Journal of Chromatography, 297 (1984) 75–82
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 341

COMPLETE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF PHENYLTHIOHYDANTOIN- AND 4-N,N-DIMETHYLAMINOAZOBENZENE 4'-THIOHYDANTOIN-AMINO ACIDS ON AN ULTRASPHERE ODS COLUMN WITH THE SAME BUFFER SYSTEM

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SUMMARY

A high-performance liquid chromatographic method is described for the separation of twenty phenylthiohydantoin (PTH)-amino acids and eighteen 4-N,N-dimethylaminoazobenzene 4'-thiohydantoin (DABTH)-amino acids. These amino acid derivatives, commonly generated after Edman degradation by the phenylisothiocyanate (PITC) or 4-N,N-dimethylaminoazobenzene 4'-isothiocyanate (DABITC)–PITC coupling method, can be separated by high-performance liquid chromatography on an Altex Ultrasphere octadecylsilane (ODS) column. PTH-degradation products of threonine and serine were unambigously distinguished with a dual detection system at 254 and 320 nm. The separation was accomplished by use of a gradient program consisting of 10 m*M* sodium phosphate buffer, pH 6.6, 0.5% *n*-butanol and methanol. It is demonstrated that only the methanol concentration in the gradient system must be altered depending on whether a PTH- or a DABTH-separation is required.

INTRODUCTION

During recent years the strategy for automated and manual Edman degradation has concentrated on two leading sequence systems generating PTH- and/or DABTH-amino acid derivatives. For the identification and quantification of the common PTH-amino acids, several laboratories described the use of high-performance liquid chromatography (HPLC) on reversed-phase columns. Most frequently, gradient elution systems were used for the separation of PTH-amino acids[1–8], whereas only a few papers have been published on isocratic separations of these derivatives[9–11].

Since the introduction by Chang *et al.*[12] of DABITC, as a substitute for PITC in Edman sequence analysis, attempts have been made to separate and quantify the

0021-9673/84/$03.00

DABTH-amino acids generated. Identification of these derivatives has been successfully performed by two-dimensional thin-layer chromatography (TLC)[12–14], but quantitative analysis is rather laborious in practice, and for some derivatives it is restricted by insufficient resolution of the spots.

Wilson *et al.*[15] first reported the separation of DABTH-amino acids by means of HPLC on silica in a two-column system. Recently, a single-column system with octadecylsilane (ODS)-coated silica packing has been introduced for these analyses[16,17]. Although both methods afford sensitive and quantitative analyses, some DABTH-amino acids were not completely resolved. Moreover, both systems were time-consuming.

Hitherto, two completely different HPLC systems had to be used in order to separate PTH- or DABTH-derivatives. This paper deals with the application of an HPLC gradient system, with a single column, and the same solvents for the separation and quantitation of both PTH- and DABTH-amino acids.

EXPERIMENTAL

Chemicals

Standard solutions of individual PTH-amino acid derivatives (Serva, Heidelberg, F.R.G.) were prepared, about 100 nmol of each in 1 ml of ethanol, methanol or ethyl acetate. The solutions were stored at −25°C.

Amino acids for the preparation of DABTH-amino acids were purchased from Serva. DABITC was purchased from Fluka (Buchs, Switzerland). Methanol was obtained from Carlo Erba (Milan, Italy). The water used for the experiments was distilled twice. All other reagents were analytical grade. All buffers were filtered through Millipore filter (0.45 μm) and degassed under reduced pressure or by sonication.

Preparation of DABTH-derivatives

Standard DABTH-amino acids were prepared by a modification of the method of Chang *et al.*[12]. A solution of 0.5 μmol of amino acid in 100 μl of buffer (0.3 *M* N,N-dimethyl-N-allylamine in 1-propanol–water, 1:1; pH 10.1) was treated with 100 μl of DABITC solution (2μmol/ml acetone) and incubated for 1 h at 50°C. The solution was then dried under vacuum. Water (200 μl) and 400 μl of 6 *M* hydrochloric–acetic acid (1:2) were added and the solution was left for 50 min at 50°C. The mixture was dried again under vacuum and the residue dissolved in 400 μl of ethanol and stored at −25°C.

