Journal of Chromatography, 613 (1993) 35–42
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6679

High-performance liquid chromatographic determination of amino acids in protein hydrolysates and in plasma using automated pre-column derivatization with *o*-phthaldialdehyde/2-mercaptoethanol

G. Georgi*, C. Pietsch and G. Sawatzki

Research Department, Milupa AG, Bahnstrasse 14–30, W-6382 Friedrichsdorf (Germany)

(First received July 30th, 1992; revised manuscript received November 12th, 1992)

ABSTRACT

A sensitive and reproducible method for the routine determination of amino acids in plasma and in protein hydrolysates based on reversed-phase high-performance liquid chromatography and *o*-phthaldialdehyde pre-column derivatization is described. The resolution of all amino acids was found to be good. The total time for analysis, including separation and reconditioning, ranged from 38 min for protein hydrolysates to 62 min for 29 physiological amino acids. The precision of hydrolysate analysis was within a relative standard deviation of 0.8–7.3% depending on the use of internal or external standards. The relative standard deviations of peak areas for physiological amino acids (standard) ranged between 1.8 and 5.6%. The relative standard deviations of retention times were less than 0.5% for all amino acids. This method can be used for routine analysis. One single column with 4-μm end-capped C_{18} material was found to be sufficient for 400–500 successive runs.

INTRODUCTION

Several methods have been published describing the analysis of protein hydrolysates and physiological amino acids by reversed-phase HPLC and *o*-phthaldialdehyde (OPA)/2-mercaptoethanol pre-column derivatization. Analyses can be done in a short time, with a high sensitivity and a usually satisfactory resolution [1–5]. However, our own experience has shown that in routine practice quantification of plasma amino acids, in particular, is difficult to achieve by pre-column derivatization. Consequently, we have developed an HPLC method based on OPA pre-column derivatization aimed at routine determination of amino acids in protein hydrolysates and in plasma. OPA reagent was chosen in order to keep the reaction times short (2 min) and to make it possible to completely automate the analytical process.

EXPERIMENTAL

Equipment

A Pharmacia LKB HPLC system (Freiburg, Germany) was used and included the following elements: HPLC pump 2248, low-pressure mixer, solvent conditioner, autosampler 2157, column oven and fluorescence detector with filters for excitation at 330 nm and emission at 408 nm. An IBM-PS/2 Model 30-286 computer was used to control the HPLC-system (HPLC manager) and to evaluate the data (Nelson software). For cen-

* Corresponding author.

0378-4347/93/$06.00

trifugation an Eppendorf centrifuge 5412 (Eppendorf-Netheler-Hinz, Hamburg, Germany) was used. Filtrations were carried out using 0.22-μm (type GV, Millipore, Eschborn, Germany) and 0.2-μm (FP 030, Schleicher & Schüll, Dassel, Germany) membrane filters. Derivatization was performed in amber glass reaction vials with crimp caps and seals (Chromacol, Trumbull, USA). Amino acids were separated on a LiChroCART column (Superspher 100 RP-18 end-capped, 125 × 4 mm I.D.; Merck, Darmstadt, Germany) combined with a LiChroCART precolumn (LiChrospher 100 RP-18 end-capped, 4 × 4 mm I.D.; Merck). At the pump–autoinjector interface a guard column (SuperPac; Spherisorb ODS2, 10 × 4 mm I.D., 5 μm particle size, Pharmacia) was placed. Some investigations were also performed with the Spherisorb ODS2 column (125 × 4 mm I.D., 3μm particle size) from Pharmacia.

