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DETERMINATION OF AMINO ACIDS IN BIOLOGICAL, PHARMACEUTICAL, PLANT AND FOOD SAMPLES BY AUTOMATED PRECOLUMN DERIVATIZATION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An easy, rapid and sensitive method for the simultaneous determination of primary and secondary amino acids in different matrices, e.g. protein hydrolysates, pharmaceutical formulations, plant extracts, food samples and physiological fluids, is described. After minimum sample preparation amino acids were derivatized with two different reagents, *o*-phthalaldehyde–3-mercaptopropionic acid for primary and 9-fluorenylmethylchloroformate for secondary amino acids, by an automated precolumn derivatization technique. With minor adjustments of separation parameters this method can also be used to determine amino acids in different matrices. Analysis time including reaction, separation and reconditioning ranged from 20 min for hydrolysates to 60 min for physiological fluids. The separation was done on a reversed-phase column with a gradient of acetate buffer–acetonitrile as the mobile phase. The precision for peak areas of the individual amino acids was within a relative standard deviation of 2% for the hydrolysate assay and 2–5% for the physiological assay, and for retention times better than 0.7%. The detection limit with the diode array detector (ultraviolet–visible) was ca. 2–5 pmol, measured at 338 nm for primary and 266 nm for secondary amino acids; with the fluorescence detector 20–50 fmol were detectable at excitation and emission wavelengths of 230 and 455 nm for primary and 266 and 310 nm for secondary amino acids, respectively.

INTRODUCTION

Traditionally, amino acids have been analysed by methods based on the technology developed by Moore and Stein [1], by which free amino acids are separated by cation-exchange chromatography and treated with ninhydrin in a postcolumn derivatization step, and the derivatives are detected in the visible region. Although this approach is reliable and the resolution for the amino acids is reasonable, the analysis time is rather long (1 h for hydrolysate and up to 4 h

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for physiological amino acids) and sensitivity is limited (ca. 150 pmol). Furthermore, postcolumn systems are difficult to operate and maintain, and these dedicated systems are limited to one type of analysis.

A general alternative to the method of Moore and Stein [1] is offered with precolumn derivatization, by which the derivatives can be separated on a reversed-phase high-performance liquid chromatographic (HPLC) column with shorter analysis time, higher sensitivity and more flexible instrumentation. Typical reagents for precolumn derivatization are *o*-phthalaldehyde (OPA) [2–5], dansyl chloride [6], dabsyl chloride [7], phenyl isothiocyanate (PITC) [8] and 9-fluorenylmethylchloroformate (FMOC) [9]. Although all these derivatives are fairly sensitive, have sufficient selectivity and result in short analysis times, there are still some limitations. Dabsyl and dansyl chloride form derivatives with primary and secondary amino acids, which are fluorescent (dansyl) or absorb in the visible wavelength range (dabsyl). Both reactions are rather time-consuming and require high temperatures, the derivatives are relatively unstable and the reaction is not very selective (other functional groups might be derivatized as well). OPA reacts within a short time with primary amino acids to form highly fluorescent isoindoles, although the derivatives are rather unstable and secondary amino acids do not react. PITC, known as the Edman reagent, reacts with both primary and secondary amino acids to yield phenylthiocarbamyl (PTC) derivatives with reasonable detection sensitivity at 244 nm. Extensive sample preparation is required and trace amounts of salts and other matrix compounds interfere with the derivatization step. Furthermore, the derivatization procedure itself is rather complex compared with other procedures. FMOC derivatives are highly fluorescent, the reaction time is quite fast (30 s) under mild conditions and derivatives are stable. The major drawback is that excess reagent is hydrolysed and must be extracted to prevent interference with other amino acids during separation. The limitations of these precolumn derivatization methods are either tedious sample preparation and lengthy or manual derivatization procedures.

