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Short Communication

Mapping of derivatised biogenic amines by two-dimensional thin-layer chromatography

A comparative study

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ABSTRACT

The analysis of amines is greatly enhanced by the formation of derivatives with improved chromatographic properties. We have used dansyl chloride, dabsyl chloride, and 7-chloro-4-nitrobenzoxazole to derivatise fourteen structurally diverse. amines which occur in biological systems, and have made a comparative study of their thin-layer chromatographic properties. Two-dimensional TIC maps of derivatised amines are presented for the three different procedures, and some factors which influence sensitivity are evaluated. On grounds of sensitivity of detection and chemical stability of the derivatives formed, dansyl chloride is the preferred reagent for chromatographic analysis of complex mixtures of biogenic amines.

INTRODUCTION

Biogenic amines, which include mono-, di-, and polyamines, catecholamines, indoleamines, and imidazoles [1] are difficult to analyse by a single chromatographic technique because of their structural diversity and lack of an easily detectable common chromophore. The usual approach, therefore, has been to derivatise free amines with an easily detected label group (for reviews, see refs. 2 and 3).

An ideal label should be specific for amino groups, forming highly coloured or fluorescent derivatives to allow sensitive detection. Excess reagent and by-products should be colourless, non-fluorescent, or easily eliminated. Non-polar derivatives are desirable to improve chromatographic properties. Chemically stable derivatives suitable for NMR and mass spectroscopic identification are also advantageous.

Three of the more common derivatising agents used are dansyl chloride [4,5], dabsyl chloride [6], and 7-chloro-4-nitrobenzoxazole (NBD chloride) [7]. Unlike fluorescamine [S] or o-phthalaldehyde [9] these have the advantage of forming derivatives with both primary and secondary amines, and all three give derivatives

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stable enough for subsequent spectroscopic analysis [10,11].

Here we describe two-dimensional (2D) TLC systems optimised to separate amines after derivatisation with dansyl chloride, dabsyl chloride, or NBD chloride. Thin-layer chromatography has the advantage of being rapid, simple, and economical. Although much has been published on the TLC separation of dansylated amines [12,13] far less information is available on dabsyl or NBD derivatives, and no comparative study has been previously undertaken.

EXPERIMENTAL

Materials

Chemicals were obtained from Aldrich (Poole, UK). Amine hydrochlorides (100 μg/ml) were prepared as standards. Silica 60 (250 μm) TLC plates (20 x 20 cm) were obtained from Sigma (Poole, UK). Glass distilled water was used throughout.

Derivatisation

For dansylation, aliquots of aqueous amine solutions (=20 μg) were saturated with sodium hydrogen carbonate and reacted for 15 h at room temperature with dansyl chloride in acetone (800 μl, 5 mg/ml), then warmed (60°C, 10 min) to complete the reaction and remove excess acetone. After dilution to 1 ml with water the dansylated amines were extracted with 3 × 2 ml toluene. When necessary the phases were separated by centrifugation. The organic phases were evaporated to dryness under a stream of air and stored in the dark at -20°C.

Derivatisation with dabsyl chloride and NBD chloride were similar to dansylation except that the derivatising agents used were dabsyl chloride in acetone (800 μl, 5 mg/ml), or NBD chloride in ethanol (800 μl, 5 mg/ml). NBD-amines were extracted with ethyl acetate rather than toluene. At all stages exposure to light was kept to a minimum.

Thin-layer chromatography

Derivatised amines were redissolved in ethyl acetate and applied to TLC plates, which were developed in the dark. Fluorescent spots were visualised under 360 nm UV light. Where appropriate plates were sprayed with triethanolamine in n-propanol (20%, v/v); glacial acetic acid in n-propanol (20%, v/v); or hydrochloric acid in methanol (3%, v/v).

Solvent systems used for TLC were as follows: (1) ethyl acetate-cyclohexane (3:2, v/v); (2) benzene-triethylamine (5:1, v/v); (3) ethanol–15 *M* ammonia (1:1, v/v); (4) ethanol-acetic acid (1:1, v/v); (5) toluene-acetic acid (1:1, v/v); (6) n-butyl acetate; (7) toluene; (8) toluene-acetic acid (2:1, v/v); (9) toluene–acetone (4:1, v/v).

