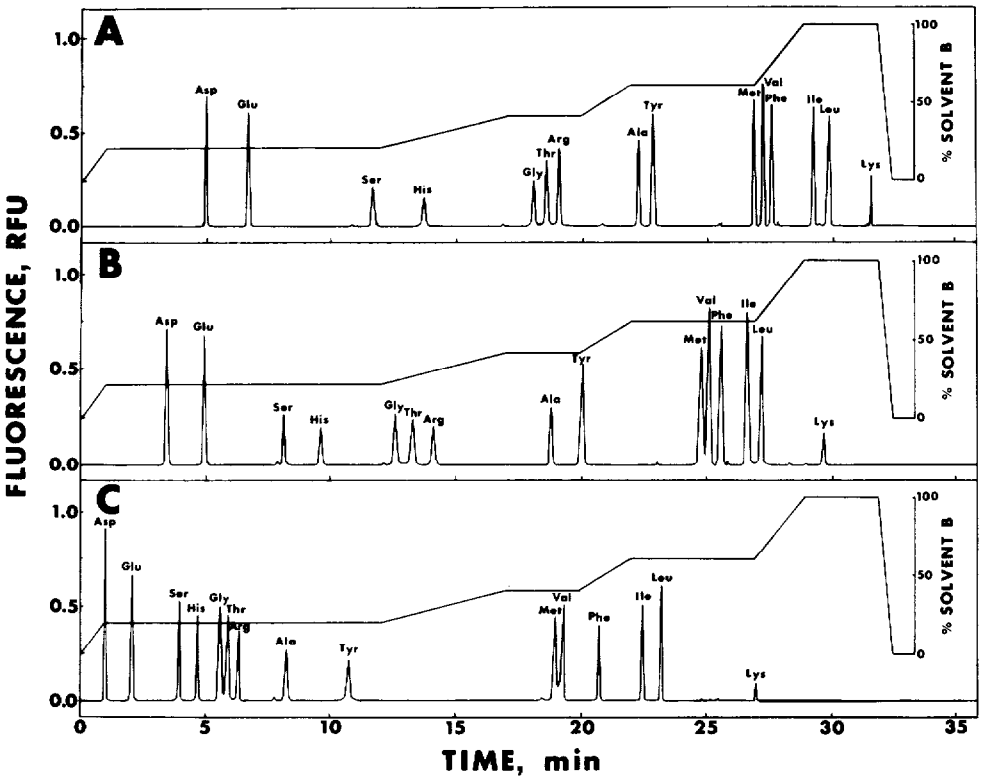

Fig. 1. Elution profiles of OPA-derivatized amino acids chromatographed on 5-μm particle size Ultrasphere ODS columns of varying length. A, 250-mm length column; B, 150-mm length column; C, 45-mm length column. All three columns were 4.6 mm I.D. Each peak represents 40 pmoles. The flow-rate (1.7 ml/min), methanol gradient and buffer (0.1 *M* sodium acetate, pH 7.2) were identical in each experiment.