The objectives of work described in this paper were to develop a reliable method (a) suitable for both primary and secondary amino acids, (b) using automated precolumn derivatization and flexible HPLC instrumentation, (c) with high sensitivity (femtomoles), (d) with short reaction and analysis time, (e) requiring a minimum of sample preparation and compatible with common matrices. For this purpose an automated two-step derivatization technique was developed [11], in which derivatization of primary amino acids by *o*-phthalaldehyde–3-mercaptopropionic acid (OPA–3-MPA) and secondary amino acids by FMOC is done within the sample injection cycle. In the first step, all primary amino acids are treated with OPA–3-MPA. In the second step, the OPA derivatives and the free secondary amino acids are mixed with FMOC to generate a mixture of OPA derivatives and FMOC derivatives (Fig. 1). All reaction steps are precisely time-controlled, to ensure reproducible quantitation. The mixture is then injected onto the reversed-phase column and detected at two different wavelengths. The combination of these two reagents has led to a system that can be totally automated with a fast reaction time (ca. 1.5 min) for both types of amino acid, with excellent sensitivity (down to 20 fmol/μl by fluorescence) and with conveniently short

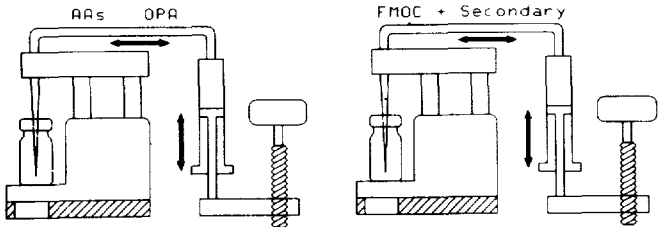

Fig. 1. Schematic layout of the automated precolumn derivatization system.

analysis time (20 min for a hydrolysate analysis including the reaction and reconditioning time of the column).

EXPERIMENTAL

Apparatus

A Hewlett-Packard HP 1090 Series M liquid chromatograph with DR5 solvent-delivery system, variable-volume autoinjector, cooled autosampler (4°C), temperature-controlled column compartment and solvent-preheating device was used. The following columns were used: Hypersil ODS, 5 μm (20 cm×2.1 mm I.D.) for amino acid analyses in hydrolysates and Hypersil ODS, 5 μm (20 cm×4.6 mm I.D.) for amino acid analyses in physiological fluids; both were supplied by Hewlett-Packard (Waldbronn, F.R.G.). A diode array detector at wavelength 338 nm for primary and 266 nm for secondary amino acids was used for detection. For trace level work in the low picomole range an HP 1046A programmable fluorescence detector was used at excitation and emission wavelengths of 230 and 455 nm for primary amino acids, switched to 266 and 310 nm, respectively, for detection of the secondary amino acids.

Reagents and chemicals

Reagents for derivatization, OPA–3-MPA, FMOC, borate buffer and standards (10, 25, 100, 250 pmol, containing sixteen amino acids, representative of a protein hydrolysate) were supplied by Hewlett-Packard (AA reagent kit high sensitivity No. 5061-3347). A second amino acid standard containing 36 amino acids, representative of a physiological sample, was prepared from the commercial Pierce standards (20086+20087) and diluted with 0.1 *M* hydrochloric acid by a factor of 1:10. Acetonitrile (HPLC grade), sodium acetate and potassium acetate (p.a. grade) were obtained from Baker (Gross Gerau, F.R.G.) and THF (silylation grade) from Pierce (Rodgau, F.R.G.). Reagents and samples were kept at 4°C in the cooled autosampler.

Derivatization procedure

The derivatization was fully automated by means of an injector program. Within one injection sequence, different volumes of sample and reagents were taken from different vials. Mixing was enhanced by moving sample and reagent volumes back and forth (one cycle) inside the injection capillary. The steps in the derivatization sequence are summarized in Table I. In the first step, borate buffer (pH 10.4) was taken (3 μl from vial 2), then 1 μl of OPA–3-MPA was drawn and mixed with the borate to adjust the pH of the OPA reagent (mix 3 μl, three cycles).

TABLE I

INJECTION PROGRAMME FOR DERIVATIZATION OF PRIMARY AMINO ACIDS WITH OPA-3-MPA AND SECONDARY AMINO ACIDS WITH FMOC

Step	Action	Amount (μl)	Details	Substance
1	Draw	3	From vial 2	Borate (pH 10.4)
2	Draw	1	From vial 0	OPA
3	Mix	3	Two cycles	
4	Draw	0	From vial 100	Water for needle wash
5	Draw	1	From sample	Amino acids
6	Draw	0	From vial 100	Water for needle wash
7	Mix	5	Seven cycles	Derivatization of primary amino acids
8	Draw	1	From vial 1	FMOC
9	Draw	0	From vial 100	Water for needle wash
10	Mix	7	Five cycles	Derivatization of secondary amino acids
11	Draw	2	From vial 3	Borate (pH 2.7)

TABLE II

CHROMATOGRAPHIC GRADIENT CONDITIONS FOR HPLC ANALYSIS OF OPA-3-MPA AND FMOC AMINO ACID DERIVATIVES IN HYDROLYSATES ANALYSIS

Buffers: A = 0.03 *M* sodium acetate, 0.5% THF (pH 7.2); B = acetonitrile–0.1 *M* sodium acetate (4:1).