TLC plates eluted with aqueous pH buffers were dried at 110°C and cooled before visualising the spots under 360 nm UV light. Buffers were prepared as follows: formic acid-acetic acid–water (4:9:70, v/v/v; pH 2); aqueous potassium hydrogen phthalate (10 g/l; pH 4); phosphate (1.13 g/l Na_2HPO_4; 8 g/l K_2HPO_4; pH 6); and borate (3 g/l boric acid; 1.5 g/l NaOH; pH 10).

pH dependent absorption maximum of dabsyl hexanolamine

Dabsyl hexanolamine (4.88 mg, 10.9 μmol) prepared from dansyl chloride and hexanolamine hydrochloride was purified by two rounds of preparative TLC on silica 60. The purity was verified by proton NMR spectroscopy (data not shown). This was dissolved in aqueous acetonitrile (60%, v/v) and split into five equal aliquots. Aqueous buffers were added (either pH 4, 3, 2.5, 2 or 1; 100 μl), and the concentrations adjusted to 0.73 μmol/ml with acetonitrile. Absorbances were recorded between 390-530 nm on a Pye Unicam SP8100 UV visible spectrophotometer.

RESULTS AND DISCUSSION

The amines chosen for study (listed in Table I) were derivatised with the appropriate reagents as described in the experimental section. Chromatographic separation was initially evaluated by the procedure of Alberts *et al.* [13]. Dansylated and dabsylated derivatives were found to have similar TLC properties and were adequately resolved. NBD-amines, however, were resolved satisfactorally in solvent 1, but not in solvent 2,

TABLE I

MOBILITIES OF NBD-AMINES ON SILICA 60 TLC PLATES

NBD-derivatised amines	Relative mobilities (R_F)[a]				
	1	5	6	8	9
Ammonia	0.26	0.06	0.27	0.45	0.27
Methylamine	0.26	0.17	0.39	0.57	0.37
Ethylamine	0.38	0.24	0.52	0.63	0.45
Ethanolamine	0.07	0.02	0.12	0.30	0.14
n-Butylamine	0.55	0.33	0.69	0.71	0.54
Isobutylamine	0.55	0.32	0.69	0.71	0.54
Futrescine	0.09	0.20	0.38	0.31	0.20
Cadaverine	0.12	0.21	0.39	0.34	0.24
p-Tyramine	0.17	0.08	0.34	0.48	0.34
3-MeO-*p*-tyramine	0.17	0.08	0.34	0.48	0.34
Tryptamine	0.46	n.d.	0.65	0.55	0.36
Spermidine	0.09	n.d.	0.32	0.27	0.17
Spermine	0.09	n.d.	0.34	0.30	0.24
Histamine	0.00	n.d.	n.d.	0.30	0.07

[a]Numbers at the head of each column refer to the solvent system used, as designated in the Experimental section. n.d. = Not determined.

where only five spots moved at all (see Table I). The NBD-amines were initially re-chromatographed in solvent 3 and in solvent 4, to compare the effects of basic and acidic conditions. Little difference was seen between these two systems, with all the spots running too fast. Mobilities in toluene-acetic acid (solvent 5) were assessed to determine the effect of a less polar solvent. Here all the components ran slowly suggesting that polarity rather than pH was the major factor determining elution rate. This is perhaps confirmed by the R_F values obtained subsequently in n-butyl acetate (solvent 6) and in toluene (solvent 7). Elution was faster in the relatively polar ester than in the more hydrophobic aromatic solvent.

Solvent 8 [toluene-acetone 2:1 (v/v)] and solvent 9 [toluene-acetone 4:1 (v/v)] were assessed, and although both systems improved resolution some spots eluted too fast in solvent 8. It was concluded that solvent 9 was the most useful solvent mixture devised. R_F values for NBD-amines in the more useful solvent systems tested are listed in Table I.

An optimised 2D-TLC system for NBD-amines was therefore assessed, run in the first direction with solvent 1 and in the second with solvent 9. R_F values were measured and a 2D-TLC map was constructed for this combination (Fig. 1). Comparing the resolving power of this new 2D-TLC system for NBD-amines with that for dansyl-amines (Fig. 2) and dabsyl-amines (Fig. 3) showed that of the fourteen amines chosen for comparison ten were resolved as their dansyl or NBD derivatives, and eight as their dabsyl derivatives. A major problem with the dansyl and dabsyl systems was inadequate separation of the ammonia and polyamine derivatives (especially spermidine, cadaverine and putrescine). NBD-polyamines were better resolved, and completely separated from NBD-ammonia. However, p-tyramine and 3-methoxy-*p*-tyramine could not be resolved as their NBD derivatives, but were separated by both the dansyl and dabsyl systems. None of the 2D-TLC systems investigated were able to separate the derivatives of the two butylamine isomers.