Chemicals and reagents

Boric acid, potassium hydroxide, formic acid 98% and sodium phosphate (all of analytical grade) were obtained from Baker (Gross-Gerau, Germany). Mercaptoethanol and OPA were purchased from Pierce (Oud Beijerland, Netherlands). Protein hydrolysate amino acid calibration standard AA-S-18, O-phospho-L-serine, L-α-aminoadipic acid, L-asparagine, L-glutamine, L-citrulline, β-alanine, taurine, γ-amino-*n*-butyric acid, L-α-amino-*n*-butyric acid, ethanolamine, δ-hydroxylysine, L-ornithine, L-norleucine, 5-methyl-DL-tryptophan (5-MTrp), S-carboxymethyl-L-cysteine, D-galactosamine, D-glucosamine and DL-dithiothreitol (DTT) were obtained from Sigma (Deisenhofen, Germany). Acetonitrile (HPLC grade) was from Johnson Matthey, Alfa Products (Karlsruhe, Germany). Methanol (LiChrosolv), 30% hydrogen peroxide, sulphosalicylic acid, sodium hydroxide, hydrochloric acid (all of analytical grade) and sodium azide (research grade) were purchased from Merck. Iodoacetic acid (analytical grade) was obtained from Fluka (Buchs, Switzerland). For the preparation of buffers and standards, deionized water (purification system Milli-Q Plus, Millipore, Eschborn, Germany) was used.

Sample preparation

Acid hydrolysis. Protein samples were hydrolysed in 6 *M* hydrochloric acid for 20 h at 105°C. Norleucine was used as internal standard. For determination of cystine as cysteic acid the protein samples were pretreated with performic acid.

Alkaline hydrolysis (for tryptophan). Protein samples or milks, with 5-methyl-DL-tryptophan added as internal standard, were hydrolysed for 20 h at 110°C in 4.2 *M* sodium hydroxide [6].

Plasma samples. Proteins were precipitated by adding one volume of sulphosalicylic acid (10%, w/v) to four volumes of plasma in an Eppendorf reaction vial (1.5 ml). Following cooling (30 min at 4°C) the samples were centrifuged for 5 min (10 000 *g*). The supernatants were diluted by a factor of 1:50 (v/v), filtered through 0.2-μm filters and analysed.

Cystine and cysteine of plasma samples. For conversion of cystine and cysteine to carboxymethylcysteine (CMC), the aforementioned supernatants were treated as follows: to 10 μl of the supernatant 150 μl of water, 10 μl of 12.5 m*M* dithiothreitol (DTT) and 20 μl of 1 *M* potassium hydroxide were added. The admixture was flushed with nitrogen and allowed to react for 30 min at room temperature. Subsequently 10 μl of 100 m*M* iodoacetic acid were added and the mixture was again flushed with nitrogen. After a further 10 min at room temperature, the chemical reaction to carboxymethylcysteine was completed.

Standards. In order to quantify amino acids present in various source materials, different standards were used. In the case of protein hydrolysates, AA-S-18 was used. For analysis of milk samples, standard AA-S-18 was also used, but with the addition of glucosamine or galactosamine and taurine. In the case of physiological (plasma) amino acids, standard AA-S-18 was extended by the addition of the following amino acids: O-phospho-L-serine, L-glutamine, L-asparagine, L-α-aminoadipic acid, L-citrulline, taurine, γ-amino-*n*-butyric acid, L-α-amino-*n*-butyric acid, ethanolamine, δ-hydroxylysine, β-alanine, L-ornithine, L-norleucine and carboxymethylcysteine. All standards were diluted with water

to a final concentration of 5 nmol/ml of each amino acid before derivatization with OPA.

Derivatization reagents

OPA stock solution. OPA (10 mg) was dissolved in a mixture of 8 ml of methanol and 1 ml of 0.5 *M* borate buffer (0.5 *M* boric acid adjusted to pH 10.4 with potassium hydroxide). Subsequently 0.1 ml of mercaptoethanol was added to the mixture. The stock solution can be kept for one week at 4°C in the dark.

OPA working solution. A 0.5-ml aliquot of the OPA stock solution was mixed with 1 ml of 1 *M* borate buffer (1 *M* boric acid adjusted to pH 10.4 with potassium hydroxide). The working solution is stable only for one day.

Derivatization procedure

The derivatization was performed automatically by a cooled (10°C) autosampler. A 30-μl aliquot of the sample solution and 30 μl of the OPA working solution were taken up with a syringe and mixed in a reaction vial. Complete mixing was achieved by taking up 50 μl of the solution and emptying the syringe. This process was repeated five times. The reaction time after mixing was exactly 2 min. Subsequently, 20 μl of the derivatized sample were injected onto the column.

Buffers and eluents

Sodium phosphate buffer. A 500-ml volume of 12.5 m*M* Na_2HPO_4 and 2000 ml of 12.5 m*M* NaH_2PO_4, containing 0.02% sodium azide, were mixed and filtered through a 0.22-μm filter. The pH of the phosphate buffer was 6.3. The compositions of eluents A, B and C are specified in Table I.