Time (min)	%B	Flow-rate (ml/min)
0.00	0	0.45
9.00	30	
11.00	50	
13.00	50	
14.00	100	
14.10		0.45
14.20		0.80
17.90		0.80
18.00		0.45
18.00	100	
19.00	0	

Next, 1 μl of amino acid sample was mixed with the OPA–3-MPA (mix 5 μl, seven cycles) to derivatize all primary amino acids. To this mixture of derivatized primary amino acids and free secondary amino acids, 1 μl of FMOC was added (mix 7 μl, six cycles). Between each step a wash cycle, to clean the surface of the needle, was performed (draw 0 μl from vial 100). The resulting mixture of primary amino acids as isoindole (OPA) derivatives and secondary amino acids as FMOC derivatives was injected.

TABLE III

CHROMATOGRAPHIC GRADIENT CONDITIONS FOR HPLC OF OPA-3-MPA AND FMOC DERIVATIVES IN PHYSIOLOGICAL FLUIDS

Buffers: A = 0.06 *M* sodium acetate, 0.6% THF (pH 8.0); B = acetonitrile–0.1 *M* sodium acetate–methanol (14:4:1).

Time (min)	%B	Flow-rate (ml/min)
0	3	0.75
13	8	
18	10	
38	33	
38		0.75
39		1.30
42	50	
45	100	
49		1.30
49	100	
50		0.75
51	3	

Gradient

The gradient programs applied are given in Table II for protein hydrolysate amino acid analyses and in Table III for physiological amino acid analyses.

RESULTS AND DISCUSSION

Applications

Automated precolumn derivatization [11] leads to a number of improvements in the analysis of amino acids. The technique is time-saving (the total analysis time is 19 min for hydrolysate amino acids and 60 min for physiological amino acids), sensitive (the detection limit is in the fmol/μl region with fluorescence) and precise [the relative standard deviations (R.S.D.) are better than 0.5% for retention times and 2–5% for areas]. The whole procedure is fully automated — derivatization, separation and data analysis are parts of a single method.

Protein hydrolysates. Fig. 2 illustrates the results from a 10-pmol standard (containing sixteen amino acids) analysed with fluorescence detection. The R.S.D. for retention times and peak areas, based on ten analyses, are given in Table IV, which also lists data obtained from the 100-pmol standard analysed with a diode array detector.

A typical protein hydrolysate used for reference data is bovine serum albumin (BSA), and Fig. 3 shows the results obtained with this material. A comparison of literature data and HPLC results based on this technique for two proteins, BSA and a light-chain fraction from the Bence–Jones protein, are presented in Table V. The analysis of the light-chain fraction was performed using 100 pmol of the hydrolysate. Excellent performance was also obtained for other "classical" proteins, such as myoglobin, glucagon and insulin. This method can also be used

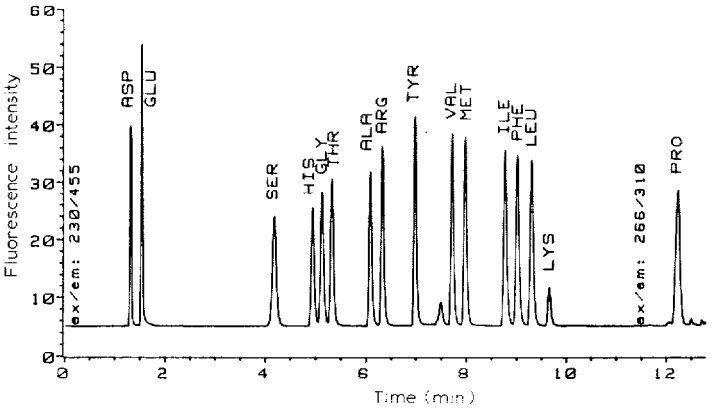

Fig. 2. Results from a 10-pmol amino acid standard. Column, Hypersil ODS (20 cm × 2.1 mm I.D., 5 μm particle size); mobile phase and gradient; see Table II; flow-rate, 0.45 ml/min; injection volume, 1 μl; oven temperature, 32°C; detection, fluorescence excitation and emission at 230 and 455 nm switched to 266 and 310 nm, respectively, after 11.5 min For derivatization see Table I.

