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Note

New high-performance liquid chromatography system for the separation of biogenic amines as their Dns derivatives

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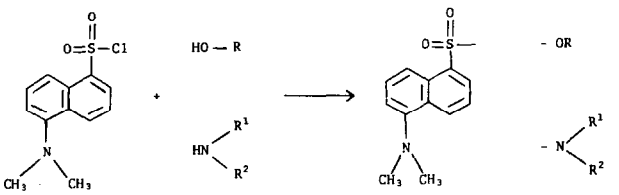
Technical advances have greatly increased the number of biogenic amines that can be demonstrated in tissue extracts and physiological fluids so a generally applicable routine technique for their separation and determination is of growing importance in animal, medical and plant biochemistry. Free amines can be separated most simply by paper electrophoresis[1] or paper chromatography[2] and thin-layer chromatography (TLC)[3,4]. However, they are highly polar substances and tend to streak, so limiting the resolution of these techniques, which are, in any case, not easy to use quantitatively. Both problems can largely be overcome by forming derivatives, which have better chromatographic properties than the free amines, and separating them by high-performance liquid chromatography (HPLC).

Several pre-column derivatization reagents have already been recommended including dabsyl[5]- and Dns[6,7] chlorides, *o*-phthalaldehyde[8,9], fluorescamine[10,11], *m*-toluoyl chloride[12] and phenyl isocyanate[13]. Dns derivatives were chosen for this work as they are chemically stable, have good chromatographic properties, can readily be freed from salts by solvent extraction, may be detected fluorimetrically at the nanogram level and can be formed from both primary and secondary amines. The relevant reagent, Dns chloride (5-dimethylaminonaphthalene-1-sulphonyl chloride), also reacts with phenolic hydroxy groups, the basic equations being:



Previous workers separating Dns amines by LC have limited themselves to a subclass, like the catecholamines[14,15] or polyamines[16,17]. The most impressive existing system has been that of Seiler *et al.*[18], which will separate up to 15 di- and polyamines simultaneously.

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EXPERIMENTAL

A Varian (Walnut Creek, CA, U.S.A.) HPLC system was used for the analytical separations. This consisted of an LC 5000 solvent delivery system and solvent programmer, and 10-μl sample loop injector, to which was coupled a Fluorichrom™ fluorescence detector and a Pharmacia dual-channel chart recorder (S-751 82, Uppsala, Sweden).

The column was a reversed-phase Spherisorb C_{18} one (5 μm ODS, 25 cm × 5 mm I.D.), supplied by HPLC Technology (Macclesfield, U.K.) protected by a guard column (5 cm × 5 mm I.D.) with Vydac reversed-phase packing.

Chemicals

Acetonitrile and the water used to prepare the buffer were normally HPLC-grade, though ordinary glass distilled water was an adequate substitute for the latter. The disodium hydrogen phosphate like all the other chemicals used, was of normal reagent-grade. Dns chloride was supplied by Sigma (Poole, U.K.) but evertything else came from BDH (Poole, U.K.).

Derivatization

The procedure was a modified form of that of Seiler and Wiechmann[19]. Aqueous solutions of amines, each at *ca.* 50 nmoles ml^{-1}, 200 μl, were "whirlimixed" with 400 μl Dns chloride (5 mg ml^{-1} in acetone) and then saturated with solid sodium carbonate. After leaving in the dark at 20 ± 5°C for 15 h, 400 μl 15% aqueous proline was added to react with residual Dns chloride. The mixture was "whirlimixed" and left in the dark as before for at least an hour. Dansylated amines were extracted with 0.5 ml ethyl acetate, leaving the Dns proline behind in the aqueous phase.