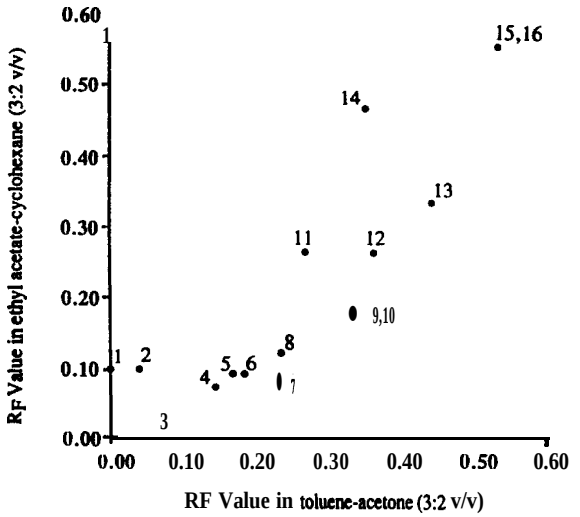

Fig. 1. Two-dimensional TLC map of NBD-amines on silica 60. NBD-derivatives are referred to by number as follows: 1 = NBD-OH; 2 = NBD-Cl; 3 = histamine; 4 = ethanolamine; 5 = spermidme; 6 = putrescine; 7 = spermine; 8 = cadaverine; 9 = p-tyramine; 10 = 3-methoxy-*p*-tyramine; 11 = ammonia; 12 = methylamine; 13 = ethylamine; 14 = tryptamine; 15 = *n*-butylamine; 16 = isobutylamine. Derivatisation and chromatographic conditions are described in the text.

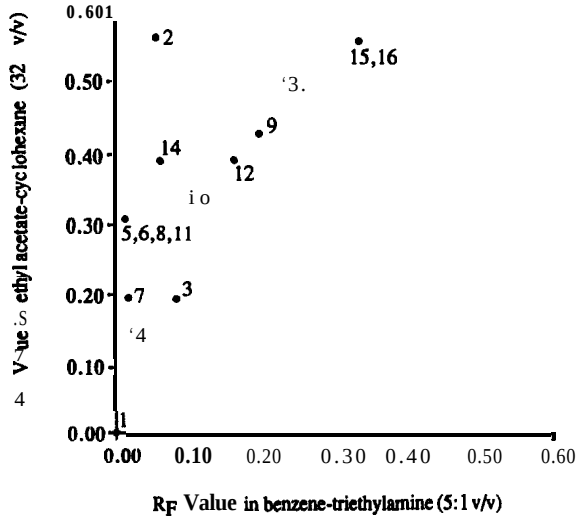

Fig. 2. Two-dimensional TLC map of dansyl-amines on silica 60. Reference numbers are: 1 = dansyl-OH; and 2 = dansyl-Cl. Dansylated amines are numbered as in Fig. 1.

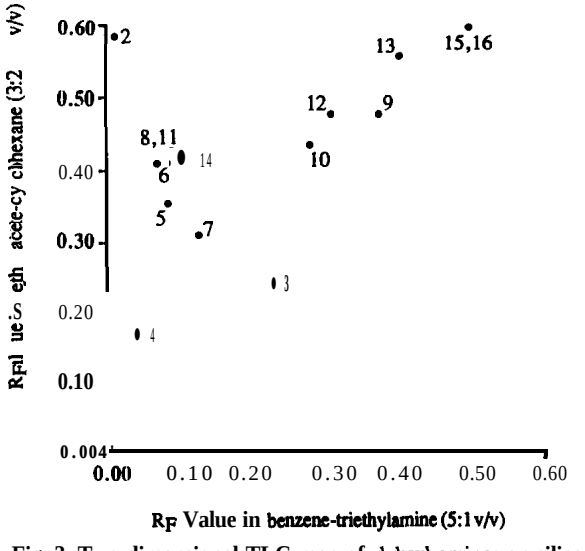

Fig. 3. Two-dimensional TLC map of dabsyl-amines on silica 60. Reference numbers are: 1 = dabsyl-OH; and 2 = dabsyl-Cl. Dabsylated amines are numbered as in Fig. 1.