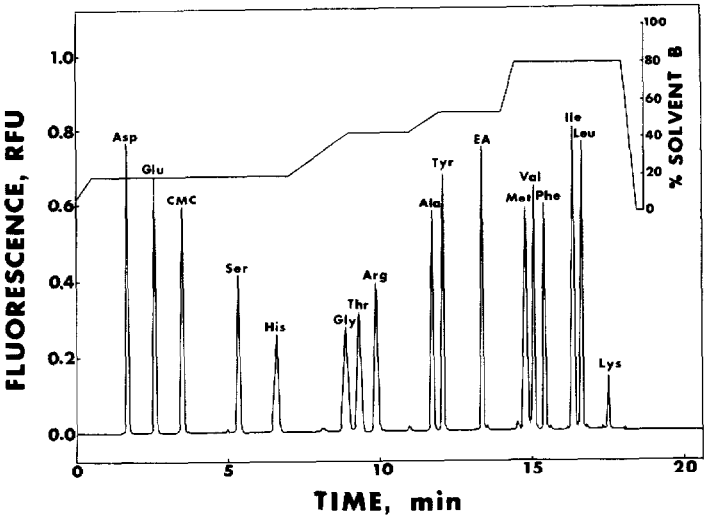

Fig. 2. Elution profile of OPA-derivatized amino acids chromatographed on a 5-μm particle size Ultrasphere ODS column (150 × 4.6 mm I.D.). Each peak represents 40 pmoles. The flow-rate was 2.2 ml/min. Non-standard abbreviations used: CMC = carboxymethylcysteine; EA = ethanolamine (internal standard).

the flow-rate through the reversed-phase column, but this was unsuccessful due to the high column pressure created by the increased solvent velocity. This problem was solved by substituting acetonitrile for the methanol used in the gradient elution; however, glycine and threonine could no longer be separated from one another[10,12,16]. Another possible solution to this problem is to use gradient elution with methanol but in reversed-phase columns of decreased length. The feasibility of

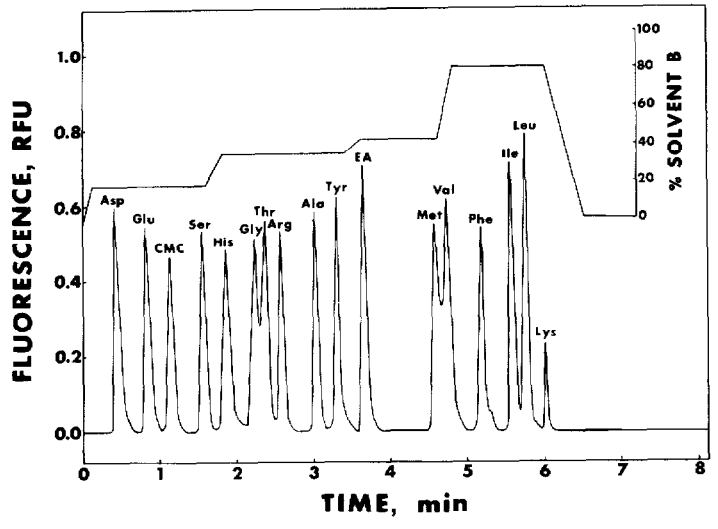

Fig. 3. Elution profile of OPA-derivatized amino acids chromatographed on a 5-μm particle size Ultrasphere ODS column (45 × 4.6 mm I.D.). Each peak represents 40 pmoles. Flow-rate was 4.5 ml/min. Non-standard abbreviations as in Fig. 2.

this approach is illustrated by the chromatograms in Fig. 1. In all three of these chromatographic runs, the flow-rate was constant, identical methanol gradients and buffers were used and all three columns were 4.6 mm in diameter and contained Ultrasphere 5-μm particle size ODS resin. The only variable was column length. In Fig. 1A, a 250-mm column was used. The maximum column pressure for this experiment was 5700 p.s.i. which is near the limit for most commercial HPLC pumps. In Fig. 1B, a 150-mm column was used and the maximum column pressure wich developed was 3300 p.s.i. In Fig. 1C, a 45-mm column was employed, which generated only 1000 p.s.i. pressure. The conclusion from this experiment was that amino acid mixtures can be resolved in a shorter analysis time and at lower column pressures by decreasing the column length. The decrease in column pressure also permits more flexibility in selecting the column flow-rate thereby making further decreases in analysis time feasible. This is illustrated in Figs. 2 and 3. The 150-mm column (Fig. 2) required an analysis time of only 18 min for the resolution of the amino acid mixture when, instead of the conditions in Fig. 1, an increased flow-rate and a modified methanol gradient were used. The analysis can be reduced to approximately 12 min by further modification of the methanol gradient but at some loss in the resolution of the amino acid derivatives. Analyses using the 45-mm column (Fig. 3) required a minimum of 6 min. Using a 2-min regeneration time, approximately seven amino acid analyses per hour can be performed using this column. The 150-mm as well as