TABLE IV

RELATIVE STANDARD DEVIATIONS FOR RETENTION TIMES AND PEAK AREAS

Values based on ten consecutive analyses. Data for 100 pmol obtained by diode array detection and data for 10 pmol obtained by fluorescence detection.

Amino acid	Retention time (min)	R.S.D. (%)	Area R.S.D. (%)	
			UV, 100 pmol	Fluorescence, 10 pmol
Asc	1.17	0.24	2.4	2.26
Glx	1.32	0.22	2.1	1.64
Ser	3.52	0.43	3.0	1.76
His	4.35	0.39	3.4	2.26
Gly	4.52	0.42	3.1	1.82
Thr	4.81	0.38	2.0	1.58
Ala	5.83	0.39	3.2	2.08
Arg	6.25	0.31	3.6	1.42
Tyr	7.21	0.31	1.6	2.48
Cys-Cys	7.91	0.31	3.8	—
Val	8.33	0.32	2.2	2.04
Met	8.62	0.30	1.8	1.86
Ile	9.55	0.24	2.0	1.42
Phe	9.72	0.24	1.8	1.18
Leu	10.04	0.21	2.2	1.68
Lys	10.42	0.14	3.4	2.68
Pro	12.46	0.11	4.8	3.12

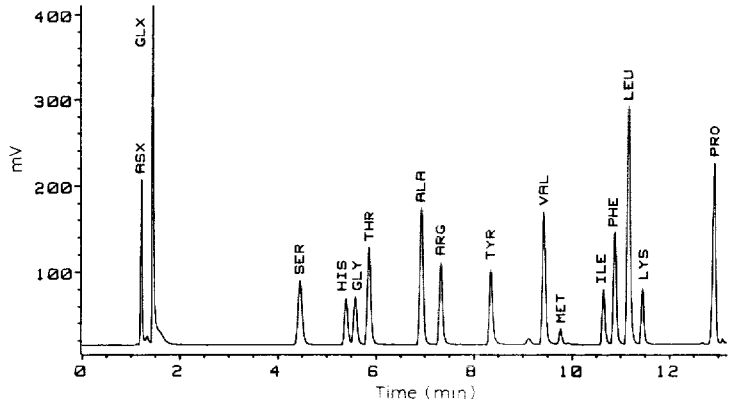

Fig. 3. Analysis of hydrolysed bovine serum albumin. Conditions as in Fig. 2.

TABLE V

COMPARISON OF HPLC DATA AND LITERATURE DATA FOR TWO HYDROLYSATES: BSA AND A LIGHT-CHAIN FRACTION FROM THE BENCE-JONES PROTEIN

Amino acid	Amino acid composition			
	Bovine serum albumin* (200 pmol)		Light-chain protein immunoglobulin** (100 pmol)	
	Literature	AminoQuant	Literature	AminoQuant
Asx	56	52.4	–	–
Glx	78	79.5	2	2.3
Ser	24	26.1	2	2.0
His	17	17.0	1	0.8
Gly	16	16.6	1	1.1
Thr	34	31.1	2	1.6
Ala	46	45.1	1	1.0**
Arg	23	23.0*	–	–
Tyr	19	18.2	–	–
Val	36	33.6	3	2.7
Met	4	3.6	–	–
Ile	14	12.4	–	–
Phe	27	26.6	–	–
Leu	61	60.9	1	1.2
Lys	59	55.8	1	1.2
Pro	28	28.3	1	1.0

*All amino acids relative to Arg. Literature reference: data bank of protein identification resource.
**All amino acids relative to Ala. Sample reference: light-chain protein of Bence-Jones immunoglobulin.

to determine the amino acid pattern in a variety of samples in different matrices e.g. for quality control of amino acids in intravenous solutions [12] (Fig. 4), for control of beer fermentation (Fig. 5), and for amino acid metabolism studies of pesticide-treated plants (Fig. 6).