Separation programmes

All separations were performed at 25°C and a flow-rate of 0.9 ml/min. The gradients are given in Table Ia–d. For regeneration the column was washed for 3–4 min with eluent C and then equilibrated for 6–8 min under starting eluent conditions. Changing from eluent C to eluent A was performed within 1 min.

TABLE I

GRADIENT PROGRAMMES FOR (a) PROTEIN HYDROLYSATE AMINO ACIDS, (b) PROTEIN HYDROLYSATE AMINO ACIDS INCLUDING TAURINE, GLUCOSAMINE OR GALACTOSAMINE AND FOR PLASMA AMINO ACIDS, (c) CYSTEIC ACID AND CARBOXYMETHYLCYSTEINE AND (d) TRYPTOPHAN

Solvents: A = 12.5 m*M* sodium phosphate pH 6.3–acetonitrile (100:1, v/v); B = 12.5 m*M* sodium phosphate pH 6.3–acetonitrile (70:30, v/v); C = methanol–water (90:10, v/v).

	Time (min)	A (%)	B (%)	C (%)
a	00.00	70	30	0
	06.00	62	38	0
	13.00	58	42	0
	16.00	30	70	0
	25.00	20	80	0
	26.00	10	90	0
	31.00	0	93	7
	34.00	0	92	8
	35.00	0	0	100
	38.00		End of chromatogram	
b	00.00	80	20	0
	36.00	48	52	0
	38.00	28	72	0
	49.00	10	90	0
	54.00	0	94	6
	59.00	0	92	8
	60.00	0	0	100
	62.00		End of chromatogram	
c	00.00	90	10	0
	10.00	60	40	0
	12.00	0	100	0
	15.00	0	0	100
d	00.00	10	90	0
	12.00	0	100	0
	13.00	0	0	100

RESULTS AND DISCUSSION

Amino acids in protein hydrolysates

For analysis of protein hydrolysates a separation programme was used, allowing the determination of the amino acids within 38 min (Table Ia). Fig. 1 shows a chromatogram of a standard amino acid mixture. All amino acids were nearly baseline resolved. When analysing special biological samples it must be taken into account that hydrolysates from milk, especially from human

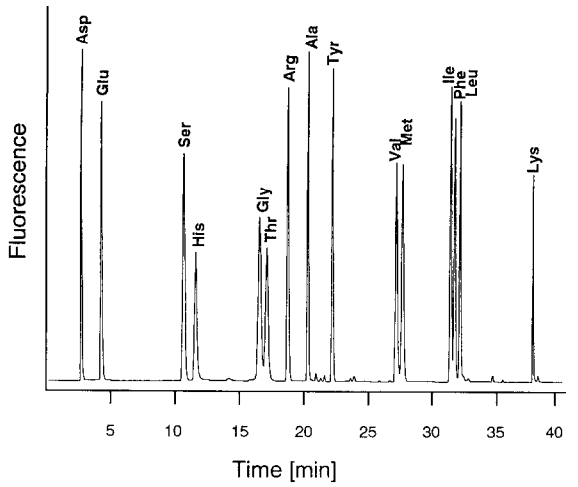

Fig. 1. Elution profile of an OPA-derivatized protein hydrolysate amino acid standard (50 pmol per amino acid). Column, LiChroCART (Superspher 100 RP 18 e, 125 × 4 mm I.D.); flow-rate, 0.9 ml/min; eluents, sodium phosphate–acetonitrile (gradient: Table Ia).

milk, contain taurine and amino sugars such as glucosamine or galactosamine in addition to protein-derived amino acids [7,8]. These components are also measured. Using the normal separation programme for amino acids of hydrolysates (Table Ia) taurine could be determined. However, glucosamine and galactosamine co-eluted with glycine and alanine and led to a false increase in the peak areas of these amino acids. Therefore, amino acid mixtures known to contain amino sugars had to be investigated by a prolonged (62 min) separation programme (Table Ib). In this way, these amino sugars are separated from the amino acids, but glucosamine and galactosamine cannot be distinguished. Furthermore, both amino sugars eluted as two individual peaks. Fig. 2 shows a chromatogram of a hydrolysed human milk sample. In accordance with standard runs, glucosamine and taurine were complete separated from the remaining amino acids. All amino acids were nearly baseline resolved.

