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APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO THE DETERMINATION OF FREE AMINO ACIDS IN PHYSIOLOGICAL FLUIDS

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SUMMARY

Multiple-step gradient systems were used for the analysis of free amino acids in physiological fluids by high-performance liquid chromatography with fluorescence detection on two reversed-phase C_{18} columns.

Standard amino acids, plasma or urine samples were subjected to derivatization with *o*phthalaldehyde in the presence of mercaptoethanol before the separation was performed.

More than 22 amino acids were separated in less than 1 h on either 5-μm Ultrasphere-ODS columns or 5-μm Resolve C_{18} columns by using a two-solvent system. The correlation of the integrated peak areas with the concentration of all amino acids showed a linear relationship between 10 and 150 pmol per 20-μl injection. The method has a lower detection limit of less than 1 pmol per 20-μl injection for all amino acids.

Quantitative analysis of micro amounts of amino acids in plasma and urine by the internal standard method gave highly reproducible results with a mean coefficient of variation of less than 3% and $r^2 = 0.999$. Because of the simplicity of the method its application provides a better means for closely monitoring the patients undergoing dialysis and treatment for renal disorders. These results are compared with those from classical ion-exchange chromatography.

INTRODUCTION

The analysis of amino acids is reckoned to be one of the most important applications in the biomedical and biochemical fields. More than 70 diseases are associated with defects in amino acid metabolism[1].

For the past three decades, ion-exchange chromatography based on the ninhydrin reaction and the principle of post-column derivatization has been used for the analysis of amino acids in physiological fluids[2–4]. Gas chromatography (GC) has also found frequent use for screening protein amino acids[5–8]. A frequent method of choice is based on the formation of a derivative of an amino acid sufficiently volatile for GC analysis. In order to fulfill this requirement, generally large amounts of sample

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and tedious extraction processes become essential before the GC analysis. This disadvantage curtails the application of this technique on a more routine basis.

These methods are complemented by high-performance liquid chromatography (HPLC) in combination with fluorescence detection based on the derivatization of amino acids with fluorescamine[9], dansyl chloride[10], dabsyl chloride[11] or *o*-phthalaldehyde (OPA)[12,13]. OPA is used for a pre-column derivatization with a wide range of reversed-phase columns and multiple-step gradient systems. It has become popular in recent years in amino acid analysis, because of the associated short analysis time and high sensitivity[14–16]. The only disadvantage is that OPA does not react with secondary amines[16].

It seemed plausible that a similar pre-column technique could be used in the analysis of primary amino acids, and this study was designed to explore the feasibility of using OPA in a pre-column derivatization procedure with subsequent separation on reversed-phase columns.

Two systems are described, one based on 5-μm Ultrasphere-ODS columns (Altex) and the other on 5-μm Resolve C_{18} columns (Waters Assoc.) with fluorescence detection at λ_{ex} = 340 nm and λ_{em} = 450 nm.

This report describes a quick general screening procedure for the determination of free amino acids at physiological concentrations in very small volumes of plasma and urine of uremic patients kept on a protein-rich diet and under dialysis conditions. The results obtained are compared with those from classical amino acid analysis (AAA).

MATERIALS AND METHODS

Chemicals

Individual crystalline samples of L-amino acids were obtained from Pierce (AMAC standard kit No. 20065). Methylated histidine, citrulline, phosphoserine, hydroxylysine, carnosine, taurine, cystathionine and β-alanine were obtained from Sigma. Individual standard stock solutions (1 μM) were prepared in 0.1 M hydrochloric acid. A standard mixture of 24 amino acids was prepared to a concentration of 0.04 μM and stored at 4°C before use. A mixture of standard amino acids was further diluted as required with 0.1 M hydrochloric acid and kept at 4°C before use. These solutions were stable for up to 2–3 months.