HPLC

Analyses were performed with a Waters HPLC system comprising two Model 6000 A pumps, a 720 system controller, a data module, an automatic WISP sample injector, a Model 440 detector (fixed wavelength at 254 nm) and a Model 480 variable-wavelength detector (Waters Assoc., Milford, MA, U.S.A.). The sensitivity of the detectors was routinely set at 0.05 a.u.f.s. Separations of PTH- and DABTH-amino acids were carried out in an Altex Ultrasphere ODS (5 μm) column (250 × 4.6 mm I.D.) (Altex Scientific, Berkeley, CA, U.S.A.). The injection volume was 10 μl (about 0.1 nmol of each PTH- or DABTH-amino acid). Detection was carried out

at 254 nm for the PTH-derivatives, 320 nm for the degradation products of PTH-Ser and -Thr and 436 nm for the DABTH-derivatives.

RESULTS

Identification of DABTH-amino acids

After the development of a micro-sequencing DABITC–PITC double-coupling method[18], the need for better qualitative and quantitative determinations than those obtained with TLC[14] led to the development of HPLC systems for separating the DABTH-deratives[15,17]. The use of DABITC as a substitute for PITC in the Edman degradation reaction, introduced by Chang *et al.*[12], has several advantages. The DABTH-derivatives formed have a maximal absorption at 436 nm. This obviates interference from UV-absorbing impurities and base-line rise due to gradient change. Moreover, their extinction coefficients ($\varepsilon_{mM}^{436\,nm} = 34$)[19] are about twice as those of their PTH-analogues ($\varepsilon_{mM}^{254\,nm} = 16$)[15]. The column type and the toxicity of acetonitrile as eluent prompted us to look for another HPLC separation system which would enable us to separate PTH- as well as DABTH-amino acid residues with the same solvent system on the same column by using only a different gradient system. An Altex Ultrasphere ODS column was therefore used, equilibrated at 32°C. Solvent A was 10 m*M* phosphate buffer, pH 6.6, containing 23% methanol and 0.5% *n*-butanol, while solvent B was pure methanol. The gradient used was 48–67% solvent B in 9 min. Thereafter, this concentration was pumped isocratically through the column for the next 21 min at a flow-rate of 1.3 ml/min and a column temperature of 32°C (Fig. 1).

Except for DABTH-Lys and -Arg, which do not leave the column, even when higher methanol concentrations are used in the eluting buffer, this chromatographic system enables us to identify all the common amino acid DABTH-derivatives. Only the peaks for DABTH-Asp, -Glu and -CMCys are broad, and the response is lower. Since the detection of amino acid DABTH-derivatives is based on the chromophore dimethylaminoazobenzene rather than the thiohydantoin ring, all the by-products of DABTH-Ser and DABTH-Thr seen in TLC[19] can be identified at the same wavelength. This leads to an unambigous distinction between DABTH-His and DABTH-Thr, since whenever the latter derivative is eluted from the column, a DABTH-ΔThr derivative is also detected about 7 min later.

DABTH-Leu and -Ile can now be separated; this was impossible with the HPLC systems described earlier. Identification of the DABTH-derivatives at 436 nm circumvents possible interference from UV-absorbing impurities in either sequence buffer or chromatographic solvents.

