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COMPLETE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF 4-N,N-DIMETHYLAMINOAZOBENZENE-4′-THIOHYDANTOIN AND 4-DIMETHYLAMINOAZOBENZENE-4′-SULPHONYL CHLORIDE AMINO ACIDS UTILIZING THE SAME REVERSED-PHASE COLUMN AT ROOM TEMPERATURE

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SUMMARY

Reversed-phase high-performance liquid chromatographic methods for the complete separation of all 4-dimethylaminoazobenzene-4′-sulphonyl chloride and 4-N,N-dimethylaminoazobenzene-4′-thiohydantoin amino acids on the same Supelcosil LC-18 column at room temperature are described. The procedures are simple and reproducible, and the systems are easily interconvertible. The use of a fixed-wavelength detector at 436 nm permits amino acid analysis at levels lower than 1 pmol with a stable baseline.

INTRODUCTION

One of the major problems in the structural analysis of red blood cell enzymes is that they are present in only small amounts. This means that only micrograms of purified protein can be obtained, as with mammalian red blood cell hexokinase (E.C. 2.7.1.1). This enzyme has been shown to exist in two or more distinct molecular forms[1]. At present, the molecular basis of hexokinase heterogeneity remains unknown, although preliminary experimental findings have suggested a post-translational modification as a possible mechanism[1]. In an attempt to characterize these modifications, we are developing methods that can be used with small amounts of pure protein. Over the last few years, many workers[2–14] have described the use of reversed-phase high-performance liquid chromatography (RP-HPLC) for the separation of 4-dimethylaminoazobenzene-4′-sulphonyl chloride (DABS) and 4-N,N-dimethylaminoazobenzene-4′-thiohydantoin (DABTH) amino acids. These methods are widely applied in microsequencing analysis, because their high sensitivity permits amino acid analysis at the picomole level with reliable results. However, the methods reported[2–14] for separating DABS- and DABTH-amino acids have certain drawbacks. In this paper, we describe the first complete separation of all DABS- and DABTH-amino acids and by-products on the same reversed-phase column at room temperature.

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EXPERIMENTAL

Chemicals

DABS-Cl and 4-N,N-dimethylaminoazobenzene-4'-isothiocyanate (DABITC) were purchased from Fluka (Buchs, Switzerland). Free amino acids for the preparation of DABS- and DABTH-amino acids were obtained from Sigma (St. Louis, MO, U.S.A.). Acetonitrile, methanol, 2-propanol and all the other reagents for HPLC and sequencing analysis were obtained from Fluka. Analytical-reagent grade potassium dihydrogen phosphate and acetate were purchased from Merck (Darmstadt, F.R.G.). Millipore filters (0.22 μm) were obtained from Millipore (Bedford, MA, U.S.A.). The water used for the experiments was doubly distilled. All buffers were filtered through a Millipore filter (0.22 μm) before HPLC analysis.

DABS-Cl and DABITC were recrystallized according to Chang[8]. A 0.5-g amount of DABITC was dissolved in 50 ml of boiling acetone (UV spectroscopy grade) and the insoluble materials were removed by passing the solution through a sintered-glass filter funnel. When the clear solution was left at −20°C overnight, needle-shaped crystals precipitated. DABITC was dried under vacuum, utilizing a Savant system concentrator (Savant, Hicksville, NY, U.S.A.).

A stock solution of DABITC (1.41 mg/ml) in acetone was prepared. Aliquots (100 and 500 μl) of the stock solution were pipetted into Eppendorf tubes, dried under vacuum and stored at −20°C over a period of months without appreciable degradation. Fresh DABITC solution was prepared by redissolving dried DABITC stored at −20°C in an appropriate volume of acetone before use. The same procedure was used for DABS-Cl, giving a 4 nmol/μg (1.3 mg/ml) stock solution in acetone.

