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COLUMN CHROMATOGRAPHIC SEPARATION OF DANSYL AMINO ACIDS

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SUMMARY

A column chromatographic separation of dansyl amino acids is described in which polyamide is used as the sorbent and three systems with different benzene–acetic acid ratios are used as the eluent. The separation method offers good possibilities for quantitative determinations. Additional correlations can be made by using the same material for identifying an unknown amino acid. The column effluent is monitored by an adapted Farrand spectrofluorimeter equipped with a flow-through cell and a reduced chart speed gear box.

INTRODUCTION

Dansylation has become the most widely used method for the determination of the free N-terminal amino acids. Although various flat-bed techniques, including one- and two-dimensional chromatography on alumina and silica gel layers, chromatography on polyamide sheets and paper electrophoresis, are commonly used today (for a review, see ROSMUS AND DEYL[1]), there is still a considerable danger of introducing errors into the method of identification of N-terminal amino acids. This can be lowered considerably by using the double-checking technique described by PATAKI[2] for dansyl derivatives and for other methods of N-terminal analysis by DEYL[3].

On the other hand, the high sensitivity of the dansylation procedure makes this technique extremely useful for the analysis of trace materials. In order to obtain the most reliable results, the technique of column chromatographic separation of these derivatives has been developed and is described this paper.

MATERIALS AND METHODS

Apparatus

Column preparation and operation. The column used had dimensions of 100 × 1 cm and was adjusted for constant-temperature operation (35°); it was filled with Woelm polyamide (15 g). As the degree of separation obtained is considerably influenced by the method of column packing used, a special device developed by KESNER[4] for uniform column filling was used. The pouring device consists of an

infusion bottle fitted with a stirrer blade that extends below the ball-joint. The infusion bottle was filled with about 50 ml of distilled benzene and the portion of polyamide suspension was added. Adequate amounts of the sorbent were retained for sample application. The infusion bottle was then closed, the lower tap joining the column head with the infusion bottle was opened and polyamide particles were allowed to sediment under gravity. The stirrer speed was adjusted to maintain a clear benzene layer above the polyamide suspension. After the level of polyamide had reached the column head, the upper tap was closed, the filling device was removed and the excess of benzene was gently aspirated out. As the quality of the column packing influences the final separation to a great extent, the packing has to be carried out carefully so as to remove particles that might adhere to the column walls during the filling procedure. It is also advisable not to touch the sedimenting layer of polyamide in order to prevent uneven particle distribution and consequent deformation of bands during chromatography.

After the column had been filled, benzene was pumped through it for about 1 h in order to pack it. During the packing procedure, the flow-rate was kept at 2.5 ml min^{-1} and during operation of the column the flow-rate was decreased to 0.1 ml min^{-1}. During the packing procedure, the thermostat was set at 35° and the column, the mixing chamber and the reservoirs were adjusted to this temperature. The outlet of the column was connected to an adapted Farrand spectrofluorimeter cell; as in most instances the fluorescence intensity was much too high for the recorder scale, a proportional pump was inserted. The excess of the outflow from the column was either discarded or retained in a fraction collector for further investigation by flat-