The reproducibility of the overall method was assessed by calculating the relative standard deviations of the amounts and retention times of all amino acids. The reproducibility of the chromatographic procedure, which includes variations of injection and derivatization, was examined by injecting a hydrolysed human milk sample eight times in series. Calculation was performed by the external standard method (Table

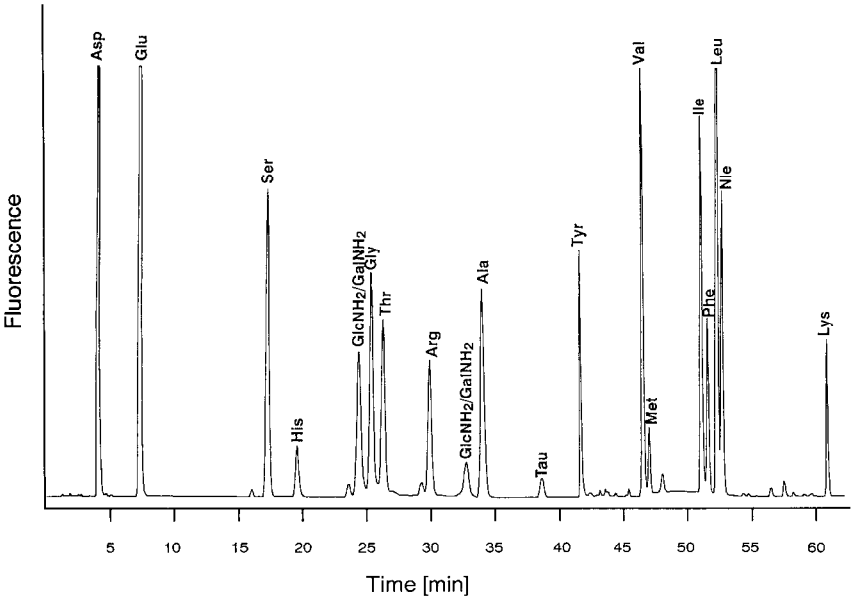

Fig. 2. Elution profile of an OPA-derivatized hydrolysate of a human milk sample. Column, LiChroCART (Superspher 100 RP-18 e, 125 × 4 mm I.D.); flow-rate, 0.9 ml/min; eluents, sodium phosphate–acetonitrile (gradient: Table Ib). Non-standard abbreviations used: $GlcNH_2$ = glucosamine; $GalNH_2$ = galactosamine.

IIA). The relative standard deviations of amounts ranged from 3.06% for methionine to 5.45% for taurine. When hydrolysing one human milk sample ten times, an additional variation occurred that could be attributed to the preparation procedure. The resulting relative standard deviations (Table IIB) were clearly increased compared with the values of the sample, which was repeatedly injected (Table IIA). The lowest value was obtained for aspartic acid, 4.13%, and the highest one for alanine, 7.34%. When calculating the amounts by the internal standard method (Table IIC), variations caused by the sample preparation procedure and injections are corrected. Consequently, the relative standard deviations were the lowest, ranging from 0.59% (isoleucine) to 3.03% (taurine). The relative standard deviations of retention times were less than 0.4% for all amino acids.

TABLE II

RELATIVE STANDARD DEVIATIONS OF THE AMOUNTS OF AMINO ACIDS IN A HYDROLYSED HUMAN MILK SAMPLE

A = Eight runs in series of one hydrolysed human milk sample; calculation was performed by the external standard method. B = Ten runs of one human milk sample, which was hydrolysed ten times; calculation was performed by the external standard method. C = Ten runs of one human milk sample, which was hydrolysed ten times; calculation was performed by the internal standard method.