Preparation of standard DABS-amino acids

Standards of mono-DABS-amino acids were prepared according to the method of Chang *et al.*[7] with slight modification. Amino acids (1 mg) were dissolved in 2 ml of 0.2 *M* sodium hydrogen carbonate buffer (pH 9.0). In this way, the large volume of buffer prevented a change in pH, as occurs with serine, threonine, aspartic acid and glutamic acid. To 100 μl of amino acid solution, 100 μl of DABS-Cl solution (2 nmol/μl in acetone) were added. The mixture was heated at 70°C for 10 min, dried under vacuum and the residue dissolved in 2 ml of 70% (v/v) ethanol. The standard DABS-amino acid solutions were stored at −20°C.

Bis-DABS-amino acids (lysine, histidine and tyrosine) (50 nmol) were dissolved in 100 μl of 0.2 *M* sodium hydrogen carbonate buffer (pH 9.0) and treated with 200 ml of DABS-Cl solution (4 nmol/μl in acetone). The mixture was heated at 70°C for 10 min, dried under vacuum and the residue dissolved in 500 μl of 70% (v/v) ethanol. The final concentration of the standard bis-DABS-amino acid solution was about 1 nmol per 10 μl.

Preparation of DABTH derivatives

Standard DABTH-amino acids were prepared according to the method of Chang[8] with slight modifications. Amino acids (0.5 mg) were dissolved in 1 ml of triethylamineacetic acid buffer (pH 10.65). To 100 μl of this solution were added 50 μl of DABITC solution (4 nmol/μl in acetone). The mixture was heated at 54°C for 1 h, dried under vacuum and the residue dissolved in 100 μl of 50% (v/v) trifluo-

roacetic acid (TFA). The acid solution was heated at 54°C for 45 min and then dried again under vacuum. The dried DABTH-amino acid derivative was dissolved in a suitable volume of 70% ethanol and stored at −20°C.

HPLC analysis

The HPLC system used (Beckman, Berkeley, CA, U.S.A.) consisted of two Model 112 pumps, a Model 420 solvent programmer, a Model 210 sample injection valve and a Model 160 fixed-wavelength (436 nm) visible-range detector, equipped with a 18.5-μl flow cell. Integration of peak areas was obtained by means of an HP 3390 A electronic integrator (Hewlett-Packard, Avondale, PA, U.S.A.). DABS- and DABTH-amino acid analyses were performed on a 5-μm Supelcosil LC-18 column (25 cm × 4.6 mm I.D.) equipped with a stainless-steel guard column (2 cm × 4.6 mm I.D.), packed with pellicular reversed-phase material (Pellicular Packing LC-18, 40 μm) (Supelco, Bellefonte, PA, U.S.A.).The sensitivity of the detector was routinely set at 0.005 a.u.f.s. The injection volume was 20 μl. The mobile phase utilized for the separation of DABS-amino acids consisted of two eluents: 25 m*M* potassium dihydrogen phosphate (pH 6.8) (solvent A) and acetonitrile–2-propanol (80:20) (solvent B); DABTH-amino acid analysis was performed utilizing 35 m*M* sodium acetate buffer (pH 5.1) (solvent A) and acetonitrile (solvent B).

The chromatographic conditions used to obtain the chromatogram shown in Fig. 1 were as follows:1 min at 20% of solvent B, 6 min up to 26% of solvent B and held for 13 min, up to 70% of solvent B. The gradient was then returned to 20% of solvent B and the initial conditions were restored in 8 min. The flow-rate was 1.0 ml/min. The chromatographic profile reported in Fig. 2 was obtained utilizing the following gradient: 8 min at 39% of solvent B, 4 min up to 53% of solvent B and held for 28 min. The gradient was then returned to 39% of solvent B and the initial conditions were restored in 10 min. The flow-rate was 1.0 ml/min.

