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RAPID AND SENSITIVE DETERMINATION OF Dns-AMINO ACIDS IN PLASMA USING HIGH-SPEED OCTADECYL LIQUID CHROMATOGRAPHIC COLUMNS

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SUMMARY

Rapid and sensitive methods are described for the separation of Dns-derivatives of twenty common amino acids using high-speed liquid chromatographic columns with 5-μm or 3-μm C_{18} packings. With the 5-μm column, the separation was completed in 37 min and with the 3-μm column in less than 20 min. The fluorimetric detection sensitivity of individual amino acids varied between 5 and 0.5 pmol. Plasma-free amino acids in mink were determined with the 5-μm column with a mean recovery of 101% for different amino acids. The mean coefficient of variation for ten subsequently analysed plasma samples was 4.8%.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (HPLC) has recently been used for the determination of amino acids. The most widely used derivatization reagents for amino acids are *o*-phthalaldehyde (OPA) and 5-diaminonaphthalenesulphonyl acid (Dns) because of the high detection sensitivity of the corresponding derivatives [1–4]. The advantages of Dns compared with OPA are that it also reacts with the secondary amino acids proline and hydroxyproline, and the Dns derivatives are more stable, which makes automatic analysis of Dns-amino acids possible with simpler equipment.

Recent progress in HPLC instrumentation has brought about shorter analysis times of amino acids owing to possibility of achieving more versatile gradients. The development of shorter columns and packing materials with smaller particle sizes has also led to faster analyses. Recently, methods have been described for the determination of the most common OPA-amino acids in less than 13 min [5,6].

This paper describes a rapid and sensitive method for the separation of Dns

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derivatives of twenty common amino acids on short columns packed with 5-μm or 3-μm C_{18} material and its application to the determination of plasma-free amino acids.

EXPERIMENTAL

Reagents and solvents

Free and Dns-derivatized amino acids were purchased from Sigma (St. Louis, MO, U.S.A.). Analytical-grade Dns chloride (Dns-Cl) and other reagents were purchased from Merck (Darmstadt, F.R.G.). A 10 m*M* stock solution of each amino acid was prepared in 40 m*M* lithium carbonate buffer (pH 9.5 adjusted with hydrochloric acid). The stock solution of Tyr was 2 m*M* because of the poor solubility of this amino acid in aqueous solutions.

Equipment

A Perkin-Elmer (Norwalk, CT, U.S.A.) Series 4 gradient liquid chromatograph was used. The pump unit was connected to Perkin-Elmer MPF 2A fluorescence spectrophotometer equipped with a micro flow-cell accessory with a cell volume of 20 μl. Chromatograms were plotted and peak data handled using a Perkin-Elmer Sigma 15 chromatography data station. Manual injections were made with a Rheodyne 7125-075 valve injector, and for automatic sample handling the chromatograph was equipped with Perkin-Elmer ISS-100 sampling system.

Chromatographic conditions

The columns used for the separation of Dns-amino acids were a Perkin-Elmer HS-5 C_{18} (125×4.6 mm I.D.) high-speed column, packed with 5-μm material and a Perkin-Elmer C_{18} (30×4.6 mm I.D.) column, filled with 3-μm particles. The wavelength settings for the fluorimetric detection were 340 nm for excitation and 540 nm for emission.

The mobile phase gradient programmes and solvent compositions used with both columns are listed in Table I. Before use, all solvents were filtered through Millipore HATF-04700 membrane filters with a pore diameter of 0.45 μm. The solvents were degassed with helium and pressurized in the pump unit with the same gas.

Dns derivatization

The dansylation procedure used was similar to that described by Tapuhi et al. [1]. To 2.0 ml of the amino acid mixture containing 25 μ*M* of Asp, Glu, Asn, Cys, Gln, Ser, His, Gly, Thr, Ala, Arg, Pro, Val, Met, Trp, Leu, Ile, Phe, and Lys and 5 μ*M* Tyr, was added 1.0 ml of Dns-Cl, dissolved in acetonitrile (1.5 mg/ml, 5.56 m*M*). Dansylation was performed in the dark at room temperature. After 45 min, the reaction was terminated by adding 0.2 ml of ethanolamine (2% solution in 40 m*M* lithium carbonate buffer, pH 9.5). Before injection of 10 μl of the sample on the column, the dansylated sample was filtered through a disposable Millipore Millex-SR syringe filter.