The mixture standard solutions were used for separation development while the individual amino acids were used for peak identification. Absolute ethanol, methanol "HPLC-grade", sodium bicarbonate "analysed reagent", sodium dihydrogen phosphate 1-hydrate and disodium hydrogen phosphate "AnalaR" grade were obtained from Fisher. Sodium hydroxide, hydrochloric acid and perchloric acid (70%) in pure form were obtained from J. T. Baker. OPA and mercaptoethanol were obtained from Sigma. Brij (30%) was obtained from Pierce.

Apparatus

A Waters Assoc. liquid chromatographic system, consisting of two high pressure pumps, M6000 and M-45, multiple sampler WISP 710B, data module, system controller 730B, and a U6K universal sample injector, was used. The fluorescence intensity of OPA–amino acid derivatives was detected with a filter fluorescence de-

tector (Waters Assoc.) model 420-AC with the monochromator set at 340 nm and a 450-nm cut-off filter. The sensitivity was set at 1 μA full scale.

Separation was carried out either on 5-μm Resolve C_{18} (150 × 4.6 mm I.D.) or on 5-μm Ultrasphere-ODS (250 × 4.6 mm I.D.) columns. The analytical column was coupled to a pre-column (50 × 4.6 mm I.D.) packed with the same material. Before initial use, both columns were conditioned with methanol–water (70:30) for 1 h. Before and after every run the columns were equilibrated with solvent A for 10 min.

OPA reagent

The derivatization reagent was prepared by dissolving 50 mg of anhydrous *o*-phthalaldehyde in 1 ml of methanol; to this solution 9 ml of 0.4 *M* borate buffer (adjusted to a pH 10.4 with 1 *M* potassium hydroxide containing 0.6% of 30% Brij) were added. This was followed by addition of 50 μl of 2-mercaptoethanol. The mixture was kept at 4°C for 24 h before use. It remained stable for one week, provided that after every three days 20 μl of 2-mercaptoethanol were added.

Buffer solution

Disodium hydrogen phosphate (7.1 g) and sodium dihydrogen phosphate 1-hydrate (6.9 g) were separately dissolved in 1 l of double-distilled water to make solutions of 0.05 *M* concentration. A pH value of 7.4 resulted when the two were mixed. To make system I this buffer was diluted with water. Fresh buffers were made every day.

Derivatization of amino acids

OPA (10 μl) was drawn into the automatic injector and kept in the needle. A similar volume of amino standard or supernatant of urine or plasma (after extraction) was subsequently drawn in. These solutions were mixed for 2 min in the needle before injection onto a column. This method is reproducible with time accuracy to avoid the time factor which otherwise gives unreliable results in recording the fluorescent intensity of the OPA–amino acid adduct. This procedure showed consistent results, with a standard deviation of 2–3% over five replicate analyses.

Extraction procedures

All extraction procedures were conducted at room temperature. A 200-μl volume of standard plasma or urine sample was homogenized by the addition of 800 μl of ice-cold 80% ethanol and centrifugation at 1500*g* for 10 min. The supernatant was collected and 100 μl were added to 400 μl of 0.2 *M* perchloric acid; the solution was centrifuged again at 1500*g* for 10 min. To 200 μl of the supernatant, 50 μl of standard amino acid solution (25 nmol/ml) were added. This solution was frozen until analysed. Our recovery rates by this procedure ranged from 83 to 94%.

A second extraction method was to take 200 μl of plasma or urine sample treated with 1800 μl of 0.2 *M* perchloric acid and to centrifuge at 1500*g* for 15 min. To 200 μl of the supernatant, 50 μl of standard amino acid solution (25 nmol/ml) were added. The solution was frozen prior to HPLC analysis. The recovery rates were similar ranging from 88 to 95% for all amino acids.

