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CHROMATOGRAPHIC SEPARATION OF DANSYL AMINO ACIDS AND DANSYL AMINES ON AMBERLITE IRC-50

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SUMMARY

A column chromatographic method for the separation of dansyl amino acids and dansyl amines is described in which Amberlite IRC-50 equilibrated with the eluent is used as the stationary phase and six solvent mixtures, which are composed of a buffer or 2% acetic acid and tetrahydrofuran, methyl ethyl ketone and acetone, are used as eluents. Neutral and acidic dansyl derivatives were separated at low pH, and those having a positive charge or present as the dipolar ion at pH 5.60 were separated at high pH. Quantitative determination of histamine as its monodansyl derivative was successful, and an analysis for histamine in a rat liver extract is described.

INTRODUCTION

1-Dimethylaminonaphthalene-5-sulphonyl(dansyl) derivatives of amino acids and amines are highly fluorescent compounds and nanogram amounts of amino compounds can be determined by converting them into their dansyl derivatives[1–3].

We have tried to use ion-exchange resins as the column material for the chromatographic separation of dansyl derivatives and a carboxylic acid type resin, Amberlite IRC-50, was found to be useful for this purpose.

EXPERIMENTAL

Reagents

The dansyl amino acids were purchased from Seikagaku Kogyo Co. (Tokyo, Japan), and dansyl chloride from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Biogenic amines were obtained from commercial sources. Dipeptides of the branched chain amino acids, Val–Val, Ile–Val, Val–Ile and Ile–Ile, were supplied by Dr. T. Inui. Citric acid monohydrate, formic acid (99% and 85%), acetone, methyl ethyl ketone, tetrahydrofuran and acetic acid were of special grade and thiodiglycol was of reagent grade for amino acid analyzer.

Dansylation of the dipeptides

The above dipeptides were dansylated according to the procedure recommended by Gray[4], and purified on a 0.6 × 10–15-cm column of Amberlite IRC-50 (H^+), using a mixture of 2% acetic acid and acetone (3:2, v/v) as the eluent.

Dansylation of amines

Serotonin and catecholamines. Ten nanomoles of each amine in 10 μl of 0.2% acetic acid were mixed with 30 μl of dansyl chloride solution in acetone (10 mg/ml) and 10 μl of carbonate buffer (pH 10, 0.4 *N*). Then 10–20 μl of acetone were added in order to clarify the reaction mixture. After incubation at 25° for 5 h, the mixture was neutralized with 60 μl of 0.1 *N* formic acid, diluted with 200 μl of eluent E (Table I) and added to the column.

Histamine. One to five nanomoles of histamine dihydrochloride in 10 μl of 0.2% acetic acid were mixed with 20 μl of dansyl chloride solution (2.5 mg/ml) and 10 μl of sodium hydrogen carbonate solution (0.4 *N*), and 10 μl of dansyl chloride solution were added in order to clarify the reaction mixture. After incubation at 25° for 2 h, the mixture was evaporated to dryness under nitrogen and treated with formic acid as described by Tamura *et al.*[5], to give monodansyl histamine. The dried residue was dissolved in eluent F and added to the column.

Dansylation of the histamine fraction from a rat liver extract was performed as described above, with the use of double amounts of the reagents.

Preparation of histamine fraction from rat liver

Four grams of rat liver were homogenized in a glass homogenizer with 20 ml of 0.4 *N* perchloric acid and centrifuged at 30,000 *g* for 30 min. One eightieth of the supernatant fraction was added to a 0.32 × 2.8-cm column of Dowex 50-X4 (Na^+) buffered at pH 5.0, washed with 5 ml of 1 *N* hydrochloric acid, and histamine was eluted with 1.6 ml of 2.5 *N* hydrochloric acid.

Preparation of the columns

Amberlite IRC-50 (A.G.) was pulverized, classified according to size and washed as described previously[6]. Particles of size ranges 35–40, 40–50 and 50–60 μm were used. The washed resin in the sodium form was buffered to the pH of the buffer used to prepare the eluent by adding either formic acid or citric acid solution to the resin suspension. The buffered resin was washed with the eluent described in Table I,