TABLE I

MOBILE PHASE GRADIENT PROGRAMMES FOR SEPARATION OF Dns-AMINO ACIDS

Buffers: A, 50 m*M* sodium acetate (pH 6.1); B, 50 m*M* sodium acetate (pH 5.0). Columns: I, 125×4.6 mm I.D., 5-μm C_{18}; II, 30×4.6 mm I.D., 3-μm C_{18}. Gradient shapes: 1, linear; 0.2, convex; 2.0, concave.

Column	Time (min)	Methanol (%)	Buffer A (%)	Buffer B (%)	Gradient shape
I	0	32	68	-	-
	5	34.5	65.5	-	1
	6	35	36	29	1
	15	41	32	27	1
	16	42	58	-	1
	25	50	50	-	1
	28	55	45	-	1
	33	80	20	-	1
	38	80	20	-	1
II	0	27	73	-	-
	5	40	60	-	0.2
	15	80	20	-	2.0
	20	80	20	-	-

Plasma samples

Plasma samples (0.2 ml) were deproteinized by addition of 1.0 ml of methanol [7]. After centrifugation (3000 *g* for 10 min), the supernatant was removed, evaporated to dryness and dissolved in 1.0 ml of 40 m*M* lithium carbonate buffer (pH 9.5); 0.5 ml of Dns-Cl was then added. After 45 min in the dark, 0.1 ml of ethanolamine was added, the samples were filtered and 10 μl were injected on to the column.

RESULTS AND DISCUSSION

Phosphate and acetate buffers were tested in the pH range 4–7 as the aqueous component of the mobile phase. The type of buffer had only a slight effect on the elution pattern of the Dns-amino acids. Because of the risk of precipitation of phosphate buffer at high methanol concentrations [8], acetate buffer was chosen. High pH values and high temperatures have been reported to increase the solubility of C_{18} bonded columns [9]; thus ambient temperature and pH below 7 were selected.

The excitation and emission wavelengths for pure dansylated samples in 40 m*M* lithium carbonate buffer (pH 9.5) were 395 and 525 nm, respectively. However, when the sample is mixed with the mobile phase during the chromatography, the wavelength maxima are shifted considerably. For the standards in Fig. 1, the maximal excitation and emission wavelengths for combined 0–20 min eluate (in acetate buffer–methanol; Table I) were 325 and 500 nm, and for 20–40 min eluate 330 and 530 nm, respectively. Thus, the detection sensitivity can be increased by careful control of the wavelength.

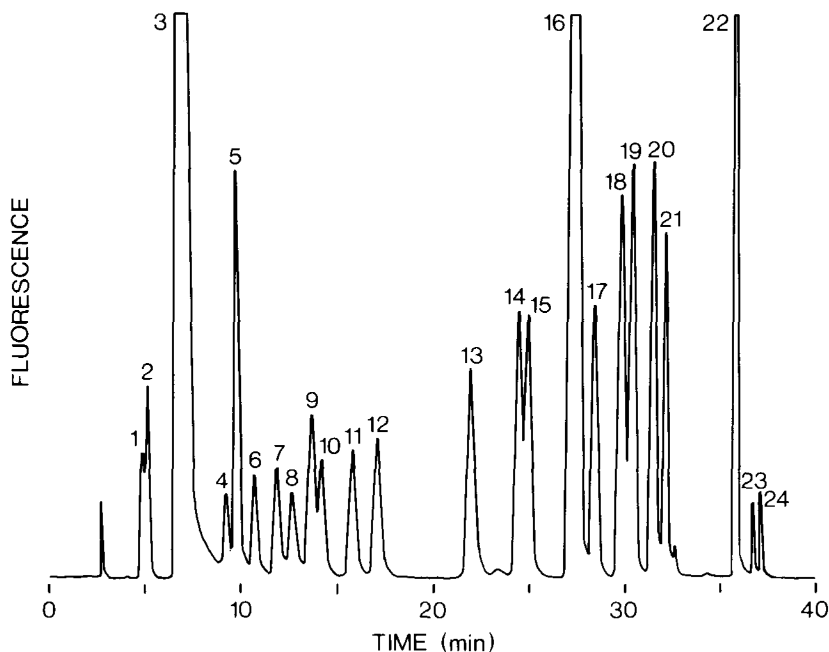

Fig. 1. Separation of a synthetic mixture of Dns-amino acids on a 5-μm C_{18} column according to the conditions described in the text. Peaks: 1 = Dns-Asp; 2 = Dns-Glu; 3 = Dns-OH; 4 = Dns-Asn; 5 = Dns-Cys; 6 = Dns-Gln; 7 = Dns-Ser; 8 = Dns-His; 9 = Dns-Gly; 10 = Dns-Thr; 11 = Dns-Ala; 12 = Dns-Arg; 13 = Dns-Pro; 14 = Dns-Val; 15 = Dns-Met; 16 = Dns-NH_2; 17 = Dns-Trp; 18 = Dns-Leu; 19 = Dns-Ile; 20 = Dns-Phe; 21 = di-Dns-Cys; 22 = di-Dns-Lys; 23 = di-Dns-His; 24 = di-Dns-Tyr.

