Journal of Chromatography, 549 (1991) 404–409
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 360

Short Communication

The effect of ^2H_2O and an ion-pairing agent on the liquid chromatographic separation of dansylated amino acids

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(First received August 24th, 1990; revised manuscript received February 12th, 1991)

ABSTRACT

The chromatographic separation of dansylated amino acids using either H_2O- or ^2H_2O-based eluent is compared. The retention time of the majority of solutes studied is found to be shorter in heavy water and this is thought to be related to the higher pK_a values of dansylated amino acids in ^2H_2O compared to H_2O solutions.

INTRODUCTION

The analysis of amino acids is of importance in the study of the constitution of proteins and peptides and in the monitoring of the concentration of physiologically active molecules [1,2] such as L-glutamic acid, L-glycine and γ-aminobutyric acid (GABA) in biological fluids. It is difficult to analyse amino acids directly by high-performance liquid chromatography (HPLC) because most of these molecules lack the appropriate chromophore necessary for detection at low concentrations. Several derivatisation procedures have been successfully used in the literature to circumvent this problem [3–6]. Four of the most common derivatisation methods involve the use of the following reagents: 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride, Dns-Cl) [3], 9-fluorenyl-methylchloroformate (FMOC-Dl) [4], *o*-phthalaldehyde (OPA) [5] and phenyl isothiocyanate (PITC) [6]. An advantage of some of these techniques is that high sensitivity can be achieved by fluorescence detection. As dansylation appears to have advantages over the other techniques (the derivatisation procedure is straightforward and the resulting derivatives are stable) we report the analysis of a number of Dns-amino acids using fluorescence detection and the use of ^2H_2O and tetra-*n*-butylammonium hydroxide (an ion-pairing agent) to improve chromatographic separation.

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EXPERIMENTAL

Reagents and chemicals

Acetonitrile used in all mobile phases was far UV HPLC-grade and was purchased from BDH. Amino acids L-Glu, L-Ser, L-Gly, L-Ala, β-Ala, Val, δ-amino-*n*-valeric acid and ε-amino-*n*-caproic acid) were 99% pure (Sigma) and were used without further purification. Tetra-*n*-butylammonium hydroxide was purchased from Aldrich as a 40% (w/w) solution in water. Deuterium oxide (^2H_2O) and deuterated acetic acid ($C^2H_3CO_2{}^2H$) were obtained 99.9 atom% deuterium from Aldrich. Dns-Cl was used as purchased from Aldrich (99%). Analar ammonium acetate (99% pure) from BDH was dissolved in distilled-deionised water (Milli-Q). All pH and p^2H measurements were recorded on a Radiometer PHM82 standard pH meter calibrated with standard pH buffers.

HPLC apparatus

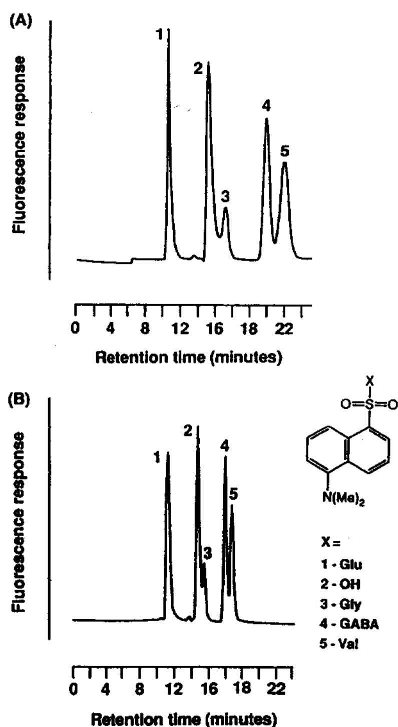
The chromatographic system (Perkin-Elmer) consisted of a series 4 liquid chromatograph controller and pumps. Sample detection was achieved using a Perkin-Elmer LS4 fluorescence spectrometer set at an excitation maximum of 330 nm (10 nm slit width) and an emission maximum of 550 nm (10 nm slit width) with a flow cell volume of 3 μl. Data were acquired using the Perkin-Elmer LIMS/CLAS system.