Physiological fluids. The most common types of physiological fluid are serum,

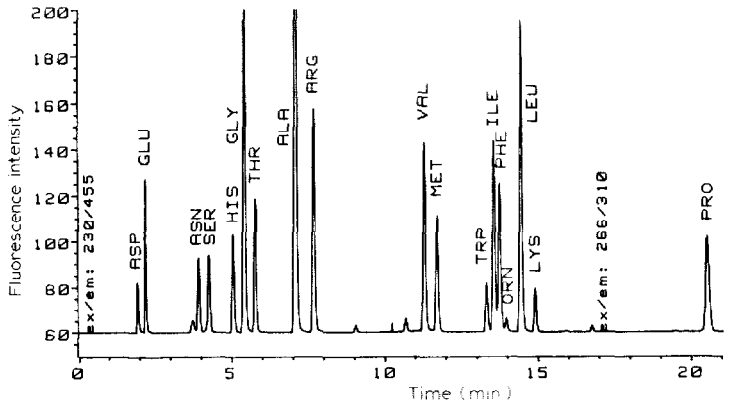

Fig. 4. Analysis of an intravenous solution diluted 1:200. Column, Hypersil ODS (20 cm×4.6 mm I.D., 5 μm); mobile phase, see Table II; flow-rate, 1.0 ml/min. Gradient: 3–40% B (20 min, linear), 40–100% B (4 min, linear), held at 100% B for 6 min, 100–3% B (1 min, linear), held at 3% B for 7 min prior to next injection. Oven temperature, 46 °C; detection, fluorescence excitation and emission at 230 and 455 nm switched to 266 and 310 nm, respectively, after 16 min. For derivatization see Table I.

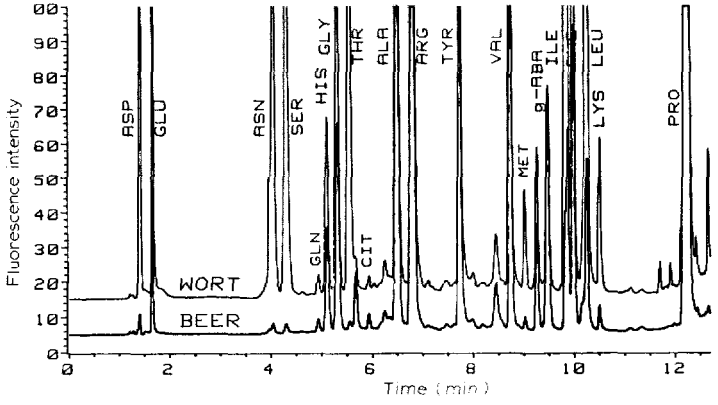

Fig. 5. Amino acid pattern in beer wort overlaid with the corresponding amino acid analysis in beer. Conditions as in Fig. 2. Valine is used to control the fermentation in wort. Lysine can be used as an indicator for the ingredients.

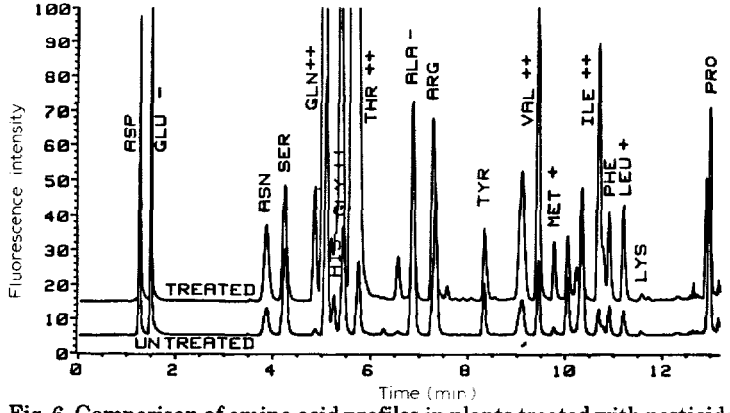

Fig. 6. Comparison of amino acid profiles in plants treated with pesticides and non-treated ones. The amino acid pattern is used for metabolism sudies. Conditions as in Fig. 2.