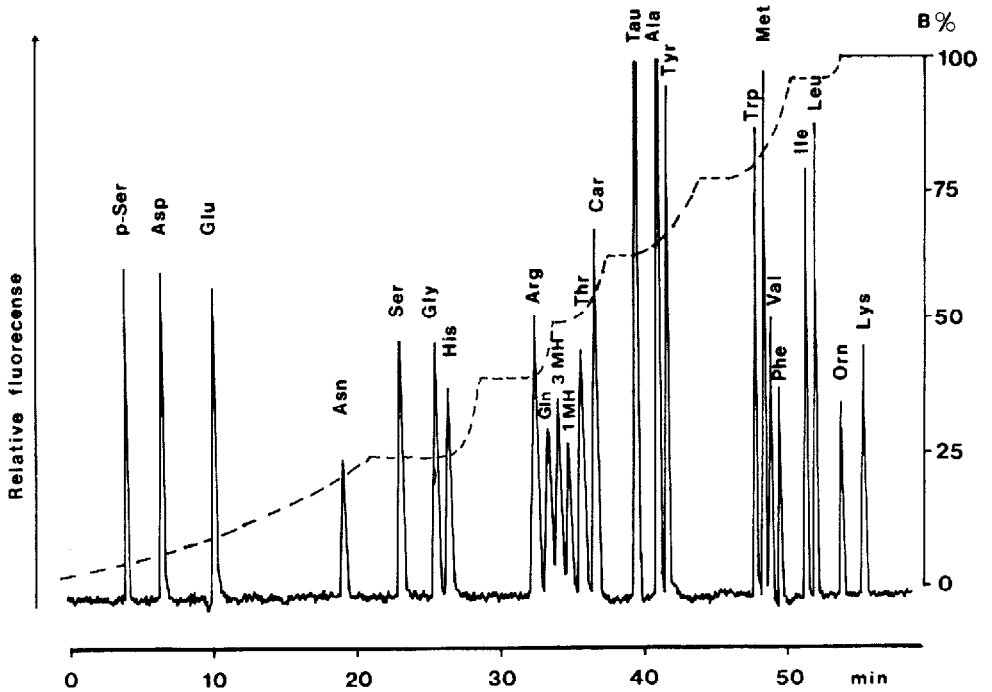

Fig. 1. Chromatogram of 24 amino acids (10 nmol/ml) as OPA–mercaptoethanol derivatives. Chromatographic system I (see Experimental).

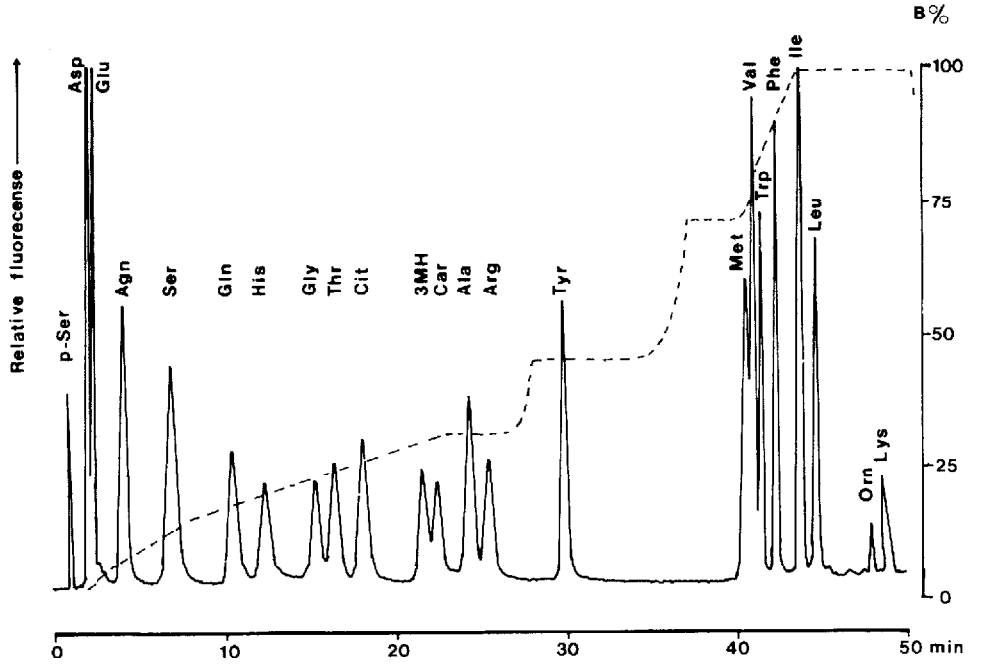

Fig. 2. Chromatogram of 23 amino acids (10 nmol/ml) as OPA–mercaptoethanol derivatives. Chromatographic system II (see Experimental).