Separation of NBD-amines with aqueous buffers

When separated in organic solvents NBD-amines tended to distribute across plates as a diagonal. However, their relatively high polarity suggested the possibility of using aqueous pH buffers as the second solvent for 2D-TLC, thereby making the separation dependent on charge rather than polarity. Dansyl and dabsyl derivatives were too non-polar to be separated by aqueous TLC systems.

NBD-amine derivatives were separated on Silica 60 plates using four different buffers (pH 2, 4, 6 or 10). The results are presented in Table II. The reagent blank gave a major non-fluorescent, yellow spot (presumably NBD-OH) that ran with the mobile phase front during chromatography. Most of the NBD-amines gave only one fluorescent spot. However, several proved to be non-fluorescent or had low fluorescent intensity, and consequently were difficult to detect. This, and the generally poor resolution obtained, particularly at high pH, suggested that the procedure was not useful for resolving NBD-amines.

Relative sensitivity of the derivatising reagents

To compare minimum detection levels spots of derivatised ethylamine (0.2-5 ng) were eluted on Silica 60 in solvent 1. Detection was assessed visually either under 360 nm UV light or, in the case of dabsylated ethylamine, under normal

TABLE II

pH-DEPENDENT TLC MOBILITIES OF NBD-AMINES ELUTED WITH AQUEOUS BUFFERS

Chromatographs were run on Silica 60 plates eluted with aqueous pH buffers. Buffers are described in the methods section. Diethanolamine, *tert.*-butylamine and di-N-butylamine gave non-fluorescent derivatives.

Amine derivative	Relative mobiities (R_F)			
	pH 2	pH 4	PH6	pH 10
Methylamine	0.66	0.28	0.37	streaked
Ethylamine	0.59	0.16	0.38	streaked
n-Propylamine	0.56	0.12	0.38	streaked
Isopropylamine	0.55	0.12	0.36	streaked
n-Butylamine	0.51	0.09	0.35	streaked
Isobutylamine	0.55	0.13	0.37	streaked
sec.-Butylamine	0.52	0.12	0.36	streaked
tert.-Butylamine	–	–		
Isoamyllamine	0.49	0.12	0.36	streaked
n-Octylamine	0.34	0.09	0.03	streaked
Ethanolamine	0.32	0.06	0.02	streaked
Dimethylamine	0.47	0.19	0.35	streaked
Diethylamine	0.43	0.13	0.34	streaked
Di-N-butylamine			– – – –	
Diethanolamine		– – –		–

daylight. The fluorescence from dansyl-ethylamine and NBD-ethylamine was visible down to 0.5 ng (approximately 27 pmol for either compound). Dabsyl-ethylamine could be detected by its yellow colour down to 3 ng (162 pmol), but this was extended to 1.5 ng (81 pmol) after spraying with 3% (v/v) HCl in methanol. Similar results were obtained for dansyl-p-tyramine (lower detection limit 0.25–0.5 ng) and for dabsyl-p-tyramine (5-12 ng) indicating that dansyl- and NBD-ethylamine can be detected about 3-5 times more sensitively than dabsyl-ethylamines.

Sensitivity of detection of other amines was assessed by visual comparison with the corresponding ethylamine derivatives, dansyl-amines or dabsyl-amines could all be detected at about the same sensitivity. However, the fluorescent intensities of NBD-amines were far more variable. Bulky amines, such as *tert.*-butylamine, di-n-butylamine, and di-ethanolamine gave non-fluorescent NBD derivatives while aromatic amine derivatives gave only low intensity fluorescence. Rather more surprisingly di- and poly-amines also gave low intensity fluorescent NBD derivatives. Ghosh and Whitehouse [14] have suggested that the fluorescence of NBD-amines decreases if the amine moiety is out of plane with the NBD ring. Bulky amines or multi-derivatised polyamines are too sterically crowded to allow planarity and therefore only NBD-alkylamines had fluorescent intensities approaching those of the corresponding dansyl-amines.

The fluorescent intensities of dansyl-amines are pH dependent, but can be stabilised by spraying with n-propanol-triethanolamine (4: 1, v/v) [15]. This reagent had little effect on the colours of dabsyl-amines and NBD-amines, although the fluorescence of the NBD-amines decreased in intensity. The intensity was restored (but not enhanced) by subsequent spraying with either acetic acid-n-propanol (20%, v/v), or methanolic HCl (3%, v/v).