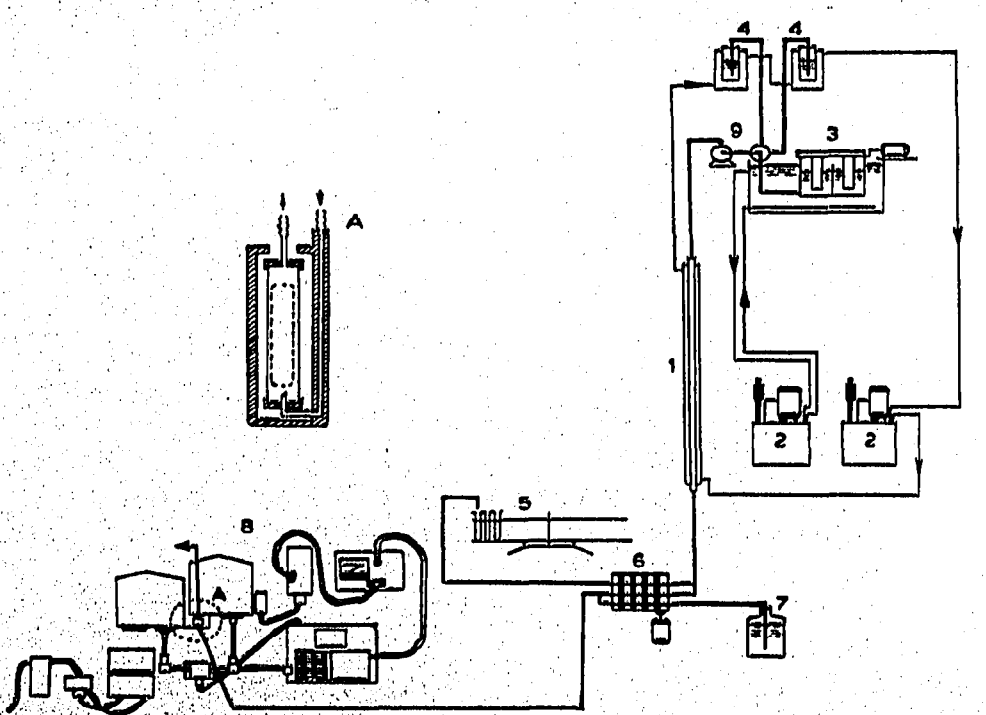

Fig. 1. The over-all assembly of the chromatographic equipment. 1 = Separation column; 2 = thermostats; 3 = gradient device and reservoirs (benzene–acetic acid gradient); 4 = reservoirs for benzene–acetic acid (9:1 and 6:4 systems); 5 = fraction collector; 6 = proportional pump; 7 = acetone reservoir; 8 = Farrand fluorimeter (detail A — flow-through cuvette); 9 = programmed three-way tap.

bed techniques. The fluorescence wavelengths were set to 340 and 500 nm for excitation and luminescence, respectively. The Farrand spectrofluorimeter was alternatively set for a decreased sensitivity (1.0 position on the sensitivity scale), and the proportional pump was by-passed. The outlet flow was diluted with acetone or methyl cellosolve from an additional reservoir. The individual parts used for the split-stream procedure and all the tubings used were parts of the Technicon amino acid analyzer. The over-all assembly of the apparatus is shown schematically in Fig. 1.

The measuring cuvette was adapted from a 5-mm round-shaped quartz tube in a manner shown in Fig. 1. The spectrofluorimeter gear box was adapted to a lower speed, 20 cm h^{-1}.

Sample preparation

The amount of sample analysed varied from 50 to 500 μl. The dansyl derivatives of both amino-terminal and standard amino acids were prepared according to the procedure described several times[6] previously. A peptide or standard amino acid mixture (0.5–5 mmoles of each component to be detected in 0.1 *M* sodium hydrogen carbonate solution) was evaporated to dryness *in vacuo*. This step removes ammonia that might otherwise result in considerable problems when the peak of dansyl amide is being eluted during column regeneration. The dry residue was redissolved in 10–15 μl of ammonia-free water and an equal volume of the dansyl reagent (a saturated solution of dansyl chloride in acetone) was added. The mixture was incubated at 37° for 1 h and the excess of the unreacted dansyl chloride was extracted with 500 μl of toluene or ethyl acetate. The extraction step was repeated three times. The whole series of operations was carried out with protection against direct light. With proteins and peptides for which hydrolysis was necessary, this hydrolysis was carried out at 105° for 18 h in a nitrogen atmosphere in a sealed tube. The sample was finally evaporated to dryness, redissolved in 1–2 ml of water and mixed with a portion of the retained polyamide to make an opaque suspension, and the mixture was evaporated to dryness at 40° *in vacuo*. The dry residue was resuspended in benzene and loaded on to the separation column by several washings with 10-ml volumes of benzene.

Elution

As the solvent system used by WOODS AND WANG[5] in thin-layer chromatography did not result in complete resolution of all the amino acid peaks, different proportions of benzene–acetic acid were examined. The most generally applicable was a mixture of benzene–acetic acid (90:5) in which, however, the fast-moving peaks of leucine and isoleucine were not separated. In order to improve this situation, elution was started with a benzene/benzene–acetic acid (9:1) gradient, composed of two 200-ml mixed reservoirs. After 300 min the inlet was switched automatically to the 9:1 benzene–acetic acid mixture and elution was carried out for the next 800 min without a gradient. In the final stage, this eluant was suddenly switched to a benzene–acetic acid (6:4) mixture, which made it possible to elute asparagine, hydroxyproline, arginine, cysteine and cysteic acid. The bluish band of dansyl amide remained uneluted and was removed during the regeneration procedure.

Column regeneration

Before re-use, the column was washed with dried acetone (1.5 h was satis-

factory). The flow-rate of the washing fluid was 1.5 ml min^{-1}. Acetone was then replaced by benzene, which was passed through the column for an additional 2 h. After this period, the column was ready for use in a new separation.