HPLC analysis

Aliquots (10 μl) of the ethyl acetate layer, containing *ca.* 0.2 nmoles of each dansylated amine, were introduced onto the HPLC column. The pH 7.17, 17.5 m*M* phosphate buffer used as one component of the mobile phase was prepared by titrating aqueous disodium hydrogen phosphate to pH with glacial acetic acid. This buffer was mixed with acetonitrile to give a gradient composed of a series of isocratic and linear steps which had the following acetonitrile concentrations (all v/v) at the times stated: 0 min, 30%; 2 min, 42%; 24 min, 42%; 25 min, 45%; 32 min, 45%; 52 min, 55%; 53 min, 65%; 64 min, 65%; 70 min, 80%; 78 min, 80%; 84 min, 30%. The shape of the gradient is critical and a smoother one will not separate so many components. This mobile phase was pumped through the column, which was maintained at 35°C, at a flow-rate of 1.5 ml min^{-1}, giving an operating pressure falling from 18–8 MPa (180–80 atm) during the run.

The system was optimised using a complete mixture of 45 dansylated amines and the peaks were identified from the retention times of derivatives chromatographed individually. Ambiguous identifications were checked by "spiking" the standard mixture.

RESULTS AND DISCUSSION

Fig. 1 shows that the system was able to separate 29 Dns amines out of the 45 tested and Table I gives the elution times of them all to the nearest 0.25 min.

One advantage of the new procedure is that each amine gives essentially only one peak. The subsidiary dansylation products that are often prominent during TLC are recorded by the quantitative detector as being so minor that they usually disappear into the base line. One exception is dopamine, which yields three derivatives: the major one elutes at 74 min while the others, appearing at 56 and 65 min, are 5% and 8% of its height respectively. Spermine also gives two subsidiary peaks, each *ca.* 7% of the main one, eluting at 63 and 73.5 min. One sample of agmatine did produce two almost equal peaks at 26.5 and 44.5 min. However, a second preparation produced only the 26.5 min component: the first sample was obviously contaminated with putrescine. In no other case was there a subsidiary peak more than 5% of the height of the main one.

Peaks representing the Dns derivatives of *ca.* 0.03 nmoles ammonia, 0.1 nmole methylamine and 0.1 nmole dimethylamine always appear. These are well known artifacts of the dansylation reaction and can even be considered useful as reference points. There is an additional, unidentified, artifact at 11.25 min, fused with but somewhat smaller than the methylamine peak. The component that elutes at 2 min is probably unreacted Dns chloride: Dns chloride certainly appears in this position when chromatographed on its own.

The minimum detectable quantities of each amine, at the instrument settings

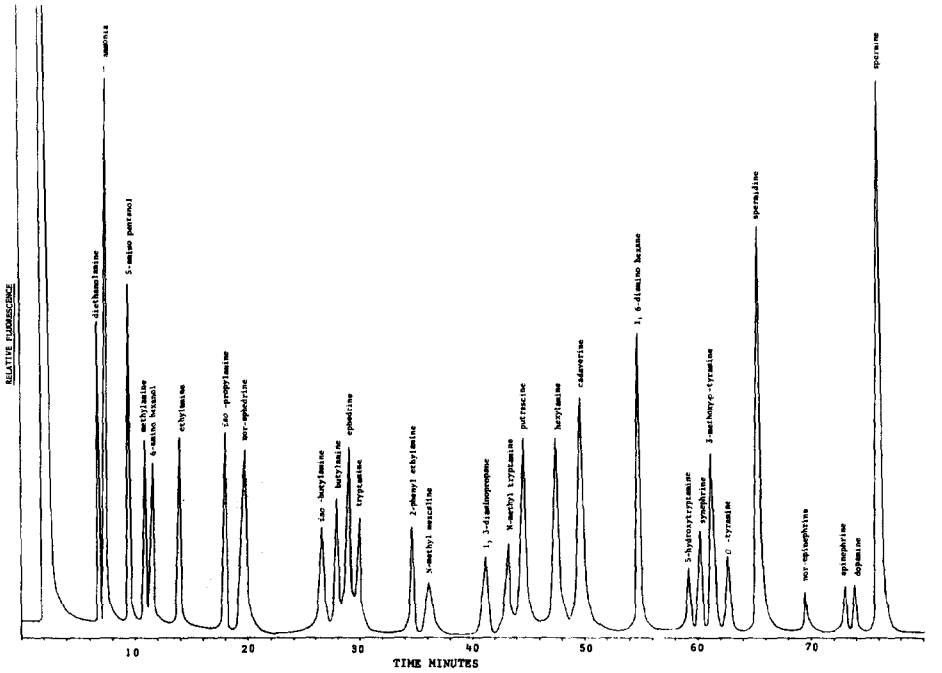

Fig. 1. Elution profile for the separation of dansylated amines on Spherisorb by acetonitrile–phosphate buffer. Experimental conditions are described in the text. Each peak represents *ca.* 0.2 nmoles.