Identification of PTH-amino acids

The HPLC system for the identification of DABTH-amino acids (column, starting buffer, column temperature and flow-rate was also used for the PTH-amino acids. Only the gradient and detection systems were changed. The linear gradient system was 0–40% solvent B in 15 min, followed by isocratic elution for the next 15 min (Fig. 2). A Waters Model 440 fixed-wavelength detector at 254 nm was used for the detection of most common PTH-amino acid derivatives, exception for the PTH-Ser and -Thr degradation products, which were detected with a Waters Model

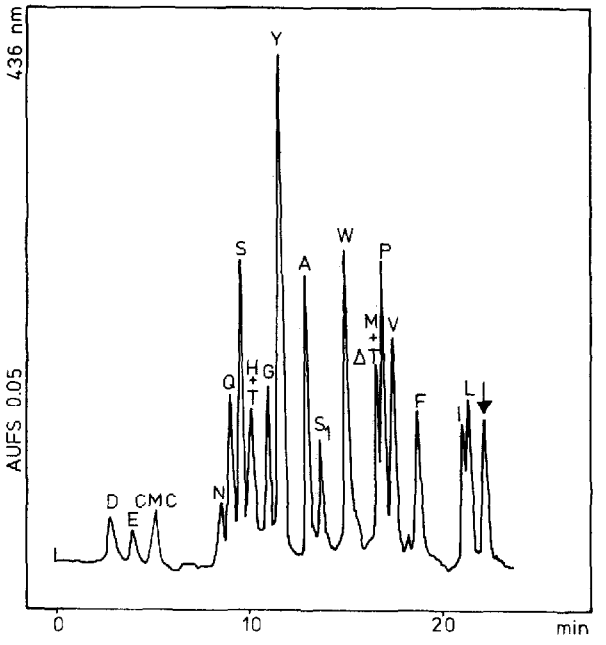

Fig. 1. HPLC chromatogram of a standard mixture of DABTH-amino acids (50 pmol each) on an Altex Ultrasphere ODS column and detection at 436 nm. Solvent A: 10 m*M* phosphate, pH 6.6, 23% methanol and 0.5% *n*-butanol. Solvent B: 100% methanol. Gradient: 48–67% B in 9 min with a flow-rate of 1.3 ml/min. Column temperature is 32°C. The arrow indicates the position of DABTH-norleucine (internal standard). For single-letter notation of amino acid residues, see IUPAC-IUB Commission on Biochemical Nomenclature, 1968.

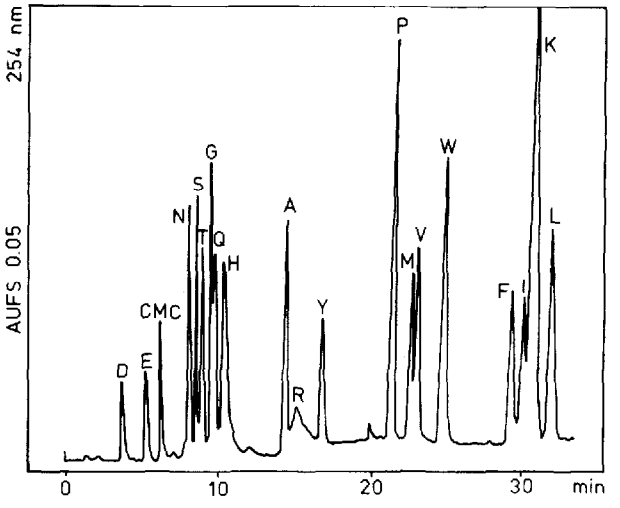

Fig. 2. Separation of a standard mixture of PTH-amino acids (± 200 pmol) on an Altex Ultrasphere ODS column and detection at 254 nm. Solvent A: 10 m*M* phosphate, pH 6.6; 23% methanol and 0.5% *n*-butanol. Solvent B: 100% methanol. Gradient: 0–40% B in 15 min with a flow-rate of 1.3 ml/min. Column temperature is 32°C.