Amino acid	R.S.D. (%) of amount		
	A (n = 8)	B (n = 10)	C (n = 10)
Asp	3.35	4.13	1.34
Glu	3.48	4.48	1.42
Ser	4.21	4.73	1.78
His	4.89	5.85	1.77
Glc-NH_2	3.75	5.97	2.43
Gly	4.28	5.00	2.89
Thr	3.71	4.92	0.95
Arg	4.24	5.59	1.65
Ala	3.70	7.34	0.94
Tau	5.45	6.68	3.03
Tyr	3.95	4.98	1.05
Val	3.57	4.81	0.79
Met	3.06	4.89	2.39
Ile	3.40	4.87	0.59
Phe	3.42	4.91	0.99
Leu	3.46	4.85	0.82
Lys	3.91	4.76	1.40

Determination of cystine and tryptophan

For quantification of cystine (cysteic acid) and tryptophan, short separation programmes were used (Table Ic and d, respectively). After oxidation of cystine, cysteic acid was eluted within 5 min. Tryptophan was quantified by the classical alkaline hydrolysis using 5-methyltryptophan as an internal standard. With the separation programme shown in Table Id both a satisfactory resolution of tryptophan and 5-methyltryptophan from other amino acids and a short separation time (10 min) were achieved. The reproducibility of both determinations was good.

Physiological amino acids

Numerous publications describe reversed-phase HPLC with OPA pre-column derivatization as sensitive, reproducible and fast methods for the determination of amino acids in protein hydrolysates and in physiological fluids [1,2,5,9–13]. However, our own investigations have shown that the use of these methods in routine practice is problematic for quantification of physiological amino acids. A major problem was to perform a sufficient number of separations on a single column. The aim of our investigations was to increase the lifetime of the column.

Fig. 3 shows the result of an optimized separation of a standard mixture of physiological amino acids. The gradient programme described in Table Ib was used. Separation was completed within 62 min. The standard mixture contains amino acids representative of plasma samples [3,11,14–18]. Nearly all amino acids were baseline resolved. The reproducibility, as determined by nine standard runs in series, was good (Table III). The relative standard deviations of peak areas ranged between 1.77 and 5.63%. The highest deviations were found for ornithine and lysine. The values for the relative standard deviations of the retention times were lower than 0.5% (0.03–0.49%).

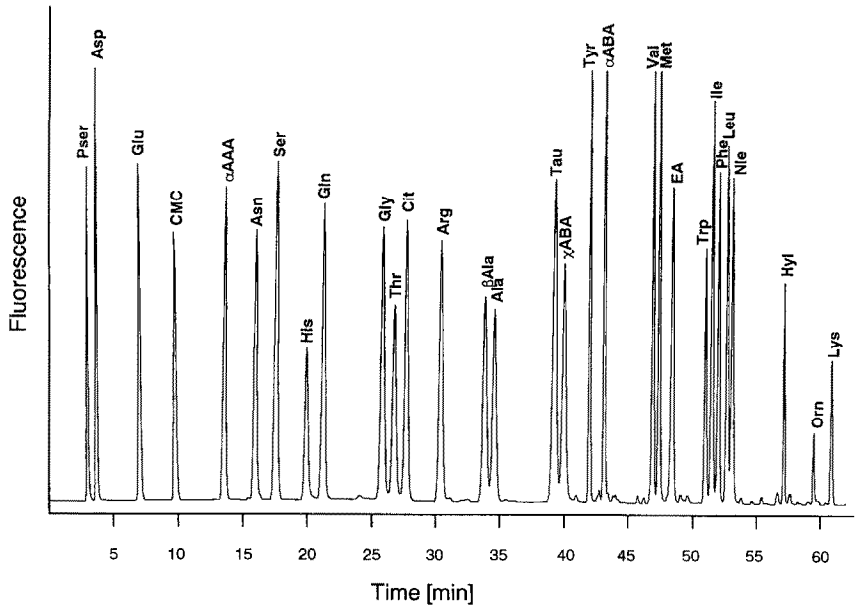

Fig. 3. Elution profile of an OPA-derivatized physiological amino acid standard (50 pmol per amino acid). Column, LiChroCART (Superspher 100 RP-18 e, 125 × 4 mm I.D.); flow-rate, 0.9 ml/min; eluents, sodium phosphate–acetonitrile (gradient: Table Ib). Non-standard abbreviations used: Pser = phosphoserine; CMC = carboxymethylcysteine; αAAA = α-aminoadipic acid; ABA = γ-aminobutyric acid; αABA = α-aminobutyric acid; EA = ethanolamine; Hyl = hydroxylysine.

