Journal of Chromatography, 529 (1990) 258–262
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5295

Letter to the Editor

Time-saving method for the reversed-phase high-performance liquid chromatography of phenylthiocarbamylamino acid derivatives of free amino acids in plasma

Sir,

Amino acid analysis by reversed-phase high-performance liquid chromatography (HPLC) after precolumn derivatization with Edman's reagent, phenyl isothiocyanate (PITC), is a well established method [1–5]. The chromatographic separation of the amino acid derivatives is a relatively fast procedure, which takes place in a matter of minutes. A time-consuming part of the complete procedure, however, is the pretreatment of plasma and the derivatization of the amino acids.

Plasma has to be deproteinized before derivatization of the free amino acids. Sulphosalicylic acid is commonly used for this purpose. Any deproteinizing substance may, however, mask early peaks in the chromatogram. This paper describes how ultrafiltration of plasma can replace deproteinization with sulphosalicylic acid as a fast and convenient procedure.

The derivatization with PITC is usually conducted as a three-step procedure [2]: freeze-drying of the amino acids after deproteinization, adding of a coupling buffer, freeze-drying, adding of the PITC derivatization solution and freeze-drying for a third time. This paper describes a single-step derivatization procedure.

EXPERIMENTAL

Precolumn preparation of samples and HPLC

The internal standard, 131.2 mg of norleucine, was dissolved in 0.1 *M* hydrochloric acid and the volume made up to 1000 ml. The solution was diluted 1:10 with 0.1 *M* hydrochloric acid before use. Venous blood was drawn into

0378-4347/90/$03.50

heparin-coated vacuum tubes and centrifuged for 15 min at 2500 *g*. One part of plasma was mixed with one part of diluted internal standard. A Millipore ultrafilter (Ultrafree®-MC 10.000 NMWL filter units, 400 µl) was filled and centrifuged at a maximum of 5000 *g* for 10 min. The yield of ultrafiltrate was ca. 100 µl.

The derivatization reagent was made fresh daily and consisted of ethanol–triethylamine–PITC (40:1:1). An 80-µl volume of derivatization reagent was added to 40 µl of ultrafiltrate and mixed on a whirlmixer for several seconds. (Some samples showed a weak opaqueness after mixing, but this did not seem to interfere with the chromatographic procedure.) The samples were allowed to stand at room temperature for 20 min for the derivatization reaction to take place before freeze-drying. The dry, derivatized amino acids could be stored at −70°C for weeks with no significant deterioration.

The HPLC apparatus included two LDC/Milton Roy Model III Constametric pumps, a Rheodyne sample injector with a 10-µl loop, a Supelcosil® LC-18, 250 mm×4.6 mm I.D. column, a Bio-Rad column heater, a variable-wavelength LDC/Milton Roy Spectra Monitor® UV detector at 254 nm and an LDC/Milton Roy Cl-10 integrator with printer. The column temperature was kept at 45°C.

Eluent A was one part of buffer solution (3.28 g of water-free sodium acetate dissolved in water and made up to 500 ml to which was added 0.4 ml of a 1:10 dilution of phosphoric acid) and one part of water. Eluent B was buffer–acetonitrile–methanol (200:150:50). The gradient was from 10% B to 50% B in 20 min, kept at 50% for 5 min, then from 50% B to 100% in 2 min, kept at 100% for 8 min (washing step), then decreased to 10% in 2 min, followed by a delay of 8 min. The separation of the amino acid derivatives takes ca. 29 min, whereas the total cycle takes 45 min.

RESULTS AND DISCUSSION

The chromatogram of free amino acids in plasma after deproteinization with sulphosalicylic acid is shown in Fig. 1. Fig. 2 shows the chromatogram from the same plasma after dilution with norleucine standard and ultrafiltration. Table I shows the values for some of the amino acids expressed in µmol/l calculated from both chromatograms on the basis of norleucine as internal standard.

Table II shows the results expressed in µmol/l from day-to-day analyses of free amino acids in the same plasma over a period of two months ($n=20$). There is a good reproducibility except for Cys, which is probably due to the well known instability of this amino acid.