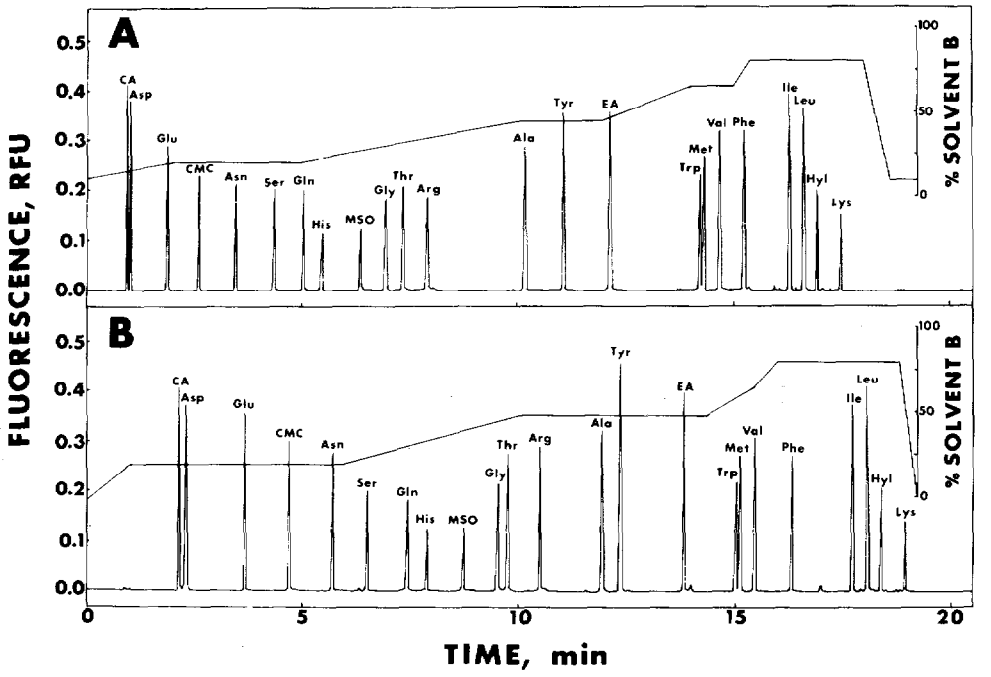

Fig. 4. Elution profile of OPA-derivatized amino acids chromatographed on 3-μm particle size reversed-phase columns. A, Ultrasphere ODS column (100 × 4.6 mm I.D.); flow-rate 1.7 ml/min; B, Microsorb C-18 column (100 × 4.6 mm I.D.), flow-rate 1.6 ml/min. Each peak represents 20 pmoles. Non-standard abbreviations used: CA = cysteic acid; CMC = carboxymethylcysteine; MSO = methionine sulfoxide; EA = ethanolamine (internal standard).

the 45-mm column gave excellent results for the quantitation of all the amino acids shown in Figs. 2 and 3 except for lysine. The quantitation of lysine was variable and not reproducible even for duplicate analyses. For this reason, the routine use of these columns in this laboratory is restricted to the screening of tryptic peptide hydrolysates.

A possible alternative to the above attempts at decreasing analysis time is to replace the 5-μm particle size column with a 3-μm particle size column. Fig. 4 illustrates the resolution obtained by employing 3-μm particle size reversed-phase columns for the separation of the OPA-derivatized amino acids. An Ultrasphere ODS column (75 × 4.6 mm I.D.) was used to obtain the chromatogram in Fig. 4A and a Microsorb C-18 column (100 × 4.6 mm I.D.) was used in Fig. 4B. Analysis times for both columns were less than 20 min and can be reduced to less than 15 min by slight modification of the gradients shown in Fig. 4. The precision of the OPA pre-column derivatization procedure was evaluated for each of the above columns by injection of a 23-component amino acid standard using the chromatographic conditions presented in Fig. 4. Six samples were consecutively injected following a reaction time of 1 min, and the resulting retention times and peak areas of the individual components measured. Analysis of the data indicated that the average deviation of the retention times was approximately ±2 sec. The average relative deviation in the peak areas was approximately 7%; however, part of this deviation is due to pipetting errors that may occur during the derivatization procedure. When the results