Fig. 4 shows the amino acid composition of a human plasma sample. Compared with the standard chromatogram (Fig. 3), neither deterioration of resolution nor baseline drift caused by possible matrix effects could be observed.

When separating plasma amino acids, selec-

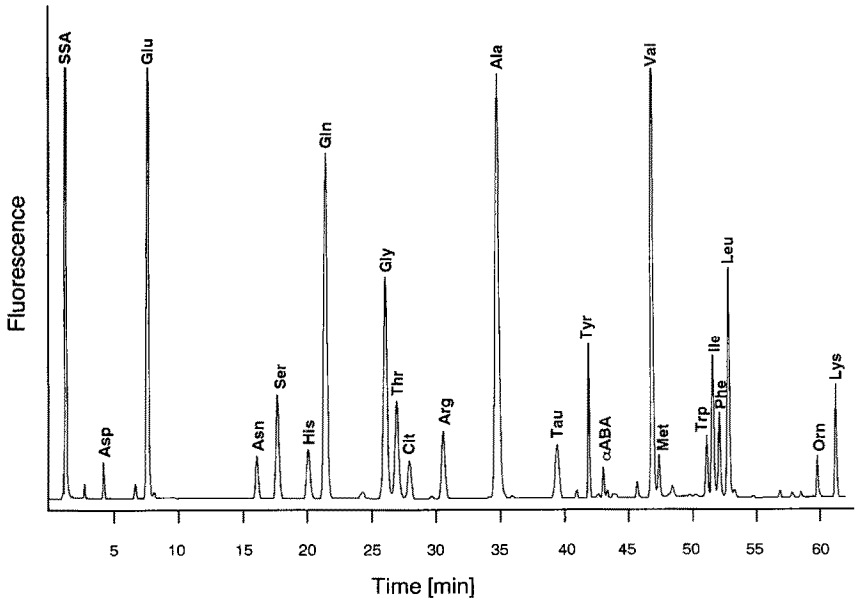

Fig. 4. Elution profile of an OPA-derivatized human plasma sample; column: LiChroCART (Superspher 100 RP 18 e, 125 × 4 mm I.D.); flow-rate, 0.9 ml/min; eluents, sodium phosphate–acetonitrile (gradient: Table Ib). Non-standard abbreviations used: SSA = sulphosalicylic acid; αABA = α-aminobutyric acid.

TABLE III

MEAN VALUES AND RELATIVE STANDARD DEVIATIONS OF RETENTION TIMES AND RELATIVE STANDARD DEVIATIONS OF AREAS (GIVEN FOR NINE RUNS OF A PHYSIOLOGICAL AMINO ACID STANDARD; 50 pmol PER AMINO ACID)

Amino acid	Retention time		Area R.S.D. (%)
	min	R.S.D. (%)	
Pser	3.54	0.49	2.67
Asp	4.25	0.23	1.87
Glu	7.64	0.41	2.63
CMC	9.85	0.49	3.22
α-AAA	13.78	0.29	2.16
Asn	16.15	0.24	1.93
Ser	17.74	0.20	3.72
His	20.10	0.14	3.02
Gln	21.37	0.14	2.24
Gly	25.98	0.12	2.86
Thr	26.88	0.11	1.87
Cit	27.84	0.11	2.38
Arg	30.53	0.11	2.14
βAla	33.96	0.09	3.46
Ala	34.70	0.09	2.62
Tau	39.40	0.09	3.16
γ-ABA	40.13	0.08	3.37
Tyr	42.08	0.03	2.25
α-ABA	43.21	0.03	2.55
Val	46.99	0.03	1.77
Met	47.48	0.06	2.39
EA	48.53	0.05	2.70
Trp	51.15	0.03	2.93
Ile	51.64	0.03	2.17
Phe	52.16	0.04	2.13
Leu	52.86	0.03	2.68
Nle	53.27	0.03	2.67
Hyl	57.40	0.03	2.93
Orn	59.79	0.04	5.63
Lys	61.21	0.03	4.03

tion of the column was most important. For amino acid determinations, reversed-phase C_{18} columns with 3, 4 or 5 μm particle sizes are available. Several investigators have demonstrated that when using 3-μm columns up to 48 physiological amino acids [9], and when using 5-μm columns 27–38 amino acids, can be separated [2,5,14]. Resolution was, however, not always satisfactory. Our own investigations drew attention to the major disadvantage of the 3-μm column (Spherisorb ODS2), which is its pronounced susceptibility to various interferences. Usually only 10–30 separations of physiological amino acids could be performed without significant deterioration of resolution. The best results were obtained with 4-μm columns, especially with the Superspher 100 column (Merck) with end-capped C_{18} material. As many as 400–500 successive runs could be performed with this 4-μm column. Only a few optimization steps were necessary when using a new column of this type.