HPLC separation

HPLC separation was carried out using a stainless-steel (25 cm × 2.1 mm I.D.) Ultrasphere octyldecylsilyl 5-μm particle size analytical column (supplied by Beckman). Column back-pressure varied from 10 to 11 MPa. Samples were loaded by a Model 7520 Rheodyne microinjector fitted with a 0.5-μl sample rotor. Initially, the HPLC separation of five Dns-amino acids was carried out under isocratic conditions using a mobile phase that consisted of [130 m*M* ammonium acetate (pH 6.8)–acetonitrile in the ratio 67:33 (v/v). The flow-rate was 70 μl/min at ambient temperature. The inclusion of 15 μ*M* tetra-*n*-butylammonium hydroxide (TBAH) improved the chromatography considerably (Fig. 1).

Gradient elution was then used to separate a larger number of amino acids using the following conditions: solvent A consisted of [130 m*M* ammonium acetate (pH 6.8) containing 15 μ*M* TBAH]–acetonitrile in the ratio 893:17 (v/v); solvent B was acetonitrile. In solvents containing ^2H_2O, the p^2H (measured from the meter reading with the addition of 0.4 units [7]) was adjusted with $C^2H_3CO_2{}^2H$ to a meter reading of 6.4, giving a p^2H of 6.8.

The following gradient program was used in all subsequent experiments: segment 1: isocratic at 0% of B for 10 min; segment 2: a linear gradient from initial conditions to 8% B in 12 min; segment 3: isocratic at 8% B for 30 min; segment 4: a linear gradient from preceding segment condition to 30% B for 8 min; segment 5: isocratic at 30% B for 40 min; segment 6: a linear gradient from preceding segment condition to 0% B for 6 min. The column was re-equilibrated to initial conditions (segment 1) for 40 min between each run. For clarification, we have summarised the above gradient profile in Fig. 2. The flow-rate was 70 μl min at ambient temperature. The gradient program was started simultaneously with injection. For both isocratic and gradient elution HPLC separations, volumes of sample injection were typically 0.5 μl.



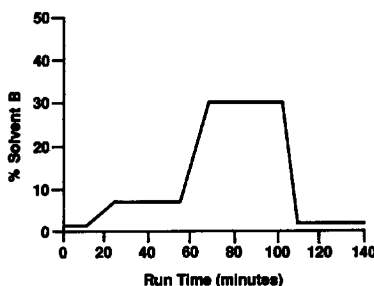


Fig. 1. A comparison of the chromatography obtained (A) without and (B) with the inclusion of 15 μM TBAH in the eluent. Conditions as in Fig. 1 except mobile phase: 130 mM ammonium acetate (pH 6.8)–acetonitrile (67:33).

Fig. 2. Gradient profile for the separation of Dns-amino acids. Gradient elution conditions: column: 25 cm × 2.1 mm I.D., 5-μm ODS (Beckman); fluorescence detector: excitation wavelength, 330 nm; emission wavelength, 550 nm; temperature: ambient; mobile phase: 140-min gradient elution; solvent A = [130 mM ammonium acetate (pH 6.8) containing 15 μM TBAH]–acetonitrile (83:17); solvent B = acetonitrile. Flow-rate: 70 μl/min.

Derivatisation procedure

Amino acids were derivatised using Dns-Cl according to the procedure by Tapuhi *et al.* [8]. The amino acid (0.001–0.1 mmol) dissolved in 40 mM sodium carbonate (pH 9.50, adjusted with hydrochloric acid), was treated with the Dns-Cl reagent (70 mM in acetonitrile) at room temperature. Some precipitation initially appeared upon reaction, but the precipitate dissolved immediately with agitation and the reaction was usually complete in 20–40 min. When not in use, solutions fo Dns-amino acids were stored at 5°C.