TABLE I

AMINO ACID COMPOSITION OF HUMAN CALCITONIN

Amino acid	*Acid hydrolysate*	*Enzymatic hydrolysate*	*From sequence*[22]
Asp	2.9*	1.1	1
Asn	–	2.1	2
Glu	2.0*	0.0	0
Gln	–	1.9	2
½Cys**	1.9	2.0	2
Ser	0.9	1.0	1
His	0.9	1.0	1
Gly	4.2	3.9	4
Thr	4.9	5.0	5
Arg	0.0	0.0	0
Ala	2.0	1.7	2
Tyr	1.0	1.1	1
Trp	–	0.0	0
Met	0.9	1.0	1
Val	1.0	1.1	1
Phe	3.0	2.9	3
Ile	1.0	1.1	1
Leu	2.0	2.1	2
Lys	1.0	1.0	1
Pro***	1.7*	0.8	1
$ProNH_2$***	–	0.6	1

* Asp + Asn, Glu + Gln and Pro + prolinamide ($ProNH_2$) for acid hydrolysate.

** Determined as carboxymethylcysteine (CMC).

*** Determined in separate analysis by the method of Umagat *et al.*[17].

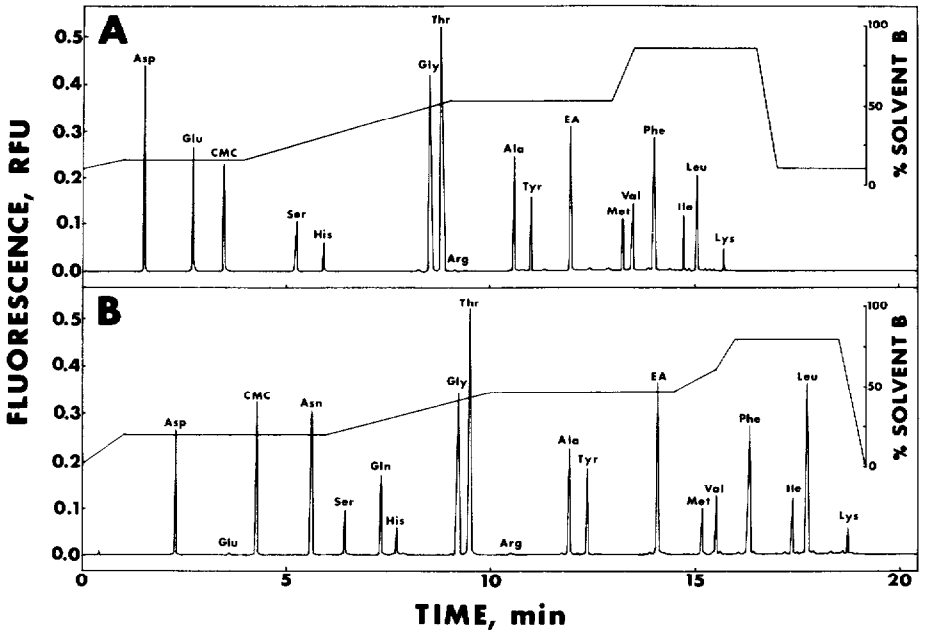

Fig. 5. Elution profiles of hydrolysates of carboxymethylated human calcitonin. A, Acid hydrolysate chromatographed on a 3-μm particle size Ultrasphere ODS column (75 × 4.6 mm I.D.) at a flow-rate of 1.8 ml/min. B, Enzymatic hydrolysate chromatographed on a 3-μm particle size Microsorb C-18 column (100 × 4.6 mm I.D.) at a flow-rate of 1.6 ml/min. The internal standard was ethanolamine (EA).