Chromatographic procedure

System I (Ultrasphere-ODS). Resolution of the peaks was achieved using gradient elution with a mobile phase in pump A consisting of 20 m*M* phosphate buffer (pH 7.4)–tetrahydrofuran–methanol (90:1:9) and a mobile phase in pump B of methanol–20 m*M* phosphate buffer (pH 7.4) (75:25). The gradient is shown in Fig. 1. The flow-rate was 1.0 ml/min and the column temperature was ambient. Before and after every run the column was re-equilibrated with 100% solvent A for 8–10 min.

System II (Resolve C_{18}). For this system, various buffers and solvent compositions were used. A suitable gradient system was: pump A, 50 m*M* phosphate buffer (pH 7.4)–tetrahydrofuran–methanol (85:1:14); pump B 50 m*M* phosphate buffer–methanol (28:72) (Fig. 2). A flow-rate of 1 ml/min was used, and the column was kept at ambient temperature. All the solvents for both systems were filtered and degassed using 0.45-μm Millipore Type HA for solvent A and HV for solvent B, respectively. Of the standard or sample 20 μl were injected onto the column for both systems.

TABLE I

RETENTION TIMES FOR INDIVIDUAL AMINO ACIDS OBTAINED BY SYSTEMS I AND II

Values are compared with retention time for leucine (r^*). A similar comparison is made between the fluorescence intensities of all the amino acids and that of leucine.

Amino acid	*Retention time*		r^*		*Sensitivity (%)*	
	System I	*System II*	*System I*	*System II*	*System I*	*System II*
p-Ser	3.90	1.92	0.077	0.040	40.8	42.3
Asp	5.68	2.26	0.112	0.047	21.7	23.1
Glu	8.17	3.24	0.161	0.068	46.0	48.0
Asn	15.16	7.40	0.299	0.155	39.3	41.0
Ser	18.82	9.14	0.371	0.192	84.5	87.0
Gly	20.76	17.38	0.409	0.365	72.2	73.0
His	21.55	14.91	0.425	0.313	67.2	60.0
Arg	27.54	27.75	0.543	0.583	109.0	130.0
Cit	27.54	20.70	0.543	0.435	94.7	98.0
Gln	28.33	12.84	0.559	0.270	62.0	68.0
3MH	28.84	23.95	0.569	0.503	62.0	68.0
1MH	29.57	23.95	0.583	0.503	43.0	48.0
Thr	30.58	18.56	0.603	0.390	77.6	78.0
Car	31.76	24.90	0.626	0.523	68.5	73.0
Tau	35.47	27.76	0.700	0.583	146.0	130.0
Ala	37.61	27.76	0.742	0.583	155.0	137.0
Tyr	38.73	34.10	0.764	0.716	72.0	81.0
Trp	46.51	43.96	0.918	0.923	72.0	76.0
Met	47.02	42.65	0.928	0.896	76.0	73.0
Val	47.54	43.04	0.938	0.904	66.0	61.0
Phe	48.22	45.44	0.951	0.955	37.2	42.3
Ile	50.02	46.80	0.987	0.983	62.3	67.0
Leu	50.67	47.60	1.000	1.000	100.0	100.0
Orn	52.51	50.20	1.036	1.055	16.4	20.3
Lys	53.98	51.28	1.065	1.077	26.0	27.2
OH-Lys	54.74	47.60	1.000	1.000	93.0	96.0

Samples

Plasma and urine were collected repeatedly from a patient with chronic renal failure over a period of 16 days; a meat-free diet was given for part of this time. Plasma samples were obtained from another patient before and 1, 2 and 3 h after the start of hemodialysis. Urine and plasma samples were frozen and kept at −70°C until subjected to HPLC and AAA.