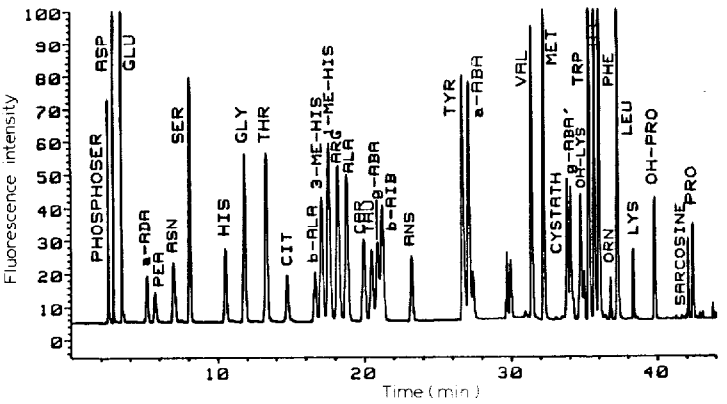

Fig. 7. Results from a 250-pmol physiological amino acid standard. Column, Hypersil ODS (20 cm×4.6 mm I.D., 5 μm); mobile phase and gradient, see Table III; flow-rate, 0.75 ml/min; injection volume, 1 μl; oven temperature, 42°C; detection, fluorescence excitation and emission at 230 and 455 nm switched to 266 and 310 nm, respectively, after 39.5 min. For derivatization see Table I.

plasma, urine, cerebrospinal fluids, amniotic fluids and faeces. The analysis of amino acids in these fluids is significant in the following-up of disorders of amino acid transport, metabolism or degradation. Early diagnosis of amino acidopathies (e.g. phenylketonuria) is very important because most can be treated in the early stages. Most of these metabolic or transport disorders are signalled by the accumulation of one or more amino acids in urine. Of course, interpretation is not always simple; additional information from plasma and sometimes cerebrospinal fluids is necessary.

Most of these amino acid analyses are still done by conventional ion-exchange chromatography [1] with an analysis time of up to 4 h and detection limits in the nanomole range. An alternative to the conventional system is precolumn derivatization and separation on reversed-phase chromatography, as described above. However, all existing systems for physiological amino acids [10] based on precolumn derivatization either separate only primary amino acids [2,5] or have limited selectivity and detection sensitivity [13].

Our method separates common physiological amino acids within 60 min (including the time for derivatization and reconditioning of the column) with detection in the low fmol/μl range. The whole procedure is automated, and injection of 1 μl of the physiological fluid is sufficient. Fig. 7 shows the analysis of a 250-pmol physiological standard and Table VI lists the R.S.D. for retention times and peak areas based on ten analyses.

In order to test the applicability of the procedure to various biological samples, serum, urine and brain fluids were analysed.

Plasma samples from different patients were analysed for physiological amino acid content. Prior to derivatization, heparinized blood samples were centrifuged and deproteinized by sulphosalicylic acid (SSA). A major drawback of this type of deproteinization is the interference of SSA with the amino acids phosphoserine, Asp and Glu. So other procedures, deproteinization with acetonitrile or ultrafiltration (Ultrasa® cell 10 Sartorius) with a cut-off filter for MW 5000, were

TABLE VI

RELATIVE STANDARD DEVIATIONS FOR RETENTION TIMES AND PEAK AREAS BASED ON TEN INJECTIONS FOR PHYSIOLOGICAL AMINO ACIDS

Amino acid standard	Concentration (pmol/μl)	R.S.D. (%)	
		Retention time	Area
Phosphoserine	50	0.28	1.86
Asp	100	0.29	0.96
Glu	100	0.28	5.13
α-Ada	24.8	0.44	3.33
Pea	50	0.43	5.87
Asn	50	0.40	1.63
Ser	100	0.35	1.29
His	100	0.45	3.15
Gly	100	0.43	2.33
Thr	100	0.39	1.86
Cit	24.8	0.42	1.90
β-Ala	50	0.39	3.57
3-Me-His	100	0.32	1.79
1-Me-His	100	0.36	1.79
Arg	100	0.36	1.56
Ala	100	0.35	1.80
Car	100	0.33	3.62
Tau	50	0.39	2.05
γ-Aba	100	0.35	3.44
β-Aib	100	0.33	2.55
Ans	50	0.26	1.75
Tyr	100	0.20	1.61
α-Aba	24.8	0.17	2.21
Val	100	0.12	1.14
EA	100	0.14	3.23
Met	100	0.12	1.08
Cystatione	100	0.10	6.4
OH-Lys	100	0.10	3.79
Trp	100	0.14	3.19
Ile	100	0.09	0.91
Phe	100	0.10	0.95
Orn	100	0.09	6.21
Leu	100	0.09	0.67
Lys	100	0.08	4.65
OH-Pro	100	0.05	3.44
Pro	100	0.02	4.82

investigated. Both methods can be applied for amino acid analysis in plasma using this technique. Fig. 8 shows an analysis of amino acids in which plasma was deproteinized with 2.5 parts of acetonitrile and 1 part of serum. The conditions for this analysis were slightly (flow and gradient) different from those of Fig. 9, and are applicable for plasma analysis if no carnosine, no γ-aminobutyric acid (γ-Aba) and no β-aminobutyric acid are present. To control the reliability of this technique, the results of the analysis of different plasma samples (analysed with