for peak areas from all six experiments were normalized to an internal standard (ethanolamine), the average relative deviation for the Microsorb and Ultrasphere columns was ± 1.6% and ± 1.5%, respectively. From these results, the incorporation of an internal standard is recommended for a quantitative analysis of an amino acid mixture. The detection limits and the linearity of response of the amino acid derivatives for our reversed-phase columns were determined by serial dilution of a stock amino acid standard solution followed by derivatization and analysis. Using the Gilson Model 121 fluorometer, peaks representing less than 100 fmoles could be detected and processed by the integrator system. The lower limit of detection for most of the amino acid derivatives was approximately 50 fmoles. When the chromatographic conditions shown in Fig. 4 were used, the peak areas were linear with concentration in the range of 1–400 pmoles for both columns. The only major performance differences between the two columns were analysis time and column lifetime. Since the Ultrasphere column was 25 mm shorter than the Microsorb column, it was able to resolve amino acid mixtures in less time and at lower column pressures than the Microsorb column. Furthermore, no performance loss was observed with the Ultrasphere column when operated at high pressures (4000–5000 p.s.i.). However, the useful lifetime of the Microsorb column was greatly reduced if the pressure was allowed to exceed 4500 p.s.i. After several analyses under these conditions, the resin became compressed at the inlet of the Microsorb column which resulted in the elution of the chromatographic peaks as broad doublets.