In addition the combination of buffer composition, pH, column temperature, flow-rate and gradient had to be optimized to obtain both a good resolution of plasma amino acids and a good column lifetime. Important factors were the composition and pH of the buffer as well as the buffering ion. Replacing the phosphate buffer by acetate buffer resulted in a deterioration of the resolution. Increasing the pH of phosphate buffer from 6.2 to 7.2 also worsened the resolution. The addition of tetrahydrofuran (THF) was disadvantageous. Concerning the reproducibility of the peak areas, great variations could be discerned, especially with regard to lysine. Omitting THF resulted in much more reproducible peak areas but retarded the elution of amino acids. Another important factor was the speed of separation of physiological amino acids. A decrease in the number of possible runs resulted when the separation time was reduced below 60 min by increasing the slope of the gradient.

CONCLUSIONS

It has frequently been emphasized that the use of HPLC pre-column derivatization procedures for the determination of physiological amino acids in routine practice causes problems. The number of runs that can be performed with one column is limited. With the HPLC method described in this paper, it is possible to determine both amino acids in protein hydrolysates and 29 plasma amino acids in routine practice. Four to five hundred successive runs are feasible without significant deterioration of resolution. The results presented are most dependent on the selection of an appropriate type of C_{18} column.

REFERENCES

1 B. N. Jones, S. Paabo and S. Stein, *J. Liq. Chromatogr.*, 4 (1981) 565.
2 R. Schuster, *J. Chromatogr.*, 431 (1988) 271.
3 S. A. Cohen and D. J. Strydom, *Anal. Biochem.*, 174 (1988) 1.
4 A. Neidle, M. Banay-Schwartz, S. Sacks and D. S. Dunlop, *Anal. Biochem.*, 180 (1989) 291.
5 G. A. Qureshi and A. R. Qureshi, *J. Chromatogr.*, 491 (1989) 281.
6 H. K. Nielsen and R. F. Hurrell, *J. Sci. Food Agric.*, 36 (1985) 893.
7 T. E. Barman, *Biochim. Biophys. Acta*, 214 (1970) 242.
8 S. A. Atkinson, C. M. Schnurr, S. M. Donovan, B. Lönnerdal, in S. A. Atkinson and B. Lönnerdahl (Editors), *Protein and Non-Protein Nitrogen in Human Milk*, CRC Press, Boca Raton, FL, 1989 p. 117.
9 B. N. Jones and J. P. Gilligan, *J. Chromatogr.*, 266 (1983) 471.
10 D. T. Blankenship, M. A. Krivanek, B. L. Ackermann and A. A. Cardin, *Anal. Biochem.*, 178 (1989) 227.
11 I. Fermo and E. De Vecchi, *J. Chromatogr.*, 534 (1990) 23.
12 U. Bütikhofer, D. Fuchs, J. O. Bosset and W. Gmür, *Chromatographia*, 31 (1991) 441.
13 T. A. Graser, H. G. Godel, S. Albers, P. Földi and P. Fürst, *Anal. Biochem.*, 151 (1985) 142.
14 D. C. Turnell and J. D. D. Cooper, *Clin. Chem.*, 28 (1982) 527.
15 W. Plenert and W. Heine (Editors), *Normalwerte*, S. Karger, Basel, 1984.
16 L. D. Stegink, L. J. Filer and G. L. Baker, *Am. J. Clin. Nutr.*, 36 (1982) 917.
17 P. H. Scott, H. M. Berger and B. A. Wharton, *Pediatr. Res.*, 19 (1985) 446.
18 D. S. Young, *Ann. Intern. Med.*, 106 (1987) 114.