RESULTS AND DISCUSSION

It is well known that the use of an ion-pairing agent can improve the chromatography of a number of molecules [9,10]. In the present study, we initially investigated the use of ion-pairing agents such as tetra-*n*-butyl ammonium hydroxide (TBAH),

tetraethyl ammonium hydroxide (TEAH) and cetyltrimethyl ammonium bromide (CTAB) on the resolution of several Dns-amino acids under isocratic elution conditions. For all ion-pairing agents, the retention time of the dansyl amino acids was increased to a varying extent compared to the case where no ion-pairing agent was used. However, only the use of TBAH gave sharper peaks and an improvement in resolution (Fig. 3). The concentration of the ion-pairing agent used was to a large extent controlled by the amount of back pressure on the column. The concentration of 15 μM TBAH was the best compromise for these effects.

Using the chromatographic conditions detailed in the Experimental Section and in Fig. 2, the chromatograms shown in Fig. 3A and B are obtained for ten

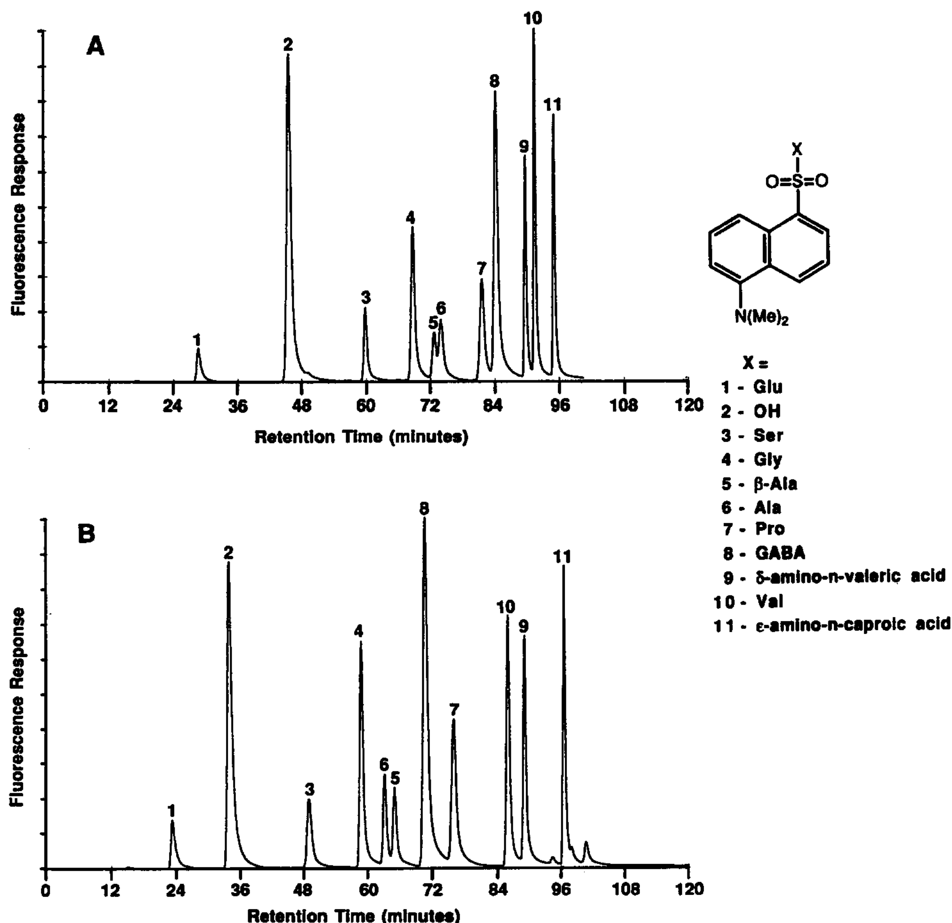


Fig. 3. A comparison of the elution order of dansylic acid and ten Dns-amino acids using either H_2O or ^2H_2O as the main component in the mobile phase. Chromatographic conditions are as detailed in the Experimental Section. (A) Chromatogram of derivatives eluted in H_2O mobile phase (pH 6.8). (B) Chromatogram of derivatives eluted in ^2H_2O-mobile phase (p^{2}H 6.8). The numbering system shown refers to (1 = Dns–Glu, 2 = dansylic acid, 3 = Dns–Ser, 4 = Dns–Gly, 5 = Dns-β-Ala, 6 = Dns-Ala, 7 = Dns-Pro, 8 = Dns-GABA, 9 = Dns-δ-amino-n-valeric acid, 10 = Dns-Val and 11 = Dns-ε-amino-n-caproic acid. 10–84 pmol of each compound was injected.