TABLE I

COMPOSITION OF ELUENTS

Eluent	*Composition**	*Proportions (v/v)*
A	Formate buffer, pH 4.00 (0.4 *N*)–tetrahydrofuran (THF)–methyl ethyl ketone (MEK)–acetone (Me_2CO)	15:1:3:3
B	Formate buffer, pH 3.15 (0.4 *N*)–THF–MEK–Me_2CO	14:1:3:3
C	2% Acetic acid–THF–MEK–Me_2CO	20:4:3:3
D	Citrate buffer, pH 5.60 (0.1 *M*)–THF–MEK–Me_2CO	14:1:3:3
E	Formate buffer, pH 4.00 (0.4 *N*)–THF–MEK–Me_2CO	9:1:2:4
F	Citrate buffer, pH 5.60 (0.1 *M*)–THF–MEK–Me_2CO	10:1:3:3

* 0.25% (v/v) of thiodiglycol was added after mixing.

poured into a tube with the eluent used to equilibrate the resin and allowed to settle under gravity. When the resin of smaller particle size was used, it was packed into short chromatographic tubes with a column adjuster and two or three of these tubes were connected in series, in which the tube packed with the smaller resin was connected to the tubes containing the larger one. By this means, development of excessive back-pressure could be avoided. The sizes of the columns are shown in Table II.

TABLE II

CONDITIONS OF THE CHROMATOGRAPHIC SEPARATION

Figure	*Eluent*	*Form of resin*	*Size of column (mm)**	*Particle size of resin (μm)*	*Temperature of column (°C)*	*Flow-rate (ml/h)*
1a	A	Na^+	Top: 155 Middle: 105 Bottom: 110	40–50 35–40 35–40	46	6
1b	B	Na^+	Top: 160 Middle: 120 Bottom: 105	40–50 35–40 35–40	37	6
1c	C	H^+	550	50–60	37	6
1d	D	Na^+	440	50–60	40	6
2	E	Na^+	Top: 100 Bottom: 100	40–50 40–50	37	9
3a	F	Na^+	200	40–50	37	6
3b	F	Na^+	200	40–50	37	6

* Diameter of column = 8 mm.

Chromatographic separation and detection

Samples were dissolved in the eluent to be used for the separation and added to the column.

The buffer change system and constant flow pump of a JEOL 6AH amino acid analyzer were utilized for the elution of the dansyl derivatives, and eluent was pumped into the column at a flow-rate of either 6 or 9 ml/h. The eluate from the column was monitored with an L.D.C. Model 1209 fluoroMonitor or a JEOL FL-detector, and a back-pressure was applied so as to prevent the formation of bubbles in the flow cell. Amounts of 10^{-9}–10^{-10} mole of the dansyl derivative could be detected with this assembly of equipment.

RESULTS AND DISCUSSION

As shown in Fig. 1a, most of the dansyl amino acids could be separated with eluent A, at 46° and a flow-rate of 6 ml/h, within 23 h.

Those dansyl amino acids which could not be separated with eluent A could be separated from each other by using eluent B (Fig. 1b), C (Fig. 1c) or D (Fig. 1d). Therefore, when the analysis of the dansyl amino acids present in the acid hydrolyzate of a dansylated protein with eluent A gave an ambiguous result, the analysis of the other aliquot of the sample with eluent B, C or D generally yielded sufficient results for the identification of the dansyl amino acid in question.