RESULTS AND DISCUSSION

The gradient elution system exhibits several advantages compared with the widely used flat-bed techniques. Firstly, it minimizes the possibility of inducing errors, as the separation is very precise and can be easily completed with an additional flat-bed check-up by using the same material, which is therefore not lost and the demands on the amount to be analyzed are consequently very reasonable. Another advantage is based on the fact that the column technique gives a good possibility of recovering unusual amino acids or hydrolysis-resistant peptides, which may be of considerable importance in special cases such as in the analysis of complex peptide mixtures. As indicated in Fig. 2, this technique offers the possibility of separating almost all common amino acids in one run, and under standard conditions the technique can also be used for quantitative determinations. These advantages are, of course, obtained at the cost of using more complicated equipment and slightly larger samples for analysis (at least twice as much as in the flat-bed technique).

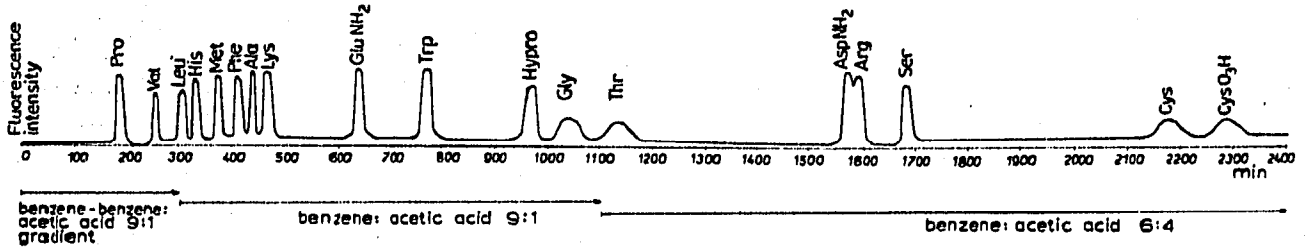

Fig. 2. Typical elution profile of dansyl amino acids on a polyamide column.

As in every separation of a complex mixture there are pairs of dansyl derivatives which are hard to separate, such as phenylalanine and a number of others with high chromatographic mobilities. In order to achieve adequate separations, which may be subjected to quantitation by using the technique common in non-derivatized amino acid analysis, one has to work in the range of *ca.* 2000 theoretical plates. An improved separation has been obtained by introducing a gradient system at the beginning of the chromatographic run. The operating times and solvent systems used are as follows: 0–300 min — gradient of benzene/benzene–acetic acid (9:1), 200 ml of each solvent; 300–1100 min—benzene–acetic acid (9:1); and 1100–2500 min — benzene–acetic acid (6:4).

Chromatographic properties of the individual solvent systems used are summarized in Table I.

Although no precise rules for predicting chromatographic mobility can be formulated, there are some general features which, in the case of an unknown derivative, may serve as a guide. An increase in the number of carbon atoms in the amino-acid side-chain decreases the retention times. Compared with a straight chain, the $-CH_2-$ difference in a branched side-chain has a much lower effect in decreasing the retention time. Hydroxylation, however, shifts retention times to much higher

values and the differences in a homologous series are increased in hydroxylated amino acids. While the presence of a second amino group makes the amino acid move with a low retention time, guanidylation considerably retards the chromatographic mobility.

TABLE I

RETENTION VOLUMES AND RELATIVE RETENTION VOLUMES OF DANSYL AMINO ACIDS

Amino acid	*Benzene–acetic acid (9:1)*		*Benzene–acetic acid (6:4)*	
	V_e[a]	V_e/V_{pro}[b]	V_e	V_e/V_{pro}
Leu	138	1.53_5	80	1.02_5
Val	124	1.38	82.5	1.05_5
His	143	1.64_5	—	—
Met	152	1.69	81	1.04
Ala	163	1.81	92	1.08
Lys	196	2.18	108	1.38
Gly	765	8.39	117	1.50
Trp	500	5.55	134	1.71_5
Thr	865	9.60	142	1.82
Ser	2540	28.21	485	6.21
Cys	—	—	980	12.57
Arg	—	—	384	4.92
Hyp	700	7.76	—	—
Pro	90	1	78	1
Phe	155	1.72	85	1.09
Gln	370	4.12	126	1.61_5
Asn	1350	15.00	370	4.74

[a] V_e = elution volume.
[b] V_e/V_{pro} = elution volume relative to proline.

The column filling is able to withstand an almost unlimited number of separations provided that not too many impurities are loaded on to the column in each run and provided that they (mainly dansyl amide) are adequately eluted during the regeneration procedure. After several separations, a grey-brown ring appears at the top of the column filling; however, this does not disturb the separation.

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