RESULTS AND DISCUSSION

Separation of DABS-amino acids

Pre-column derivatization of amino acids with DABS-Cl followed by reversed-phase HPLC[4–7,9,12] permits the detection of DABS-amino acids at the picomole level in the visible region. Many workers [7,9,12] have discussed the advantages of this method with respect to ninhydrin[15], *o*-phthaladehyde (OPA)[16–18] or dansyl chloride[19] detection. Chang *et al.*[6,12] reported the complete separation of all DABS-amino acids at high temperatures (50°C). Recently, Winkler *et al.*[9] described a good separation of all DABS-amino acids at room temperature, except for leucine and isoleucine, which are not completely separated. Further, the pH is very critical; slight changes cause fusion of the peaks of some DABS-amino acids and a loss of resolution. In order to obtain a simple and reproducible method that allows the complete separation of all DABS-amino acids at room temperature, we tested various reversed-phase columns (Ultrasphere ODS, Micropak MCH and Supelcosil LC-18) and different buffers (acetate, phosphate), organic solvents (acetonitrile, ethanol, methanol, 2-propanol), organic modifiers (dimethylformamide, triethylamine), pH values, flow-rates and gradient forms. The best results were obtained with a 5-μm Supelcosil LC-18 column (25 cm × 4.6 mm I.D.), 25 m*M* potassium dihydrogen phosphate buffer, pH

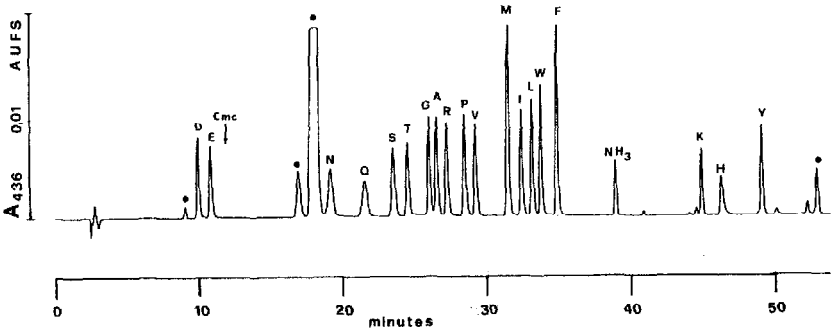

Fig. 1. Complete separation of all DABS-amino acids (*ca.* 50 pmol) by reversed-phase HPLC on a 5-μm Supelcosil LC-18 column (25 cm × 4.6 mm I.D.). Solvent A, 25 m*M* potassium dihydrogen phosphate buffer (pH 6.8); solvent B, acetonitrile–2-propanol (80:20). Chromatographic conditions as described under Experimental. Flow-rate, 1 ml/min; temperature, ambient. ●, By-products originating from the excess of reagent.

6.8 and acetonitrile–2-propanol (80:20). Fig. 1 shows the complete separation of a standard mixture of DABS-amino acids and by-products originating from the excess of reagent. For each new Supelcosil LC-18 column only a slight modification of the conditions described under Experimental are necessary in order to obtain the optimal separation. The decisive factor was the addition of 20% of 2-propanol to the acetonitrile, which permitted the complete resolution of DABS-Ser, DABS-Thr, DABS-Gly, DABS-Ala and DABS-Val. The procedure described here is the first complete separation of about 30 DABS-amino acids and by-products at room temperature.

Separation of DABTH-amino acids

Of great importance has been the recent development of the sensitive manual Edman micro-sequencing analysis of peptides and protein by the double coupling method, in which DABITC is followed by phenyl isothiocyanate (PITC) for complete reaction of all terminal amino groups[2,3,8,10,13]. The DABTH-amino acids obtained after each cycle can be identified by thin-layer chromatography or, more sensitively, by reversed-phase HPLC . Chang[8] reported the complete separation of DABTH derivatives on a Zorbax ODS column, except leucine and isoleucine, which were eluted together.

Recently, many workers[9–11,13,14] have proposed various RP-HPLC procedures for the separation of all DABTH-amino acids, but they have certain drawbacks. A better separation of DABTH-Ile and DABTH-Leu was obtained by Winkler *et al.*[9], who used as a decisive factor a 3-μm Ultraphere ODS column and the addition of 2% of triethylamine to the eluent. Yang and Wakil[11] described a good separation of all DABTH derivatives except DABTH-Ser and DABTH-Gln.

Similarly, the method proposed by Lu and Gracy[13] does not allow the resolution of two pairs of DABTH-amino acids, DABTH-Pro/DABTH-Phe and DABTH-Ile/DABTH-Leu. For the above reasons, we performed experiments similar to those described for DABS-amino acids in order to find a procedure that permits the complete separation of all DABTH derivatives at room temperature.