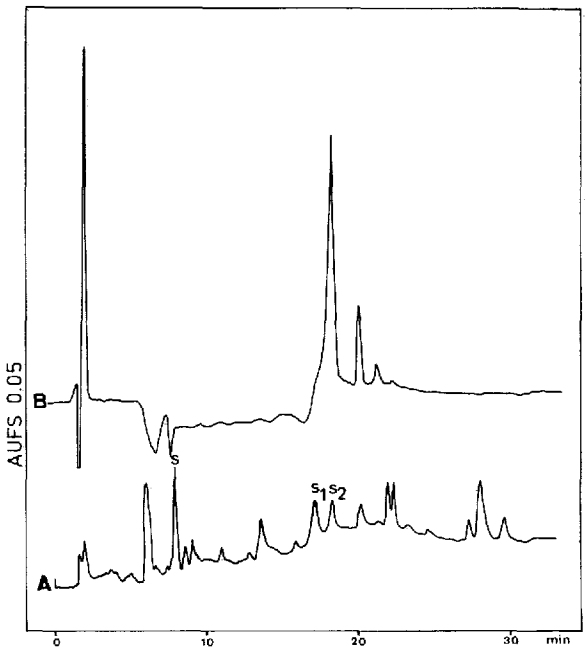

Fig. 3. HPLC chromatogram of PTH-Ser and its degradation products (S_1 and S_2) obtained after automated Edman degradation. Chromatographic conditions as in Fig. 2. (A) Detection at 254 nm; (B) detection at 320 nm. Only S_2 absorbs better at 320 nm.

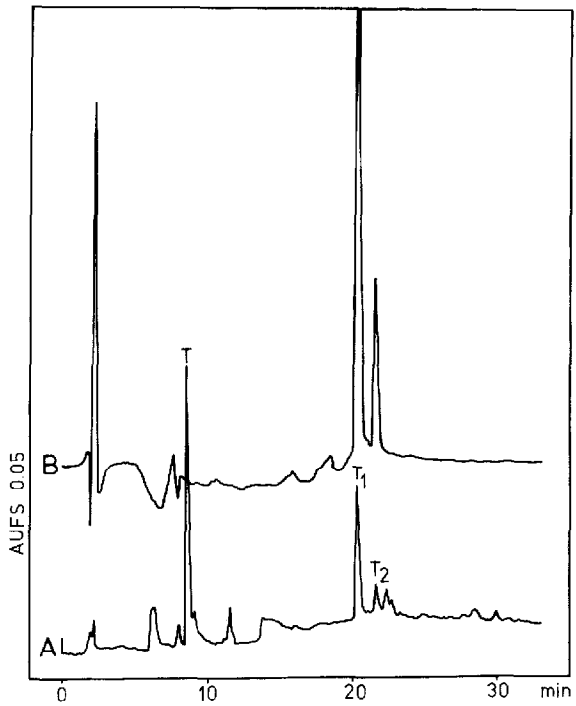

Fig. 4. HPLC chromatogram of PTH-Thr and its degradation products (T_1 and T_2) obtained after automated Edman degradation. Chromatographic conditions as in Fig. 2. (A) Detection at 254 nm; (B) detection at 320 nm.

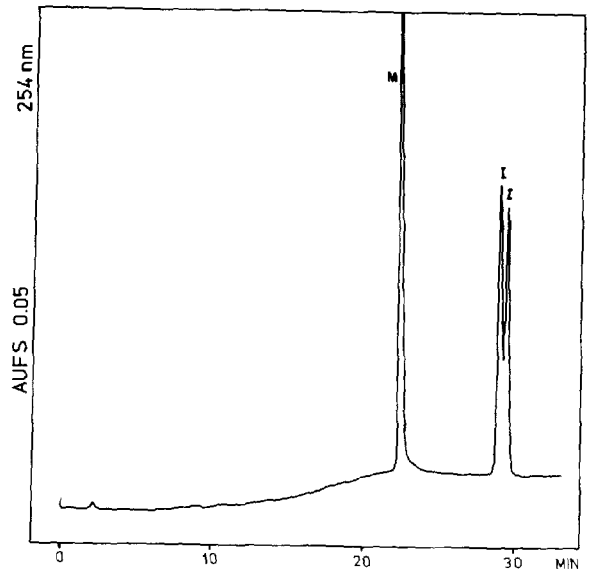

Fig. 5. HPLC chromatogram of PTH-Ile and PTH-Met standard solutions. Chromatographic conditions as in Fig. 2.