RESULTS AND DISCUSSION

The elution order of the amino acid derivatives of OPA on a hydrophobic column appears to be influenced mainly by the balance between the hydrophobic and hydrophilic groups of the derivatives. Thus, the order of elution is dependent on the column material used. Figs. 1 and 2 show chromatograms of OPA–mercaptoethanol derivatives of standard amino acids with systems I and II, respectively. System I separated 24 amino acids, and system II separated 23 amino acids. The retention times of the amino acids in relation to that of leucine are listed in Table I.

The ability to reproduce retention times is a very important factor in the analysis of free amino acids in physiological samples. Thus, internal addition of standard amino acids proved to be a suitable and sure method to accomplish such a complex separation. The method can also be used to quantitate the difference between the integrated areas obtained with the standard alone and the standard plus sample, so that the amount of amino acids in a physiological sample may be determined. Fig.

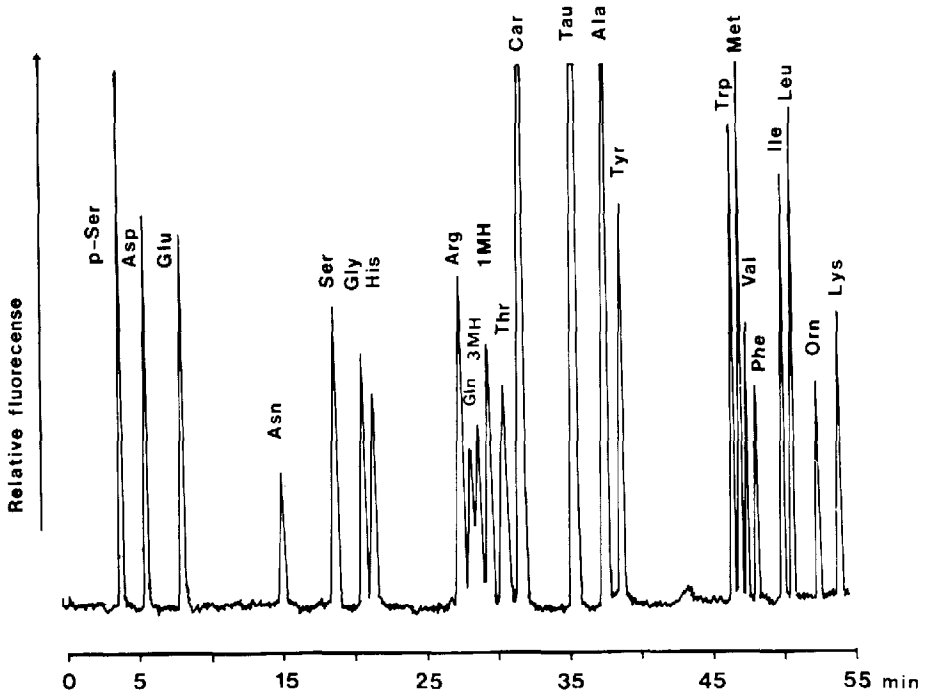

Fig. 3. Chromatogram of plasma of a uremic patient containing standard 24 amino acids (5 nmol/ml) as OPA mercaptoethanol derivatives. Chromatographic system I.

3 shows a chromatogram of a plasma sample with 5 nmol/ml of the mixture of 24 amino acids.

All amino acids in plasma and urine samples are compared with the relative retention times of standard amino acids. With both systems the average variation in the retention times for amino acids in standard, plasma and urine samples was ± 1–2% over five experiments. Table I shows the retention times for both systems as the average values after five runs under similar conditions.