TABLE I

RETENTION TIMES OF DANSYLATED AMINES

Dns amine	*Retention time (min)*	*Dns amine*	*Retention time (min)*
Diethanolamine	6.75	1,2-Diaminopropane	41.50
Ethanolamine	7.00	N-Methyltryptamine	43.00
Ammonium chloride	7.50	Putrescine	44.50
5-Aminopentan-1-ol	9.25	*n*-Hexylamine	47.00
Methylamine	10.75	Cadaverine	49.50
6-Aminohexan-1-ol	11.50	Normetanephrine	54.00
Ethylamine	14.00	Cystamine	54.50
Isopropylamine	18.00	1,6-Diaminohexane	55.00
Dimethylamine	19.00	Octopamine	55.75
n-Propylamine	19.75	5-Hydroxytryptamine	59.00
Norephedrine	20.00	Metanephrine	59.25
Isobutylamine	26.50	Synephrine	60.00
Agmatine	26.50	*n*-Octylamine	60.50
Lysine	27.25	3-Methoxy-*p*-tyramine	61.25
n-Butylamine	27.50	*p*-Tyramine	62.25
Benzylamine	27.50	*m*-Tyramine	62.25
6-Methoxytryptamine	28.50	*o*-Tyramine	62.75
Ephedrine	29.00	Spermidine	65.00
Tryptamine	30.00	Norepinephrine	69.50
2-Phenylethylamine	34.50	Epinephrine	73.00
Isoamylamine	35.50	Dopamine	74.00
N-Methylmescaline	36.00	Spermine	75.50
1,3-Diaminopropane	41.00		

used, averaged 20 pmoles, ranging from 100 pmoles in unfavourable cases, as for the catecholamines, to 5 pmoles for favourable ones, like the polyamines. However, it would be easy to substantially improve on these sensitivities.

The system gives uniformly sharp peaks for amines of many types. It adequately separates the catecholamines, which are found in a characteristic region of the elution profile. Similarly it will resolve the homologous series of aliphatic *n*-monoamines up to at least C_8 and straight-chain aliphatic diamines up to C_6. It has some capacity to discriminate between straight- and branched-chain isomers but, at least in the case of the tyramines, is insensitive to the position of substituents on aromatic rings. Despite this, aromatic amines, like the aliphatics, are well spread out through the elution profile.

There is a tendency for the simple aliphatic amines and amino alcohols to be eluted first, followed by the more complex and more physiologically active components. This is fortunate as the former are often present in tissues at much higher concentrations than the latter: initial attempts to apply the system to plant extracts have been encouraging.

Dns derivatives that run close together on TLC plates, like those of benzylamine–phenylethylamine and ammonia–1,2-diaminopropane are often well separated by this system so TLC and HPLC complement each other.

CONCLUSION

The system still has some development potential: it would almost certainly be possible to separate more dansylated amines in one run, given longer elution times, and some of the rarer biogenic amines may well fall in the gaps in the elution pattern. However, even in its present form, the procedure confortably exceeds the resolving power of any previously published LC system for the separation of free or derivatised amines. The best competing systems are those of Mitchell and Gray[20] and Villaneuva and Adlakha[21] which can separate 20 and 18 free biogenic amines in 28 and 3 h, respectively. Mayer and Pause[22] achieved an equivalent performance by separating amines from wine as their *o*-phthalaldehyde derivatives. So far LC methods for other types of amine derivative have been even less successful.

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