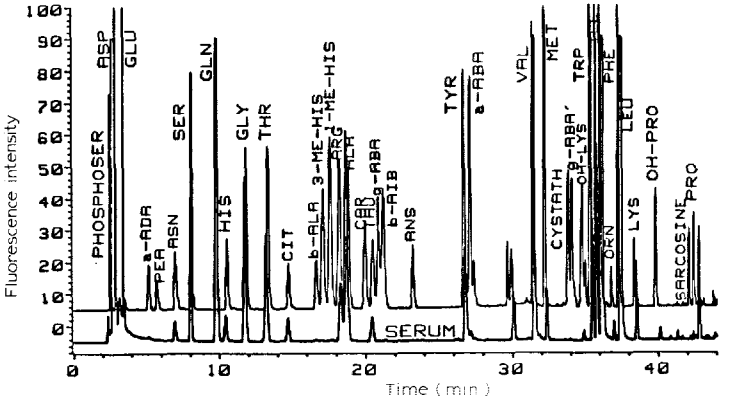

Fig. 8. Plasma deproteinized with SSA overlaid with 250-pmol standard. Conditions as in Fig. 7.

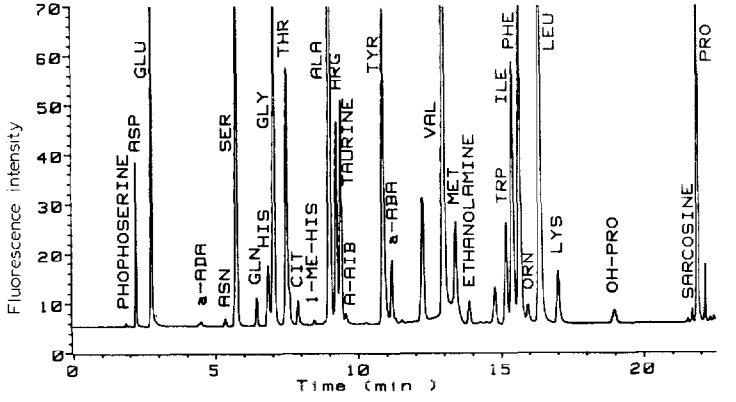

Fig. 9. Plasma deproteinized with acetonitrile. Column, Hypersil ODS (20 cm×4.6 mm I.D., 5 μm); mobile phase, see Table II; flow-rate, 1.0 ml/min. Gradient: 0–18% B (8 min, linear), 18–35% B (10 min, linear), 35–100% B (5 min, linear), held at 100% B for 6 min, 100–0% B (1 min, linear), held at 0% for 5 min prior to next injection. Oven temperature 43°C; detection, fluorescence excitation and emission at 230 and 455 nm switched to 266 and 310 nm, respectively, after 18 min. For derivatization see Table I.

the conditions for physiological fluids) have been compared with data from classical amino acid analysers. The quantitative result for one plasma compared with the "classical" ion-exchange result is shown Table VII. No data are given for Asp and Glu because SSA was used for deproteinization. The large difference in the amount of Trp found with this method, compared with ion-exchange analysis, was the same for all plasma samples tested (about twenty different samples).

Urine samples are rather difficult to analyse, mainly due to matrix interferences caused by compounds with an amino function, e.g. small peptides. These are derivatized with OPA like primary amino acids and can co-elute with some of the physiological amino acids. Samples should be prepared by the addition of acetonitrile or by ultrafiltration, as described for plasma. The quantitative result and the separation of a urine sample are shown in Fig. 10.