Linear regression analysis of the peak area of each amino acid *versus* concentration over a concentration range from 10 to 150 pmol per 20-μl injection gave a correlation coefficient of 0.999 for all amino acids, with a coefficient of variation for the peak areas in the range of 1–2%. Fig. 4 shows a standard curve for eight rep-

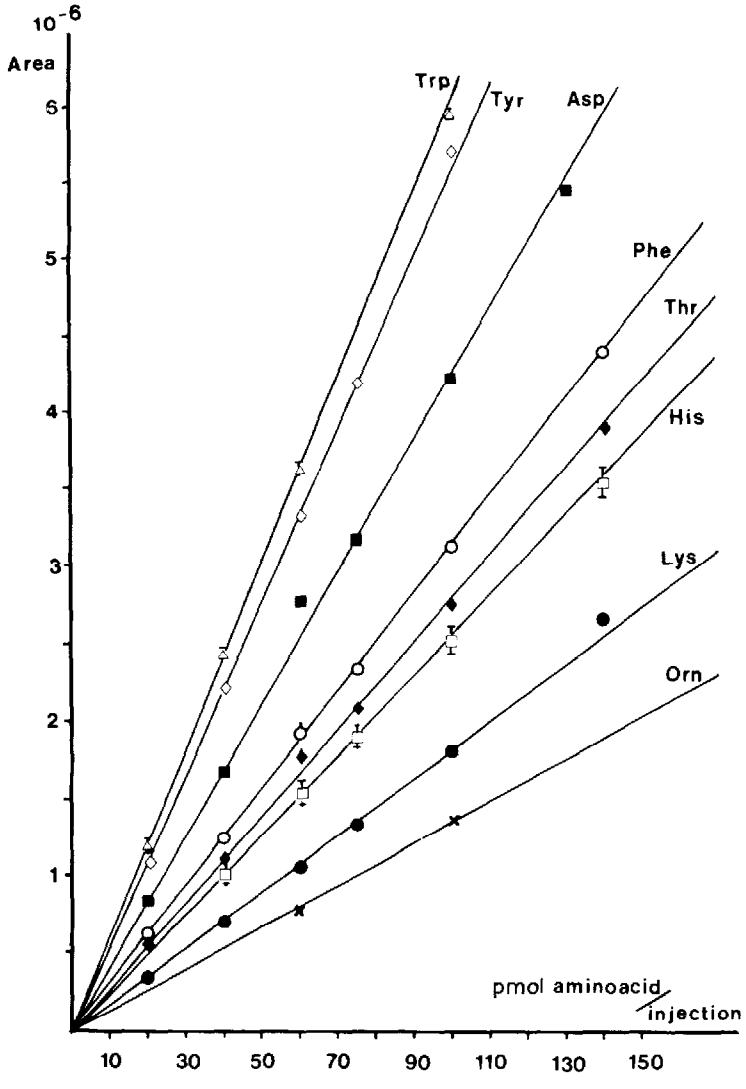

Fig. 4. Representative standard curves for OPA mercaptoethanol derivatives of amino acids. Amino acid response *versus* concentration.