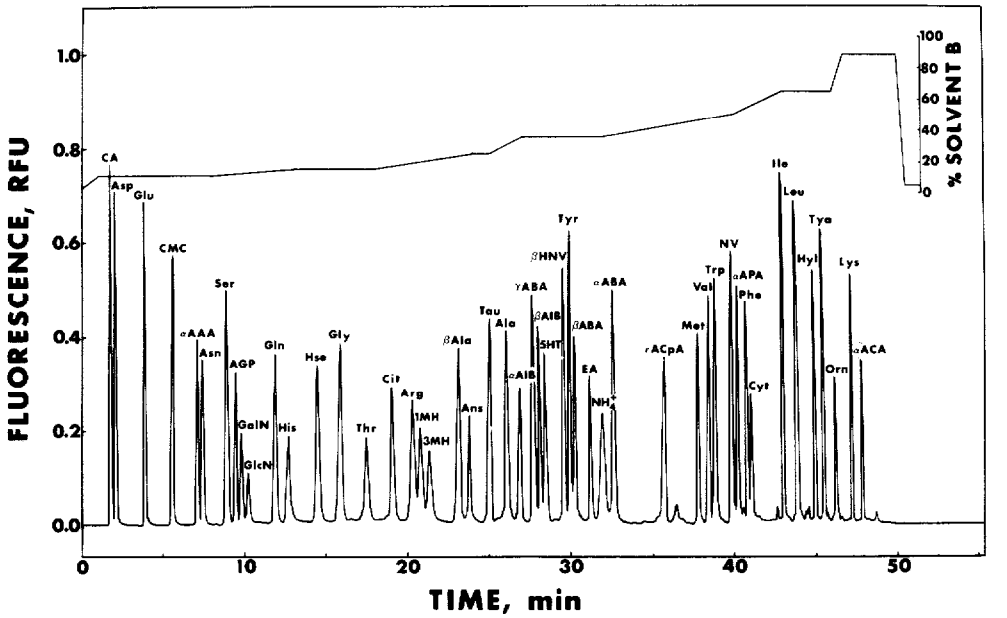

Fig. 6. Elution profile of an OPA-derivatized standard mixture of amino acids and biological amines chromatographed on a 3-μm particle size Ultrasphere ODS column (75 × 4.6 mm I.D.). Each peak represents 80 pmoles except for the ammonia peak which represents 300 pmoles. The flow-rate was 1.5 ml/min. Non-standard abbreviations used: CA = cysteic acid; CMC = carboxymethylcysteine; αAAA = α-aminoadipic acid; AGP = α-amino-β-guanidinopropionic acid (internal standard); GalN = galactosamine; GlcN = glucosamine; Hse = homoserine; Cit = citrulline; 1MH = 1-methylhistidine; 3MH = 3-methylhistidine; βAla = β-alanine; Ans = anserine; Tau = taurine; αAIB = α-aminoisobutyric acid; 5HT = 5-hydroxytryptophan; βHNV = β-hydroxynorvaline; βABA = β-amino-*n*-butyric acid; EA = ethanolamine; αABA = α-amino-*n*-butyric acid; γABA = γ-amino-*n*-butyric acid; εACpA = ε-amino-*n*-caproic acid; NV = norvaline (internal standard); Cyt = cystathionine; Tya = tyramine; Orn = ornithine; αACA = α-aminocaprylic acid; αAPA = α-aminophenylacetic acid.

The feasibility of adopting the 3-μm particle size columns for the determination of the amino acid composition of polypeptides was evaluated. Two different hydrolysis procedures, hydrochloric acid hydrolysis and total enzymatic hydrolysis with aminopeptidase M, were tested and found to be compatible with the OPA derivatization technique[17]. The results obtained for the amino acid analysis of carboxymethylated human calcitonin after each of these hydrolysis procedures are summarized in Table I. The elution profile of the acid hydrolysate is illustrated in Fig. 5A and the elution profile of the enzymatic hydrolysate is shown in Fig. 5B. The determined compositions are in good agreement with the reported amino acid sequence of human calcitonin[22]. It should be noted that the acid/amide content of calcitonin was readily obtained by the enzymatic hydrolysis procedure. Determination of asparagine and glutamine content usually requires special buffers (lithium citrate) and modified chromatography conditions[23] when analysis is performed by classical ion-exchange methods.

Recent reports have demonstrated that amino acids in physiological fluids can also be analyzed by OPA pre-column derivatization and reversed-phase HPLC[24,25].