The time required for dansylation of the different amino acids at room temperature was 45 min, as observed previously under similar conditions [1]. The dependence of peak area on the amount of amino acids in the reaction mixture was studied at various amino acid concentrations using Dns-Cl concentrations of 1.07 and 1.74 m*M* in the reaction mixture. At the higher Dns-Cl concentration, the relationship between the amount of Dns-amino acid and the peak area was linear up to higher amino acid concentrations. Thus, the absolute concentration of Dns-Cl, and not only the Dns-Cl/amino acid concentration ratio in the reaction mixture, is an important factor for successful labeling, as pointed out by Gray [10]. The dansylation procedure with 1.74 m*M* Dns-Cl gave a linear area response up to a total amino acid concentration of 0.6 m*M* in an equimolar amino acid mixture, except for Lys whose area response declined slowly above a total amino acid concentration of 0.15 m*M*. These results are in agreement with previous findings with respect to the optimal concentration ratio of Dns-Cl to amino acid. In the present study, the concentration ratios of Dns-Cl to amino acid for a total amino acid content of 0.6 and 0.15 m*M* were 2.9:1 to 11.7:1, and the previously recommended concentration ratios of Dns-Cl to amino acid were between 5:1 and 20:1 [1,11,12].

Fig. 1 illustrates the separation of a synthetic mixture of twenty common protein amino acids on a 5-μm column. Dansylation was carried out with a total

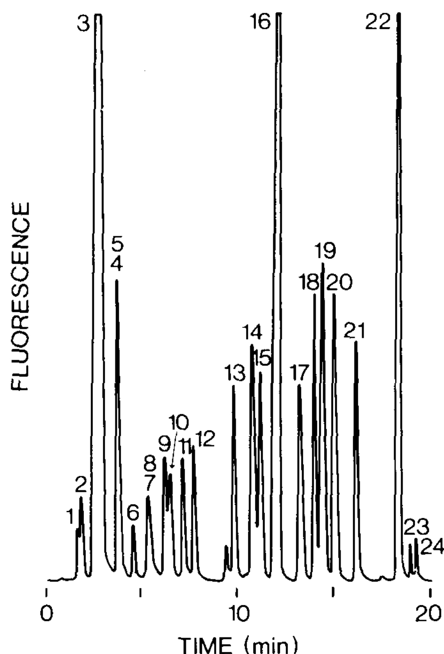

Fig. 2. Separation of a synthetic mixture of Dns-amino acids on a 3-μm C_{18} column according to the conditions described in the text. For peak identification, see legend to Fig. 1.

amino acid content of 0.15 m*M*, which gave a Dns-Cl/amino acid concentration ratio of 11.7:1. Under these circumstances, Cys formed both mono- and di-Dns derivatives. Lys formed almost exclusively its di-Dns derivative, and only a small peak of Dns-Lys was observed with a retention time of 23 min. His formed mostly the mono-Dns derivative but ca. 10% of di-Dns-His was also formed. Tyr existed only in its di-Dns form.

Methylamine has often been used to terminate the dansylation reaction [1]. Under the present conditions, methylamine formed a peak that co-eluted with the Leu and Ile peaks. Ethanolamine was found to be more suitable in this respect because the retention time of the formed Dns-NH_2 peak was shorter and it was well separated from other peaks (Fig. 1). The area of the Dns-NH_2 peak could also be used to check if sufficient excess of Dns-Cl was present in the reaction mixture. In addition to the Dns-NH_2 peak, the use of the Dns-Cl label causes also another contaminating peak in the chromatogram, i.e. Dns-OH. However, this peak was also well separated from other peaks, as shown in Figs. 1–3 (Peak No. 3).

The pH of the mobile phase had an influence on the elution pattern of the Dns-amino acids, and with the 5-μm column the best resolution was achieved with the gradient programme presented in Table I. The most suitable pH for the separation of the peaks from Asn to Arg was 5.6, and pH 6.1 for the rest of the chromatogram (Fig. 1). With the 3-μm column and the mobile phase of pH 6.1 used throughout the analysis, the Asn/Cys and Ser/His pairs were not separated (Fig. 2).