TABLE II

COMPARISON OF AMINO ACID CONCENTRATIONS DETERMINED BY HPLC AND AAA IN PLASMA AND URINE OF UREMIC PATIENTS

Amino acid	*Plasma (μmol/l)*			*Urine (μmol/l)*		
	n	*HPLC*	*AAA*	*n*	*HPLC*	*AAA*
p-Ser	4	37.0 ± 1.7	37.4 ± 2.5	10	74.0 ± 6.2	76.1 ± 6.5*
Asp	12	33.4 ± 4.0	28.3 ± 3.6	10	71.8 ± 4.6	77.8 ± 4.7
Glu	12	53.8 ± 5.3	55.2 ± 3.3	10	16.6 ± 1.6	18.7 ± 2.0**
Asn	12	70.1 ± 8.2	66.2 ± 7.0	10	88.4 ± 6.3	89.0 ± 6.7
Ser	12	102.1 ± 8.5	105.7 ± 10.1	10	133.1 ± 14.5	172.7 ± 8.0**
Gly	12	347.6 ± 24.1	346.6 ± 20.1	10	542.0 ± 37.0	556.3 ± 34.0
His	12	60.2 ± 7.5	57.2 ± 5.7	10	166.5 ± 7.1	171.2 ± 5.2
Arg	4	57.0 ± 2.6	58.4 ± 2.0	10	15.1 ± 1.9	16.0 ± 2.1*
Cit	12	95.2 ± 3.4	95.3 ± 3.5	10	36.6 ± 2.3	37.6 ± 1.1
Gln	12	304.6 ± 14.4	310.3 ± 14.0	10	94.5 ± 10.2	101.8 ± 10.3
3MH	12	51.6 ± 6.4	24.0 ± 2.3***	10	106.0 ± 10.2	112.0 ± 9.4
1MH	4	17.2 ± 0.8	27.7 ± 2.1*	9	18.2 ± 2.7	99.3 ± 11.0***
Thr	12	128.2 ± 8.3	126.0 ± 7.7	10	121.2 ± 8.0	122.4 ± 8.4
Tau	12	79.4 ± 5.9	80.4 ± 3.8	–	–	–
Ala	12	358.6 ± 22.8	364.8 ± 25.3	10	201.0 ± 7.4	202.2 ± 7.3
Tyr	12	57.8 ± 4.8	38.0 ± 5.2*	10	19.4 ± 1.7	19.5 ± 1.7
Trp	12	38.5 ± 3.0	41.1 ± 3.0*	–	–	–
Met	12	17.8 ± 1.5	19.3 ± 1.5**	10	19.1 ± 2.0	21.6 ± 2.4*
Val	4	145.1 ± 13.4	139.5 ± 9.7	10	29.7 ± 4.0	30.3 ± 4.1
Phe	12	34.6 ± 4.6	35.5 ± 4.5	9	14.0 ± 1.0	15.0 ± 1.2
Ile	12	31.7 ± 2.8	34.0 ± 2.5*	8	8.3 ± 1.2	14.0 ± 1.2
Leu	12	98.4 ± 7.5	58.5 ± 5.3***	10	41.6 ± 1.5	21.6 ± 1.2***
Orn	12	62.6 ± 4.2	61.1 ± 4.3	10	22.5 ± 1.2	22.8 ± 1.3
Lys	12	163.0 ± 6.7	159.8 ± 7.3	10	66.0 ± 5.3	64.0 ± 6.0

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

resentative amino acids. The curve obtained for ornithine and lysine shows a lower slope value than those for the other amino acids. Similar results have been obtained by other groups[17–19], and it has been suggested that it could be due to the presence of two fluorescent isoindole structures from the reaction of OPA with lysine or ornithine. We further studied plasma and urine samples from a patient with chronic renal failure who was kept under various diets (see Experimental) and a patient kept under hemodialysis; the plasma samples were collected at the beginning and after 1, 2 and 3 h of the dialysis process. All these samples were analysed both by HPLC and by classical AAA; the values obtained are given in Table II, which shows that the two sets of results are similar for most of the amino acids. There are some notable exceptions, such as 3MH, 1MH, Tyr and Leu, the level recorded by AAA being higher or lower despite the fact that the reproducibility was the same for both methods. This may perhaps be due to the different standards used in the two methods; the area factors generated by two different integrators could also create such differences.

In our experience, pre-column derivatization of amino acids with OPA is a

highly reliable method but the reproducibility and the separation is very dependent on the age and the batch of the column material. The separation efficiency of the column deteriorates after more than 200 physiological samples have been analysed.

Other methods have been used for similar purposes, *e.g.* phenylthiohydantoin (PTH) and dansyl chloride[19–27]. However, these techniques contain many variables, and mostly require temperature control with lengthy preparation and analysis times and multiple solvents. It has been shown that coelution, non-separations and noisy baselines also occur with PTH derivatization[17,18]. Considering the disadvantages of other systems, our method offers better separation and quantitation of amino acids. Both our systems achieve separation within 1 h, and the derivatization and separation procedures are conducted at ambient temperature. Also, we use an automatic injection system based on mixing OPA and the amino acids in the needle for 1–2 min prior to injection onto the column. This procedure avoids the time-dependent stability problem usually encountered with OPA adducts[8].

