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Short Communication

Rapid routine determination of amino acids in plasma by high-performance liquid chromatography with a 2–3 μm Spherisorb ODS II column

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ABSTRACT

A rapid fully automated method for the determination of amino acids is described based on high-performance liquid chromatography and pre-column *o*-phthaldialdehyde derivatization. Using a 150 mm × 4.6 mm I.D. HPLC column filled with a recently introduced 2–3 μm Spherisorb ODS II packing material, 30 physiological amino acids could be determined within 28 min (injection to injection), while 95 samples could be processed unattended within 45 h. For most amino acids, the coefficient of variation (C.V.) for the peak areas measured was below 3%, both in aqueous standards and in plasma. Providing a pre-column change every 200–300 runs, the separation remained unaltered for about 1500–2000 runs on a single column.

INTRODUCTION

Numerous methods have been described for the determination of amino acids using *o*-phthaldialdehyde (OPA) pre-column derivatization and high-performance liquid chromatography (HPLC). However, only a few describe automated systems which can run unattended on a routine basis [1–8]. Although protein hydrolysates could be analyzed in these systems within half an hour [5,8–10], the analysis of plasma samples still required about one hour [4–8].

Recently, a new batch of Spherisorb ODS II was introduced with a particle size between 2 and 3 μm, exhibiting 30–40% more theoretical plates, compared to the standard 3-μm material. Using this highly efficient packing material, a shorter column was prepared, enabling the development of a 28-min (injection to injection) plasma amino acid analysis without loss in resolution.

EXPERIMENTAL

Equipment

The separation column was a custom made Bischoff Spherisorb ODS II column (2–3 μm particles) 150 mm × 4.6 mm (I.D.), equipped with a 10 mm × 4.0 mm (I.D.) pre-column, filled with the same packing material (Applikon, Schiedam, the Netherlands). The analytical column was selected by the manufacturer (Bischoff) using the

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criteria that, in the Bischoff test system, employing acetonitrile–water 85:15 (v/v) and a flow-rate of 1.1 ml/min, a minimum of 150 000 theoretical plates/m should be measured for a toluene containing Bischoff test mixture, while the back-pressure may not exceed 100 bar. In our system, using a column temperature set at 25°C, this resulted in a back-pressure of 22 MPa at the start and 25 MPa at the end of the gradient.

Our HPLC system consisted of a Model 2248 pump, a Model 2252 controller, a Model 2256 solvent conditioner, a Model 2248-201 low pressure binary mixing panel and a model 2155 column oven, all from Pharmacia (Woerden, the Netherlands). For the automated pre-column derivatization a WISP 715 sample processor (Millipore/ Waters, Etten-Leur, the Netherlands) was used, equipped with a cooled sample storage compartment and a 96 position sample tray. Fluorescence was monitored with a Jasco Model 820FP fluorescence detector (B&L systems, Zoetermeer, the Netherlands) equipped with a xenon lamp and a 12 μl flow-cell. Measurements were made at an excitation wavelength of 335 nm and an emission wavelength of 440 nm. Data were collected on-line by a model 900 interface (Perkin-Elmer/Nelson, Gouda, the Netherlands) and processed by a Tandon Model MCS 486/50 personal computer (Amsterdam, the Netherlands) running Model 2700 (Turbochrom: version 3.2) software (Perkin-Elmer/Nelson, Gouda, the Netherlands) under Microsoft Windows, version 3.1.

Reagents and solvents

All solutions were prepared with ultra-pure water, generated by a Super-Q system (Millipore/ Waters). All chemicals used were of analytical grade (Pierce, Oud Beijerland, the Netherlands), solvents of chromatographic grade (Janssen Chimica, Amsterdam, The Netherlands).

The derivatization reagent was prepared by dissolving 15 mg of OPA in 0.25 ml of methanol, adding 2.25 ml of potassium borate buffer (1.0 mol/l, pH 10.4) and 15 μl of 3-mercaptopropionic acid (3-MPA). This reagent was placed in a 1.2-ml crimp-cap WISP vial with a self-sealing silicone rubber, teflon-coated stopper (Alltech, Breda, the Netherlands). Solvent A was a 12.5 mmol/l sodium phosphate buffer, pH 7.0, containing 7 ml of tetrahydrofuran (THF) per liter. Solvent B consisted of 12.5 mmol/l phosphate buffer, acetonitrile and THF, 57:40:3 (v/v).