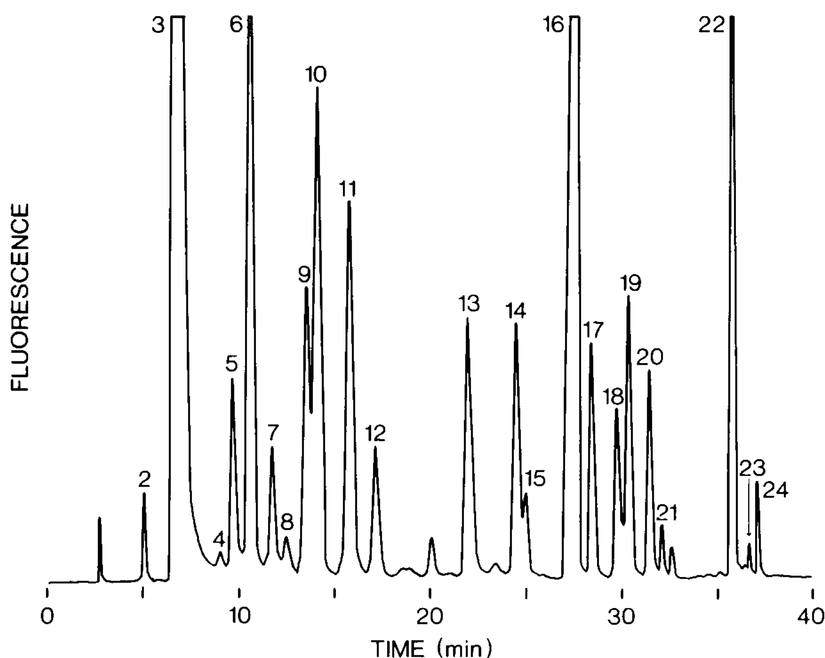

Fig. 3. Chromatogram of a plasma sample of mink obtained using a 5-μm column and the same conditions as in Fig. 1. For peak identification, see legend to Fig. 1.

Aqueous solutions cause fluorescence quenching of the Dns-amino acids [13]. The quenching phenomenon probably decreased slightly the detection sensitivity of the first ten amino acids eluted, but the sensitivity of the present method was still between 5 pmol for Asp and ca. 0.5 pmol for Lys. Each peak except Tyr (20 pmol) in the chromatogram presented in Fig. 1 contained 100 pmol of a given amino acid.

Several compounds have been used as internal standards in the HPLC analysis of Dns-amino acids: Nle or Nva [11] and 3-aminobutyrate [14]. By using the same conditions as in Fig. 1, the retention times of Dns-3-aminobutyrate and Dns-Nva were 20.4 and 26.5 min, respectively. Thus, both of these substances can be used as internal standards in the present method.

A decrease of the column particle size from 5 to 3 μm, and a simultaneous reduction of the column length from 125 to 30 mm, shortened the analysis time from 37 to 19.5 min (Fig. 2). The elution patterns observed with the 5-μm and 3-μm columns were very similar, but poor separation of Asn/Cys and Ser/His on the 3-μm column was probably due to the use of a pH 6.1 eluent only. With the 3-μm column, the concentrations of Asn/Cys and Ser/His can be calculated from the di-Dns peaks of Cys and His, which are well separated from other peaks (Fig. 2). It should be pointed out that the lifetime of the 3-μm column was considerably shorter than that of the 5-μm column, when the purity of solvents and samples was the same.

A typical chromatogram of a plasma sample of mink analysed using a 5-μm column is shown in Fig. 3. The chromatographic conditions were the same as

those in Fig. 1. As can be seen, all the common protein amino acids were well separated. The mean recovery of different amino acids determined from six independent analyses was 101.4 ± 5.8%. The range of the recovery was 89% for His and 108% for Lys. The repeatability of the determination of plasma-free amino acids was tested with ten subsequent analyses of the same plasma sample using the automatic sampling system. The coefficient of variation was 4.8 ± 2.5% (mean ± S.D.). In the present study, the quantitative analysis in plasma was performed by external calibration: a standard mixture of twenty amino acids (Fig. 1) was injected after every five unknown samples. For internal standardization Dns-Nva can be used, because in plasma samples no interfering peaks eluted with its retention time of 26.5 min (Fig. 3).

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