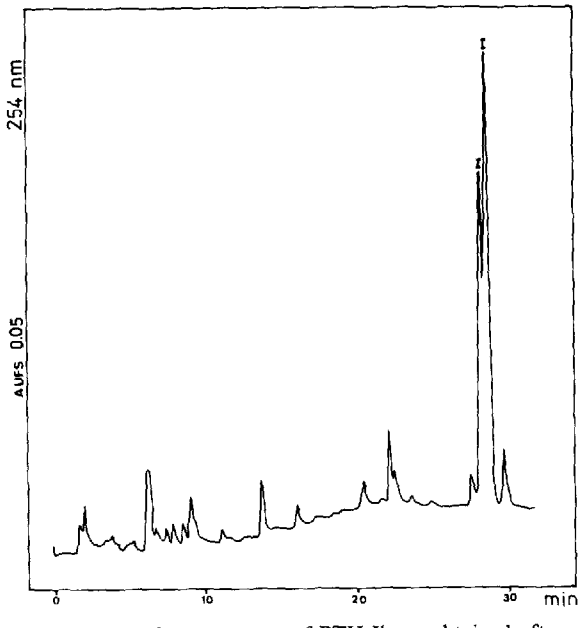

Fig. 6. HPLC chromatogram of PTH-Ile as obtained after automated Edman degradation. Chromatographic conditions as in Fig. 2.

480 variable-wavelength detector set at 320 nm (Figs. 3 and 4). It is difficult to predict the chromatographic behaviour of PTH-Arg and -His, since slight variations in ionic strength and pH influenced their relative retention time to some extent.

Our separation of the PTH-amino acids matches that of Henderson *et al.*[20] where a μBondapack phenylalkyl stationary phase was used and the elution time was 60 min for all the derivatives. Although a similar separation procedure for PTH-amino acids was described by Bhown *et al.*[7], no clear separation was shown for PTH-His, -Arg and -Lys, the latter being eluted together with PTH-Ile. PTH-Ile and -Lys are eluted together in our system too, but as PTH-Ile gives a double peak in sequencing as well as in standard solutions (Figs. 5 and 6), the two residues can be easily distinguished.

With our buffer system and methanol (*pro analysi*) obtained from Carlo Erba, neither expensive solvents nor acetone additions were necessary to depress rise in absorbance during HPLC analysis.

DISCUSSION

It is clear that an Altex Ultrasphere ODS column is extremely useful for the determination of DABTH- and PTH-amino acids. In our experience, 20 picomol of DABTH-amino acid and 80 picomol of PTH-amino acid appeared to be the optimum amount when the absorbance unit of the detector was set at 0.02 in the visible region at 436 nm and in the UV region at 254 nm, respectively. However, as little as 5 picomol of DABTH-amino acid and 20 picomol of PTH-amino acid can be detected.

Although serious attempts were made to use an isocratic separation system in order to separate all the common PTH-amino acids[11], a gradient system is still necessary in order to achieve near-baseline separation and sufficient distance between peaks to avoid confusion.

The gradient systems described in Figs. 1 and 2 have given reproducible elution profiles of amino acid DABTH derivatives (except for DABTH-Arg and -Lys) and PTH derivatives on at least three commercial Altex ODS Ultrasphere columns. For each new column the elution positions of PTH-His and -Arg can be optimized by small changes in pH[4]. Increasing the pH prolongs the retention time of PTH-Arg and decreases the retention of PTH-His.

The use of the same column and almost unchanged conditions for the identification of PTH- and DABTH-amino acids allows fast and easy switching from one separation to another.

ACKNOWLEDGEMENTS

This work was supported by F.G.W.O. grant nr. 3.0066.81 and N.F.W.O. grant nr. S 2/5-MV.E. 115.

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