Amino acid standards were prepared by dissolving pure amino acids in water to a final concentration of 250 μmol/l each. These were then calibrated against a physiological standard (Sigma/Brunschwig, Amsterdam, the Netherlands), divided into 1-ml portions and stored at −80°C.

Sample preparation

Heparinized blood samples were obtained from unrestrained, conscious female pigs [11] and collected on ice followed by immediate centrifugation at 8500 *g* in a Eppendorf model 5413 centrifuge for 10 min at 4°C. Next, plasma was deproteinized with 5-sulfosalicylic acid (SSA), 4 mg/100 μl plasma, frozen immediately in liquid nitrogen and stored at −80°C. Before analysis, samples were thawed at 4°C, vortex-mixed vigorously and centrifuged as described before. Ten microliters of the clear supernatant were, together with 10 μl of a 1000-μM norvaline solution as internal standard, diluted with 980 μl ice-cold water in a 1.2-ml WISP vial.

Automated pre-column derivatization

Employing the WISP "auto-standards" method, with the OPA-reagent placed at position 1 of the tray, the automated pre-column derivatiza-

TABLE I

GRADIENT CONDITIONS

Time (min)	Flow-rate (ml/min)	B (%)	Time (min)	Flow-rate (ml/min)	B (%)
0	0	0	15.0	1.500	30
2.0	0.020	0	18.0	1.500	36
2.5	0.050	0	20.5	1.500	48
3.5	0.100	0	22.0	1.500	100
3.9	0.750	0	23.0	1.500	100
4.0	1.500	0	24.0	1.500	0
6.0	1.500	11	27.5	0.750	0
8.0	1.500	15	28.0	0	0