Acid-sprayed dabsylated amines all became easier to detect as the orange-yellow spots deepened in colour to red. To investigate this further the absorption spectra of a known concentration of purified dabsyl-hexanolamine (0.73 μg) were measured in five different pH buffers.

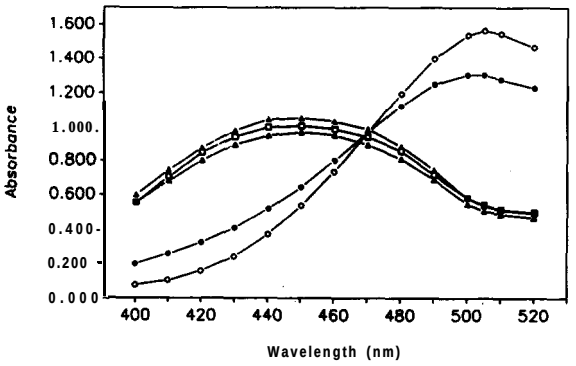

Fig. 4. pH-dependent shift of absorption maximum of dabsyl-hexanolamine. Samples of dabsyl-hexanolamine dissolved in aqueous acetonitrile (60%, v/v) were adjusted to the appropriate pH with the following buffers: (□) pH 4: potassium hydrogen phthalate (10.2 g/l); (●) pH 2: formic acid-acetic acid-water (4:9:70 v/v/v); (0) pH 1: 0.1 M HCI. (A) pH 3 and (A) **pH 2.5 buffers were prepared by acidifying pH 4 buffer with HCI. Absorbance measurements were scanned from 390 to 530 nm.**

The absorbance maximum changed sharply at pH 2 from 450 nm to 505 nm (a shift from yellow to red) and, at the same pH, the molar extinction coefficient increased by 50%, suggesting a possible doubling of minimum detection limits (Fig. 4). In fact spraying dabsyl-ethylamine on TIC plates with the acidic reagents did lead to an approximate two-fold increase of sensitivity suggesting that the improvement in detection is due to the increased absorptivity of protonated dabsylated amines, rather than red spots simply being an easier to distinguish against a white background.

CONCLUSIONS

Two-dimensional thin-layer chromatography has proved to be a very reliable method for separating amine mixtures as their dansyl derivatives [12,16]. Alberts et *al.* [13] have published an extensive 2D-TLC map showing the positions of 81 dansyl-amines after separation on silica. However, dansyl chloride is not specific to amines but will also derivatise phenols and thiol compounds [15], and also gives rise to fluorescent by-products which can obscure spots of interest. In particular dansyl-ammonia often

elutes at the same rate as dansylated polyamines, and can interfere with their analysis.

Less is known of the side reactions of dabsylation, but dabsyl chloride, its hydrolysis product dabsyl-sulphonic acid (dabsyl-OH), and dabsyl-ammonia are all coloured bright red and therefore potential sources of interference. Dabsyl chloride also reacts with thiol and phenolic compounds.

NBD chloride appears to have many advantages as an analytical derivatising agent. It is selective towards amines because although phenols, anilines, and thiols also react they only give weakly fluorescent derivatives. NBD chloride itself is non-fluorescent, presumably because the electron withdrawing chloro group causes heavy ion quenching.

It was noted that NBD-amines tended to run across 2D-TLC plates in a diagonal regardless of the attempt described to find solvents capable of breaking this pattern. Dansyl and dabsyl derivatives were more randomly distributed when an aliphatic solvent was used in the first direction, and aromatic in the second. The elution rates of the NBD-amines are apparently less sensitive to the aromatic nature of the solvent system used, presumably because the nitrobenzoxazole ring system is less aromatic in character than either the dansyl or dabsyl groups.

Here a comparative investigation of the thin-layer chromatographic properties of various derivatised amines is presented. New 2D-TLC systems for dabsyl- and NBD-amines were developed, and new separating systems for NBD-amines based on pH were investigated. On merit dansyl chloride appears to be the most useful derivatising agent for thin layer chromatographic separation of amines. It fulfils the criteria outlined above, in terms of both sensitivity and, to a lesser extent, resolution. Dabsyl chloride is less suitable because detection was considerably less sensitive, and NBD chloride because of the variable fluorescent intensities, which were dependent upon amine structure.

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