We obtained the best results by utilizing a 5-μm Supelcosil LC-18 column, as

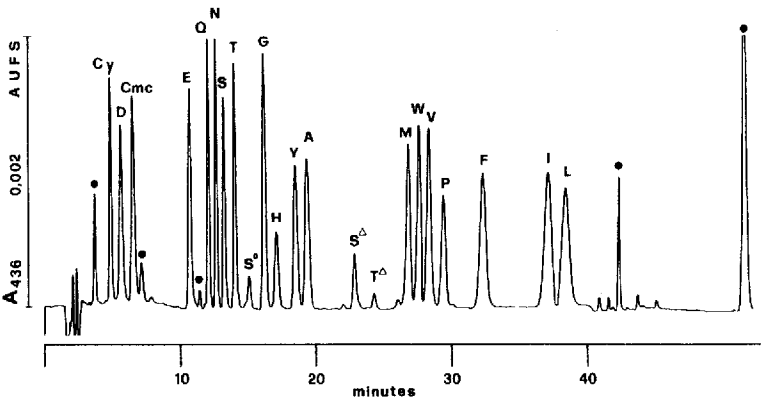

Fig. 2. Complete separation of *ca.* 2–3 pmol of standard DABTH-amino acid derivatives on a 5-μm Supelcosil LC-18 column (25 cm × 4.6 mm I.D.). Solvent A, 35 m*M* acetate buffer (pH 5.1); solvent B, acetonitrile. Chromatographic conditions as described under Experimental. DABTH peaks are identified by a single-letter code according the IUPAC-IUB Commission on Biochemical Nomenclature. Flow-rate, 1 ml/min; temperature, ambient. Cy, cysteic acid; Cmc, S-carboxymethylcysteine; S°, DABTH-Ser° (a probably hydrated DABTH-Ser with a hydroxy group at the 2-position); T^{Δ} and S^{Δ}, dehydrated derivatives of threonine and serine. ●, Decomposition product of DABTH-amino acids.

shown in Fig. 2. The complete resolution of all DABTH derivatives (about 30), including cysteic acid and S-carboxymethylcysteine, can be achieved at room temperature by the use of the gradient system described under Experimental, where 35 m*M* acetic acid (pH 5.1) was solvent A and acetonitrile was solvent B.

The lower pH of 5.1 does not affect the separation of DABTH-amino acids, except DABTH-Glu, which increases its retention time towards that of DABTH-Gln. A commercial Supelcosil LC-18 column shows a very highly reproducible retention time. In our experience, only a slight modification of the gradient form is necessary for each new column. It is sufficient to modify the last part of the gradient by increasing or decreasing slightly the percentage of solvent B to obtain the complete separation of the DABTH-amino acids. The DABTH-amino acid profile reported in Fig. 2 shows the original chromatogram, obtained from the Beckman Model 160 with a fixed wavelength, set at 0.002 a.u.f.s. It can be seen that there is no appreciable rise in the baseline due to the gradient change. However, we performed all routine analyses of DABTH amino acids at 0.005 a.u.f.s. with perfect stability of the baseline, demonstrating the major advantage of the method that less than 1 pmol of DABTH can easily be measured. Because of the high sensitivity of the method, only small volumes and amounts of samples need be injected. Under these conditions, the column has a long lifetime. Over 2000 analyses have been performed up to now without observing any irreversible deterioration effects. The guard column needs to be changed after every 200–300 injections when 20-μl aliquots are analysed. The only fault we have observed after prolonged use of the same column (several months) is a slight decrease in the complete resolution of DABTH-Met and DABTH-Trp.

CONCLUSIONS

The methods described here, based on RP-HPLC, have permitted the first

complete separation of all DABS- and DABTH-amino acids on the same column at room temperature. Under the conditions described, the procedures proposed are simple and reproducible and the systems are easily interconvertible. The use of the Beckman Model 160 fixed-wavelength detector with a sensitivity limit of 0.001 a.u.f.s. permits amino acid analyses with stable baselines at levels lower than 1 pmol.We believe that the methods will be very useful for the microsequencing analysis of pure proteins obtainable in only small amounts.

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