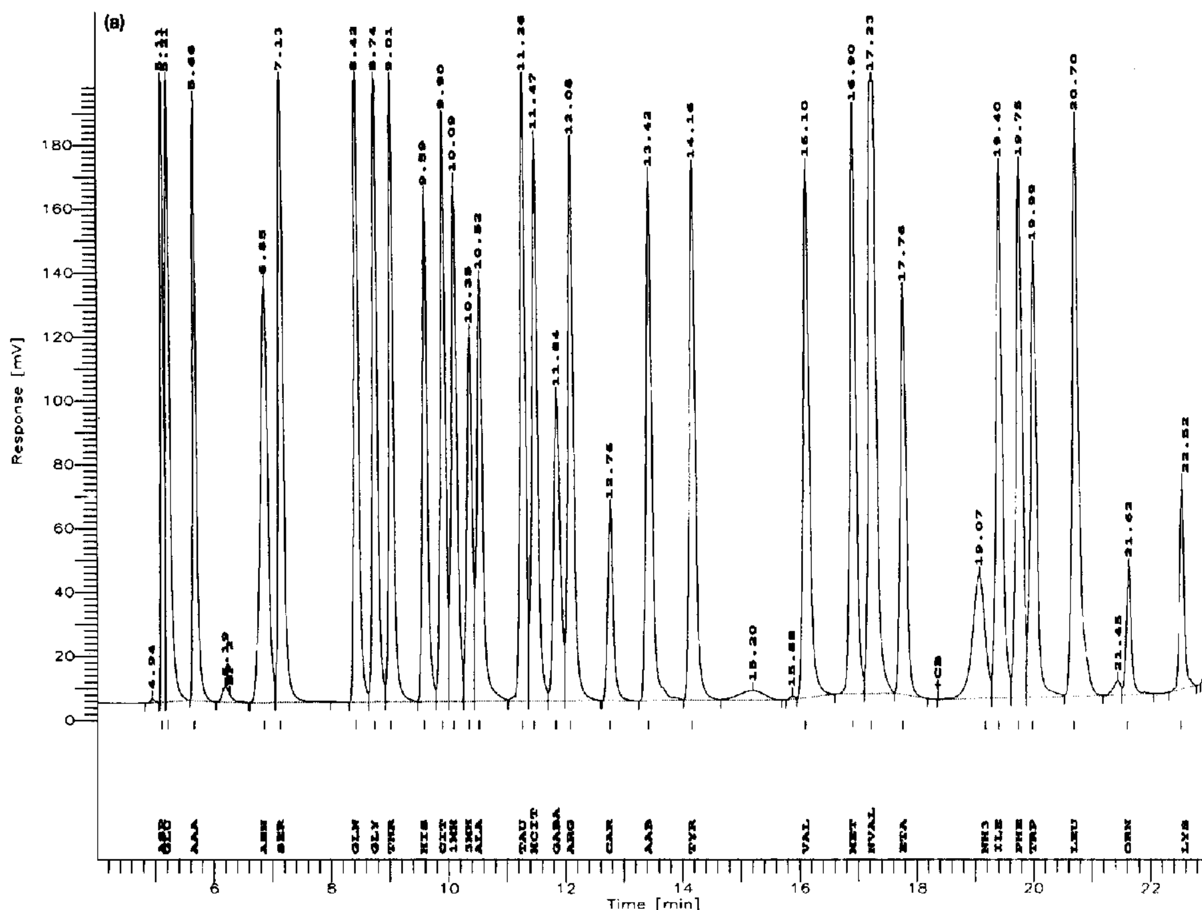

Fig. 1. (a) Separation of 30 physiological amino acids. This standard contains *ca.* 35 pmol of each amino acid.

tion was executed during the first 4.0 min of the run under stop-flow conditions. First, 5 μl of the OPA reagent was injected into the sample loop. One min later, 5 μl of a sample was injected into the loop, at which point the flow-rate was slowly increased to 1.5 ml/min, thus allowing sample and reagent to mix and react for 2 min before the gradient elution started. Gradient conditions are described in Table I.

RESULTS

In our system [4], we replaced the analytical column by the new high-efficiency column. Using a steeper gradient (Table I) and by increasing the flow-rate from 1.2 ml/min to 1.5 ml/min, we were able to separate all 30 amino acids present in our aqueous standard (Fig. 1a) within 28 min. In plasma, aspartic acid coëluted with the sulfosalicylic acid used for the deproteinization (Fig. 1b).

The samples were placed in a WISP's 96 position tray and cooled to 7°C in the sample compartment of the system. This system processed the tray unattended within 45 h, during which 84 samples and 11 standards were analyzed.

In order to determine the coefficient of variation (C.V.), 40 vials containing the aqueous standard and 20 vials containing a pooled plasma sample were randomly placed in a sample tray. No change in detector response or resolution was found between the first and last chromatograms (not shown). The C.V. for the peak areas measured (and thus the concentration) was below 3% for most amino acids both for standard and plasma, while the C.V. for most retention times was below 0.5%. The present method was compared