The positions in the elution pattern of dansyl-arginine, ε-dansyl-lysine, mono-

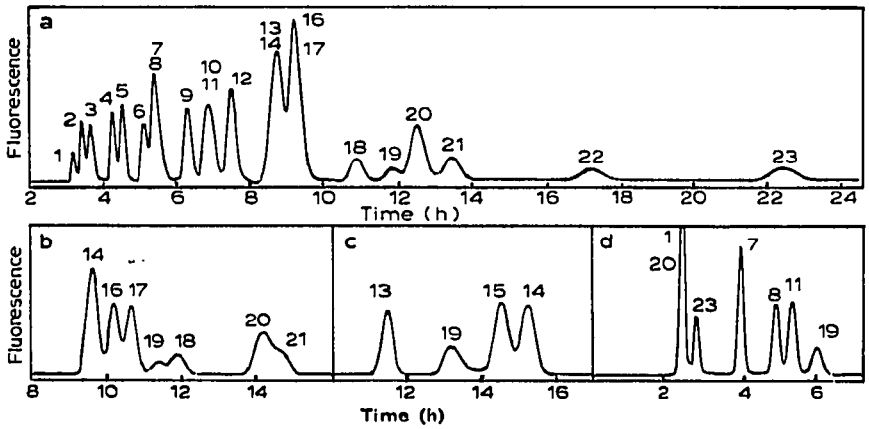

Fig. 1. Elution of dansyl amino acids. Chromatographic conditions are given in Table II. The numbers on the peaks represent the dansyl derivatives of aspartic acid (1), glutamic acid (2), serine (3), threonine (4), glycine (5), alanine (6), lysine (7, ε-dansyl), histidine (8, monodansyl), methionine (9), proline (10), arginine (11), valine (12), ammonia (13), leucine (14), tryptophan (15), isoleucine (16), phenylalanine (17), Val–Val (18), tyrosine (19, *o*-dansyl), lysine (20, didansyl), Ile–Val (21), Ile–Ile (22) and tyrosine (23, didansyl). Dansyl-tryptophan overlapped with dansyl-leucine and dansyl-Val–Ile overlapped with dansyl-Ile–Val under the chromatographic conditions of Figs. 1a and 1b. 1-Dimethylaminonaphthalene-5-sulphonic acid was eluted ahead of all of the above dansyl derivatives.

dansyl-histidine, dansyl-amide and *o*-dansyl-tyrosine relative to the other dansyl amino acids were very sensitive to changes in the pH of the eluent. In this chromatographic system, therefore, the pH of the buffer of eluent A was adjusted so as to permit the elution of *o*-dansyl-tyrosine ahead of didansyl-lysine, and the pH of the buffer of the eluent B was adjusted so as to permit the elution of *o*-dansyl-tyrosine just after dansyl-phenylalanine.

Dansyl derivatives of serotonin and catecholamines could be separated with eluent E containing a larger proportion of the organic solvents (Fig. 2). Peaks that eluted faster than dansyl-serotonin were also found in the dansylation mixture without amines, and these are probably the dansylated products of impurities present in the reaction mixture. The small peak that was eluted after dansyl-dopamine is the

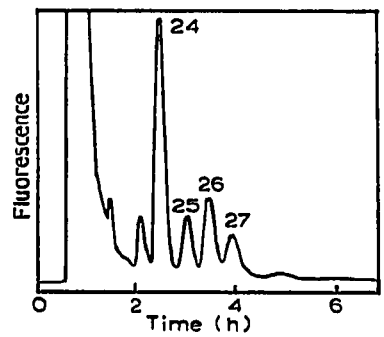

Fig. 2. Elution of dansyl amines. Chromatographic conditions are given in Table II. The numbers on the peaks represent the dansyl derivatives of serotonin (24), noradrenaline (25), adrenaline (26) and dopamine (27).

product derived from noradrenaline when it was dansylated with a large excess of dansyl chloride.

Monodansyl-histamine has a positive charge at pH 5.60, so that it could be separated from other dansyl amino acids and dansyl amines, which have a negative charge or no net charge at all (Fig. 3a).

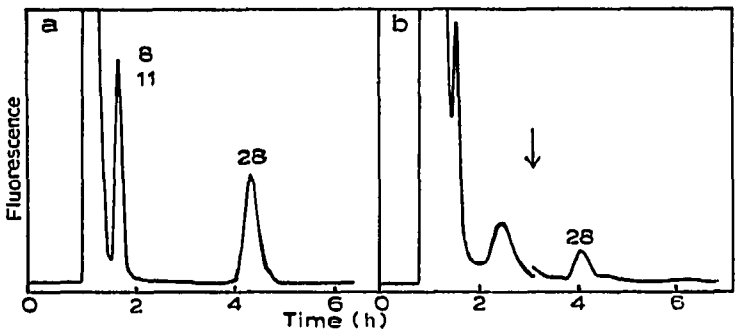

Fig. 3. (a) Elution of some dansyl amino acids, dansyl-catecholamines and monodansyl-histamine. Dansyl catecholamines were eluted in the first peak. (b) Elution of dansylated histamine fraction of rat liver. Chromatographic conditions are given in Table II. The numbers on the peaks represent the dansyl derivatives of histidine (8, monodansyl), arginine (11) and histamine (28). Sensitivity of the detector was doubled at the time indicated by an arrow.

From the elution patterns shown in Figs. 1–3, it may be inferred that non-polar interaction between the solute and the resin matrix, and ion exclusion from the resin phase of more negatively-charged dansyl derivatives, play an important role in this type of separation.

The application of this method to the determination of dansyl amino acids in the acid hydrolyzate of dansylated peptides and proteins is in progress. Its application to the quantitative determination of catecholamines in extract of mouse brain has not been successful so far, but the quantitative determination of histamine as its monodansyl derivative has been successful. The height of the peak of monodansyl-histamine was proportional to the amount of histamine dansylated (1–5 nmole). Analysis of the dansylated histamine fraction from a rat liver extract showed a peak of monodansyl-histamine well separated from the larger preceding peak.

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