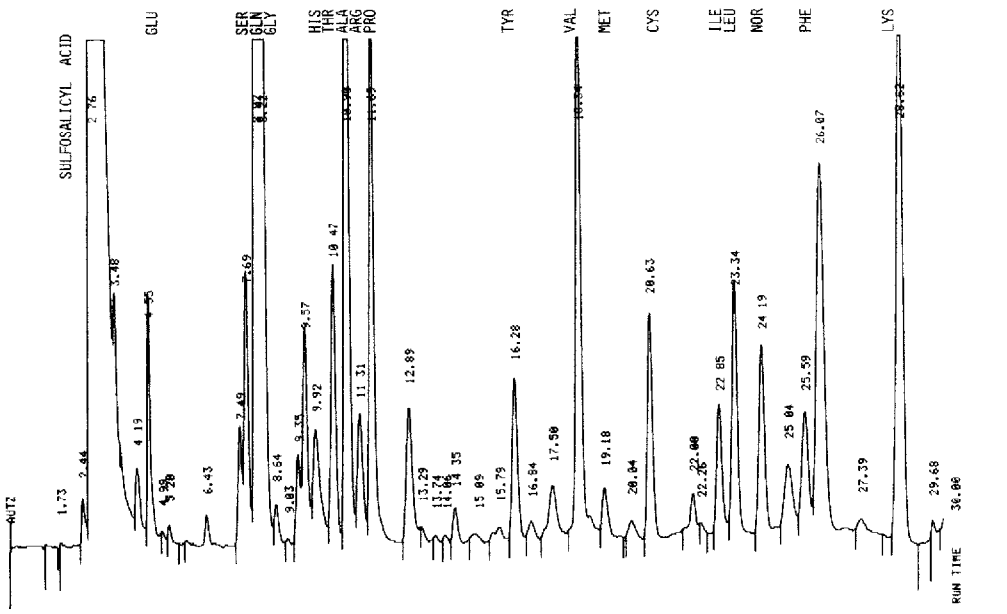

Fig. 1. HPLC of free amino acids in plasma deproteinized with sulphosalicylic acid. Amino acids were derivatized with PITC in a single step. Norleucine (200 pmol) was added as internal standard.

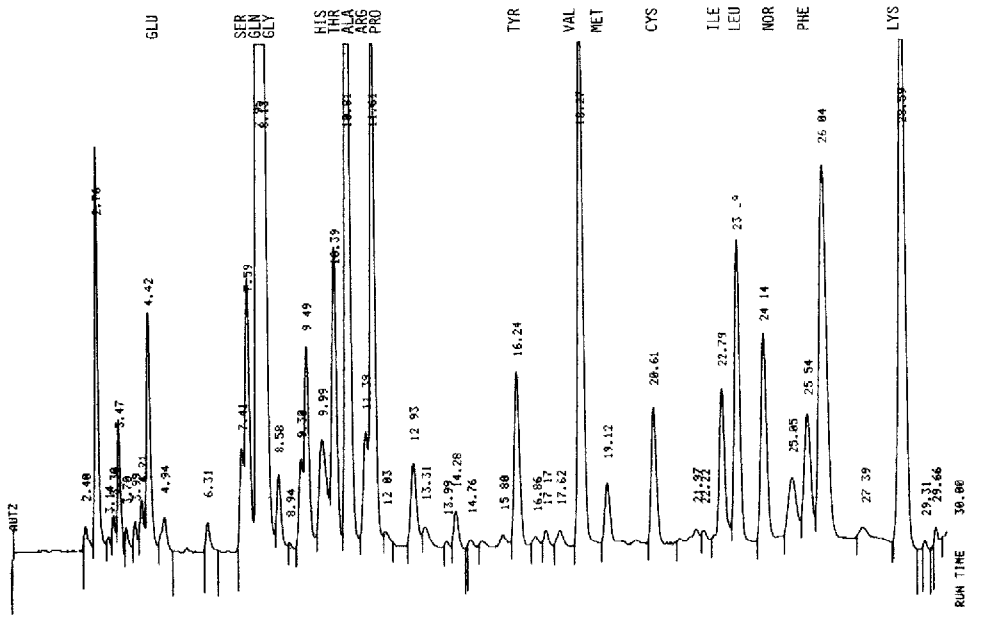

Fig. 2. HPLC of free amino acids in plasma deproteinized by ultrafiltration. Amino acids were derivatized with PITC in a single step. Norleucine (200 pmol) was added as internal standard.

TABLE I

RESULTS OF ANALYSES OF FOURTEEN FREE AMINO ACIDS IN THE SAME PLASMA, DEPROTEINIZED WITH SULPHOSALICYLIC ACID (SSA) AND ULTRAFILTERED (U), RESPECTIVELY. SINGLE-STEP DERIVATIZATION

Amino acid	Concentration (μmol/l)		Difference in per cent of SSA
	SSA	U	
Glu	88	87	−1.1
Ser	115	117	+1 7
Gln	819	754	−7.9
Gly	310	293	−5.5
Ala	363	378	+4.1
Arg	64	68	+6.3
Pro	197	192	−2.5
Tyr	79	77	−2.5
Val	243	240	−1.2
Met	22	25	+13.6
Ile	64	65	+1.6
Leu	122	134	−9.8
Phe	56	62	+10.7
Lys	189	190	+0.5

TABLE II

RESULTS OF DAY-TO-DAY ANALYSES OF ELEVEN FREE AMINO ACIDS IN THE SAME PLASMA OVER A PERIOD OF TWO MONTHS

Amino acids were calculated on the basis of peak heights with norleucine as internal standards. $n = 20$.

Amino acid	Concentration (mean ± S.D.) (μmol/l)	Coefficient of variation (%)
Ala	415 ± 31.0	7.5
Arg	86 ± 5.7	6.6
Pro	192 ± 10.8	5.6
Tyr	58 ± 2.4	4.1
Val	222 ± 9.7	4.4
Met	27 ± 1.6	6.0
Cys	77 ± 15.8	20.4
Ile	68 ± 1.9	2.8
Leu	136 ± 4.4	3.3
Phe	57 ± 3.8	6.6
Lys	183 ± 7.4	4.0

The results indicate that ultrafiltration of plasma may replace chemical deproteinization in the HPLC analysis of free amino acids, and that the precolumn derivatization of the amino acids can be performed in a single step.

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(First received December 29th, 1989; revised manuscript received March 9th, 1990)