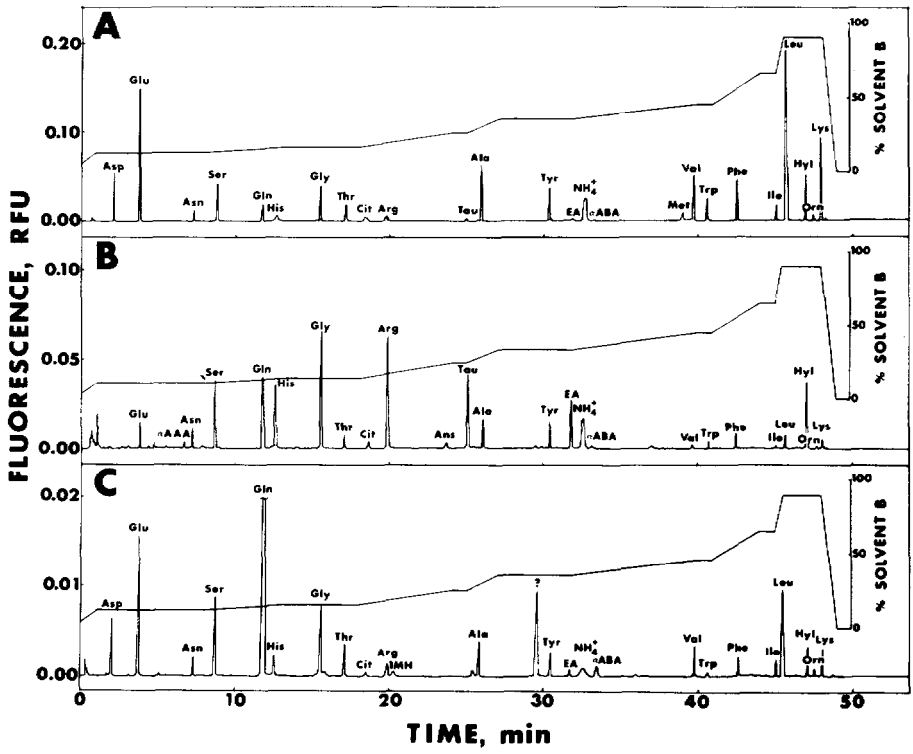

Fig. 7. Elution profiles of OPA-derivatized amino acids and biological amines from adult serum (A), adult urine (untimed) (B) and rat CSF (C). The chromatography was performed on a 3-μm particle size Ultrasphere ODS column (75 × 4.6 mm I.D.) at a flow-rate of 1.5 ml/min. Non-standard abbreviations used: see Table II.

Therefore, the use of the 3-μm particle size column for the analysis of free amino acids and other biological amines in physiological fluids was investigated. Fig. 6 shows some of the components which can be resolved on the 3-μm particle size Ultrasphere ODS column by the OPA pre-column derivatization procedure. These include amino acids and intermediates in their biosynthesis and degradation as well as dipeptides, amino sugars and biogenic amines. Fig. 7 illustrates the application of our procedure to the analysis of serum, urine and CSF. The quantitations obtained from these analyses are summarized in Table II. The mean analytical recovery for these procedures was greater than 95%, as estimated from the analysis of serum samples supplemented with known quantities of amino acids. Our methodology should also be applicable to the analysis of other biological samples such as amniotic fluid, plasma, synovial fluid, blood cell lysates and tissue extracts.

ACKNOWLEDGEMENT

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TABLE II

QUANTITATION OF BIOLOGICAL AMINES IN PHYSIOLOGICAL FLUIDS

Amine	*Serum (nmol/ml)*	*Urine (nmol/ml)*	*CSF (nmol/ml)*
Aspartic acid (Asp)	38.85	*	2.78
Glutamic acid (Glu)	155.87	4.43	21.79
α-Aminoadipic Acid (αAAA)	*	3.95	*
Asparagine (Asn)	15.47	17.16	4.28
Serine (Ser)	63.65	73.49	27.46
Glutamine (Gln)	*	104.32	398.94
Histidine (His)	29.23	248.30	12.41
Glycine (Gly)	94.51	253.39	34.75
Threonine (Thr)	54.01	23.59	24.53
Citrulline (Cit)	14.73	35.17	1.67
Arginine (Arg)	22.36	356.20	5.83
1-Methylhistidine (1MH)	*	*	0.60
3-Methylhistidine (3MH)	*	*	*
β-Alanine (βAla)	*	*	*
Anserine (Ans)	*	30.49	*
Taurine (Tau)	3.33	322.82	*
Alanine (Ala)	176.72	49.89	20.44
γ-Amino-*n*-butyric acid (γABA)	*	*	*
β-Aminoisobutyric Acid (βAIB)	*	*	*
Tyrosine (Tyr)	40.51	19.96	6.61
Ethanolamine (EA)	4.66	41.23	1.13
α-Amino-*n*-butyric acid (αABA)	0.71	6.14	2.12
Methionine (Met)	9.03	*	*
Valine (Val)	107.59	10.25	8.80
Tryptophan (Trp)	28.15	11.71	1.13
Phenylalanine (Phe)	66.72	9.56	5.71
Cystathionine (Cyt)	*	*	*
Isoleucine (Ile)	15.73	5.67	3.12
Leucine (Leu)	176.72	11.70	11.22
Hydroxylysine (Hyl)	62.81	56.90	46.80
Ornithine (Orn)	41.20	20.58	5.74
Lysine (Lys)	206.82	37.44	29.79

* Measurable peak not detected.

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