The only disadvantage is that OPA does not react with secondary amines to give detectable response, but this can be overcome by treating them with sodium hypochlorite[28] or dabsyl chloride[29]. Work is in progress in our laboratory to apply this technique to quantitate amino acids in muscle samples of uremic patients.

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1 H. J. Bremmer, M. Duran, J. P. Kamerling, H. Przyrembel and S. K. Wadman, *Disturbance of amino acid metabolism. Clinical Chemistry and Diagnosis*, Urban and Schwarzenber, Baltimore, 1981.
2 P. H. Spackman, S. Moore and W. H. Stein, *Anal. Chem.*, 30 (1958) 1190.
3 P. E. Hare, in S. B. Needleman (Editor) *Protein Sequence Determination*. Springer Verlag, Berlin, New York, 1975, pp. 204–231.
4 P. E. Hare, *Methods Enzymol.*, 47 (1977) 3.
5 C. W. Gehrke and D. L. Stalling, *Separ. Sci.*, 2 (1967) 101.
6 J. Graff, J. P. Wein and M. Winitz, *Proc. Fed. Amer. Soc. Exp. Biol.*, 22 (1963) 244.
7 C. W. Moss, M. A. Lambert and F. J. Diaz, *J. Chromatogr.*, 60 (1971) 134.
8 R. F. Adams, F. L. Vandemark and G. J. Schmidt, *J. Chromatogr. Sci.*, 15 (1977) 63.
9 W. Valter and K. Zeck, *J. Chromatogr.*, 112 (1975) 643.
10 T. Yamabe, N. Takai and H. Nakamura, *J. Chromatogr.*, 104 (1975) 359.
11 L. Lammens and M. Verzek, *Chromatographia, 11 (1978) 376.*
12 *K. V. J. Swedas, J. I. Galaev, I. L. Borislow and I. V. Berezin, Anal. Biochem.*, 101 (1980) 188.
13 J. C. Hodgin, *J. Liquid Chromatogr.*, 2 (1979) 1047.
14 D. W. Hill, F. H. Waters, T. D. Wilson and J. D. Stuart, *Anal. Chem.*, 51 (1979) 1338.
15 R. Schuster, *Anal. Chem.*, 52 (1980) 617.
16 N. B. Jones, S. Pääbo and S. Stein, *J. Liquid Chromatogr.*, 4 (1981) 565.
17 P. Lindroth and K. Mopper, *Anal. Chem.*, 51 (1979) 1667.
18 M. Roth, *Anal. Chem.*, 43 (1971) 880.
19 J. R. Benson and P. E. Hare, *Proc. Nat. Acad. Sci. U.S.*, 72 (1975) 621.
20 W. S. Garner and W. H. Miller, *Anal. Biochem.*, 101 (1980) 61.
21 E. W. Mattews, P. G. H. Byfield and I. MacIntyre, *J. Chromatogr.*, 110 (1975) 369.
22 C. L. Zimmerman, E. Appealla and J. J. Pisano, *Abal. Biochem.*, 75 (1976) 77.
23 C. L. Zimmerman, E. Appealla and J. J. Pisano, *Anal. Biochem.*, 77 (1977) 569.
24 N. D. Johnson, M. W. Hunkapiller and L. E. Hood, *Anal. Biochem.*, 100 (1971) 335.
25 J. van Beeuman, J. van Damme and J. deLey, *FEBS Lett.*, 93 (1978) 373.

26 W. D. Annan, *J. Chromatogr.*, 173 (1979) 194.
27 E. Bayer, E. Grom, B. Kaltenegger and R. Uhman, *Anal. Chem.*, 48 (1976) 1106.
28 Y. Ishida, T. Fujita and K. Asai, *J. Chromatogr.*, 204 (1981) 143.
29 A. Casini, F. Martini, S. Nieri, D. Ramarli, F. Franconi and C. Surrenti, *J. Chromatogr.*, 249 (1982) 187.