TABLE VII

FREE AMINO ACID CONCENTRATIONS IN PLASMA AS DETERMINED BY HPLC AND BY ION-EXCHANGE CHROMATOGRAPHY

Amino acid	HPLC (μM)	Ion exchange (μM)
Phosphoserine	–	–
Asp	–	6
Glu	–	35
α-Ada	–	–
Pea	–	–
Asn	110	102
Ser	127	125
Gln	660	666
His	54	67
Gly	176	186
Thr	130	139
Cit	18	18
β-Ala	–	–
3-Me-His	–	–
1-Me-His	–	–
Arg	64	70
Ala	275	296
Car	–	–
Tau	54	65
γ-Aba	–	–
β-Aib	–	–
Ans	–	–
Tyr	64	56
α-Aba	–	–
Val	208	205
EA	–	–
Met	21	19
Cystathione	–	–
OH-Lys	–	–
Trp	42	84
Ile	78	78
Phe	45	40
Orn	64	50
Leu	121	116
Lys	135	149
OH-Pro	13	11
Pro	180	217

Neurotransmitters are an important group of compounds investigated in brain fluids. One of these neurotransmitters is γ-Aba, a primary amino acid occurring in very low concentrations (nmol/l) in brain fluids. Fig. 11 shows a dialysed rat brain fluid overlaid with a standard containing some amino acids in the low pmol/μl range (1 and 5 pmol). For this analysis a fluorescence detector was used, and γ-Aba was detected at a concentration of 150 fmol/μl.

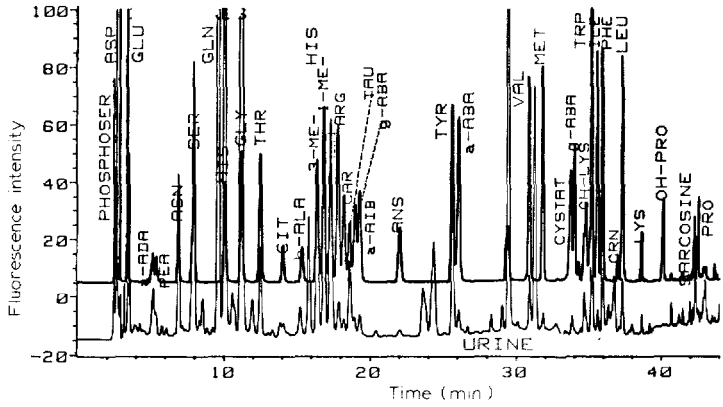

Fig. 10. Amino acids in urine overlaid with 250-pmol standard. Conditions as in Fig. 7.

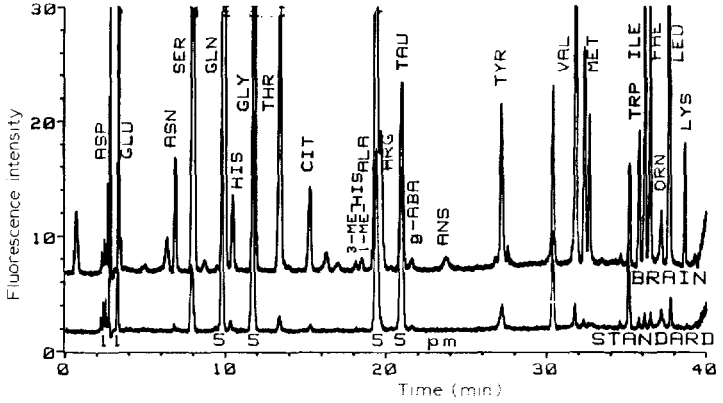

Fig. 11. Amino acids in rat brain fluid. The neurotransmitter γ-Aba was detected at 150 fmol/μl with fluorescence. The overlaid standard contains 1 pmol Asp and Glu and 5 pmol Gln, Gly, Ala and Arg. Conditions as in Fig. 7.

CONCLUSION

A rapid, easy and sensitive method for the analysis of primary and secondary amino acids has been developed. The analysis is based on the automated precolumn derivatization technique, by which the amino acids are derivatized in a two-step reaction using OPA for the primary amino acids and FMOC for the secondary amino acids. The derivatization is done within the injection cycle. Parameters for derivatization separation and data analysis are stored on a disc and handled as a single method. The detection limit is 2–5 pmol/μl with a diode array and 20–50 fmol/μl with fluorescence detection. The R.S.D. is less than 0.5% for retention times and 1–5% for peak areas. The method can be used for different sample types, including hydrolysates, pharmaceuticals, food samples, plant extracts and physiological fluids. Most of the samples can be injected directly or extracted using a single procedure (physiological fluids) without interference from matrix

compounds. The analysis time ranges between 20 and 60 min, depending on the chromatographic parameters.

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