Dns-amino acids and dansylic acid (1-dimethylaminonaphthalene-5-sulphonic acid) using either water (H_2O) or deuterium oxide (^2H_2O) in the mobile phase. The retention times [t (H_2O) and t (^2H_2O)] for the compounds analysed in these two solvents are listed in Table I. It is clear from this table that t (^2H_2O) values are lower than the corresponding t(H_2O) values. The shorter retention times in ^2H_2O compared to H_2O are probably due to a combination of two effects. The use of a counter-ion makes the ion pair more hydrophobic than the unpaired Dns-amino acid at pH values close to neutral. A second effect is related to the pK_a values of the Dns-amino acids in H_2O compared to ^2H_2O solutions. pK_a values are expected [11] to be higher in ^2H_2O than in H_2O so that slightly less dissociation is expected in the former mobile phase at pH 6.8 compared to pH 6.8 in H_2O. For instance, the pK_a value of N-acetylglycine is reported [12] as 3.7 (no pK_a values for the Dns-amino acid derivatives could be found in the literature). This will increase by about 0.4 units in ^2H_2O. The retention times of the Dns-amino acids studied decrease to a varying extent in ^2H_2O compared to H_2O (see Fig. 3A and B, and Table I). We have not studied the variation of retention times over a wide range of pH values. The order of elution is even changed in some cases in ^2H_2O *versus* H_2O solutions. We are aware that in some cases quoted in the literature [13,14] retention times of substrates measured using reversed-phase chromatography (RP-HPLC) and ^2H_2O or H_2O in the mobile phase gave longer retention times in the former solvent. In contrast, under our chromatographic conditions, shorter retention times of the Dns-amino acids are observed in ^2H_2O (Table I). This may be due to the more complex equilibria occurring when an ion pair reagent such as TBAH is used in the mobile phase in RP-HPLC. Two mechanisms can be involved [15,16]. The ion pair can be adsorbed onto the surface of the reversed-phase resulting in a situation close to ion-exchange chromatography or it can be closely associated with the negatively charged substrate in a neutral complex held together by electrostatic attraction. As the pK_a of the Dns-amino acids in ^2H_2O is higher than in H_2O due to the smaller extent of ionisation at equivalent pH and ^2H values (equal acidity) shorter retention times are expected in ^2H_2O when either mechanism is operative.

The procedure detailed was used in the determination of the concentration of

TABLE I

THE EFFECTS OF H_2O AND ^2H_2O ON THE RETENTION TIMES OF DANSYLIC ACID AND SOME Dns-AMINO ACIDS

Dns derivative	Peak No.	(H_2O) (min)	t(^2H_2O) (min)	t(H_2O)-t(^2H_2O) (min)
Glu	1	28.1	22.8	5.3
Dansylic acid	2	45.0	33.3	11.7
Ser	3	59.4	48.2	11.2
Gly	4	68.3	58.0	10.3
β-Ala	5	73.4	62.4	11.0
Ala	6	72.2	64.2	8.0
Pro	7	81.1	70.0	11.1
GABA	8	83.7	75.1	8.6
γ-Amino-*n*-valeric acid	9	89.3	88.5	0.8
Val	10	91.0	85.2	5.8
ε-Amino-*n*-caproic acid	11	94.6	96.4	− 1.8

Glu and GABA in plasma and brain tissue fluids from rats. In this analysis, the use of H_2O gave adequate resolution and sensitivity. No recourse to using ^2H_2O in place of H_2O in the buffer eluent was necessary, especially as this would be costly due to the large number of samples to be analysed.

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