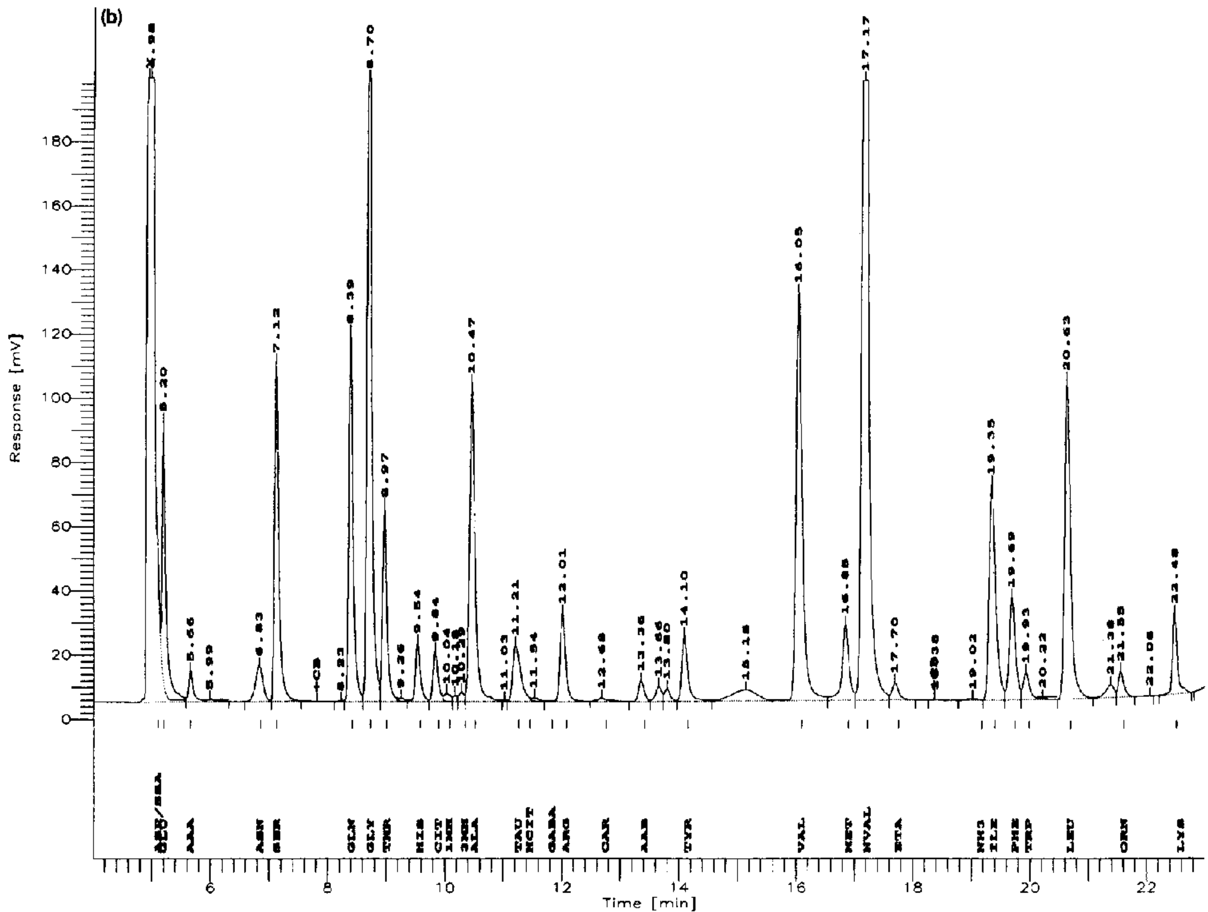

Fig. 1. (b) Free amino acids in arterial plasma obtained from a conscious unrestrained pig [11].

with our previously described technique [4], by determining the amino acid concentrations in arterial blood of normal unrestrained pigs [11]. The data were in perfect agreement with each other (Table II).

Using a single column, the separation was unaltered during 1500–2000 runs, provided that the pre-column was changed after every 200–300 runs.

DISCUSSION

Previously, we used a 250 mm × 4.6 mm I.D. analytical column packed with Spherisorb ODS II 3 μm to separate 30 amino acids within 55 min [4]. The introduction of a newly developed highly efficient Spherisorb ODS II packing material, with a particle size between 2 and 3 μm enabled the production of columns with 30–40% more theoretical plates per meter. Using these columns, we expected an equal reduction in column length and thus analysis time to be possible. In order to obtain an optimum resolution we used the method described by Eslami *et al.* [10] to develop the gradient for this new column and achieved an excellent separation within 35 min. The lower back-pressure of the column enabled us to increase the flow-rate from 1.2 ml/min to 1.5 ml/min resulting in a further reduction of the analysis time to 28 min.

For routine applications the separation obtained must also be reproducible. Because it has been described that a rapid decrease in column efficiency may be found when columns with 3 μm

TABLE II

REPRODUCIBILITY AND NORMAL VALUES

Determination of the coefficient of variation (C.V.) for both aqueous standards and plasma. The normal values are arterial amino acid concentrations of conscious unrestrained pigs.

Amino acid	Aqueous standard (n = 40)						Plasma (n = 25)			Normal values (μmol/l)			
	Retention times (min)			Peak area (mV s)			Conc (μmol/l)			This study (n = 15)		Ref. [11] (n = 9)	
	Mean	S.D.	C.V. (%)	Mean	S.D.	C.V. (%)	Mean	S.D.	C.V. (%)	Mean	S.E.M.[a]	Mean	S.E.M.
Aspartic acid	5.06	0.01	0.25	631.3	21.2	3.4							
Glutamic acid	5.19	0.03	0.56	567.7	17.4	3.1	248	13.1	5.3	238	9	207	22
α-Aminoadipic acid	5.73	0.03	0.44	556.8	13.1	2.4							
Asparagine	6.89	0.03	0.39	754.8	28.6	3.8	38	1.3	3.5	38	2	35	2
Serine	7.07	0.04	0.61	677.6	24.9	3.7	150	6.2	4.1	144	4	126	7
Glutamine	8.43	0.04	0.51	753.3	17.1	2.3	351	19.4	5.5	365	16	335	18
Glycine	8.72	0.03	0.33	634.0	17.9	2.8	804	15.5	1.9	685	35	650	57
Threonine	8.97	0.02	0.21	673.9	20.3	3.0	98	2.0	2.0	97	5		
Histidine	9.56	0.03	0.29	595.0	12.1	2.0	59	1.9	3.2	53	3		
Citrulline	9.86	0.03	0.28	708.2	15.1	2.1	62	2.2	3.7	61	4	67	6
1-Methylhistidine	10.04	0.03	0.29	624.5	16.2	2.6							
3-Methylhistidine	10.29	0.03	0.29	489.7	16.6	3.4							
Alanine	10.46	0.03	0.28	539.7	15.2	2.8	346	6.5	1.9	276	18	239	19
Homocitrulline	11.28	0.03	0.29	745.2	19.8	2.7							
Taurine	11.45	0.03	0.30	723.2	16.4	2.3	52	2.4	4.7	56	7	55	4
GABA	11.84	0.04	0.31	487.2	12.4	2.6							
Arginine	12.15	0.03	0.27	748.2	15.1	2.0	87	4.3	4.9	81	5	85	6
Carnosine	12.79	0.04	0.28	243.1	6.3	2.6							
α-Aminobutyric acid	13.42	0.04	0.32	707.6	16.3	2.3	19	1.3	6.8	18	2		
Tyrosine	14.24	0.04	0.26	720.1	11.8	1.6	36	2.0	5.6	38	2	33	3
Valine	16.17	0.04	0.26	767.1	14.4	1.9	256	6.1	2.4	241	7	233	9
Methionine	17.03	0.04	0.23	806.5	13.3	1.7	59	5.2	8.7	51	2		
Norvaline (i.s.)	17.34	0.04	0.23	1499.0	25.9	1.7	1001	17.1	1.7	993	4		
Ethanolamine	17.89	0.04	0.22	569.9	21.6	3.8							
Isoleucine	19.59	0.04	0.22	799.4	13.9	1.7	127	4.5	3.5	110	4	115	5
Phenylalanine	19.97	0.04	0.21	780.6	13.4	1.7	65	2.7	4.1	60	2	52	3
Tryptophan	20.26	0.04	0.20	699.6	12.9	1.9	16	1.3	8.0	16	1		
Leucine	20.94	0.04	0.20	973.8	32.3	3.3	129	4.0	3.1	120	3	109	7
Ornithine	21.89	0.03	0.12	192.6	5.9	3.1	65	4.4	6.7	45	6	55	5
Lysine	22.73	0.02	0.10	251.5	7.4	2.9	118	6.2	5.3	84	12	87	11

[a] S.E.M. = standard error of the mean.

Spherisorb ODS II packing material [8] are used, the constancy of the resolution was tested. Indeed, a slight increase in back-pressure and/or the occurrence of "ghost peaks" was found when analyzing plasma samples. However, this problem could be avoided by changing the pre-column regularly.

In conclusion, we achieved an excellent resolution of all major plasma amino acids within 28 min. Combined, with the automated pre-column derivatization, the method has a good reproducibility of both retention times as well as peak areas, making it suitable for routine plasma amino acid analysis.

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