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CHROMATOGRAPHY OF Dns DERIVATIVES ON PRE-COATED HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHIC PLATES

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SUMMARY

5-Dimethylaminonaphthalene-1-sulphonyl (Dns) derivatives of amino acids and amines have been separated on high-performance thin-layer chromatographic (HPTLC) plates. Due to interaction of the matrix, especially with polar compounds and solvents, relative mobilities on HPTLC plates differ from those on silica gel G layers; as these differences are small for non-polar compounds, HPTLC plates can be used for the determination of aliphatic polyamines and allied compounds when using known solvent systems for separation. This paper describes (i) solvent systems for the two-dimensional separation of Dns-amino acids that are suitable for HPTLC and normal silica gel plates, (ii) a horizontal-development technique that allows preparation of several two-dimensional chromatograms on a 10 × 10 cm plate, and (iii) an extraction procedure for the optical evaluation of compounds separated by thin-layer chromatography. The obvious advantages of HPTLC plates are rapid development, high capacity and increase in the signal-to-noise ratio.

INTRODUCTION

5-Dimethylaminonaphthalene-1-sulphonyl chloride (Dns-Cl) is currently among the most important reagents for fluorescent labelling of low-molecular-weight compounds[1]. Since the introduction of thin-layer chromatography (TLC) for the separation of Dns-amino acid and -amine derivatives[2,3], many separations of Dns derivatives have been carried out on silica gel-coated plates and many solvents have been devised for the separation of Dns-amino acids[4–6] and Dns-amines[7,8]. An improvement in the detection of amino acids was the introduction of polyamide sheets[9], on which the small spots that can be produced allow the detection sensitivity to be improved; the separation procedure of Woods and Wang[9], or versions of this method, have become a standard technique for end-group work[10]. However, the low capacity of this layer material, together with the restriction to distribution-chromatography systems, limits the usefulness of polyamide sheets in the analysis of biological materials. Nevertheless, they have been used for the detection of amino acids and certain amines in tissues[11,12].

The recently developed high-performance thin-layer chromatography (HPTLC)

pre-coated plates combine the high capacity and versatility of normal silica gel plates with the small spot-size typical of polyamide layers. It was therefore of interest to ascertain to what extent the experiences of and procedures for assaying biogenic amines and amino acids could be applied to these plates.

MATERIALS

The silica gel 60 F_{254} pre-coated plates (HPTLC) (silica gel pore size 60 Å, layer thickness 200 μm), silica gel G and solvents were obtained from Merck (Darmstadt, G.F.R.); An automated spreader (Camag, Muttenz, Switzerland) was used to prepare 20 × 20 cm TLC plates (layer thickness 300 μm). The Dns-Cl and Dns derivatives of amines were prepared in our laboratory according to known procedures[4,8]. Dns-amino acids were obtained from Sigma (St. Louis, Mo., U.S.A.), spermine phosphate, spermidine phosphate and putrescine hydrochloride from Fluka (Buchs, Switzerland) and 4-aminobutyric acid from Serva (Heidelberg, G.F.R.); monoacetyl putrescine was prepared as described by Tabor *et al.*[13].

RESULTS

Some characteristics of HPTLC plates

The purity of HPTLC plates is inadequate for quantitative work. As shown in Fig. 1, a considerable amount of absorbing and fluorescing material can be moved with methanol to the solvent front, even if the plate is developed immediately after it has been taken out of a fresh package. Washing with methanol is, however, sufficient to purify the plate to an extent adequate for the determination of Dns derivatives. In addition to optical characteristics, mass spectrometry was used as a criterion for adequate purity of the thin layer. Mass spectra were recorded of 1-nmole amounts of bis-Dns-putrescine, which were eluted with 50 μl of ethyl acetate. The background of the spectra was low, especially in the region of significance ($m/e > 150$); it was only negligibly higher in the spectrum from the spot of a methanol-washed HPTLC plate as compared with that of a normal (untreated) silica gel G plate.

The data summarized in Fig. 1 show that the layer thickness is homogenous if the plate edge is disregarded. *In situ* evaluation of the spots can therefore be used even by transmittance measurements.

The electropherograms of non-derivatized biogenic amines, obtained with use of 0.5 *M* pyridinium acetate buffer of pH 4.8 (see ref. 14), show considerable interaction of the HPTLC layer with the amines (see Fig. 2). Even the order of relative electrophoretic mobilities is reversed on the HPTLC, as compared with normal silica gel (and cellulose) layers. Interaction of the Dns derivatives with the gel matrix is much weaker, so that the relative mobilities are similar on all types of silica gel plates (see Fig. 3), provided that the solvent mixture contains only components of low, or relatively low, polarity (aliphatic or aromatic hydrocarbons, ethers, esters or halogenated hydrocarbons). As polar solvents (such as triethylamine, methanol or water) are preferentially adsorbed by the highly active HPTLC layer, the order of relative mobilities can be changed (see Fig. 4); binders present in commercial plates have much less influence in this respect. Nevertheless, most solvents that have been suggested for the separation of Dns-amines[4,8] can still be used for chromatography

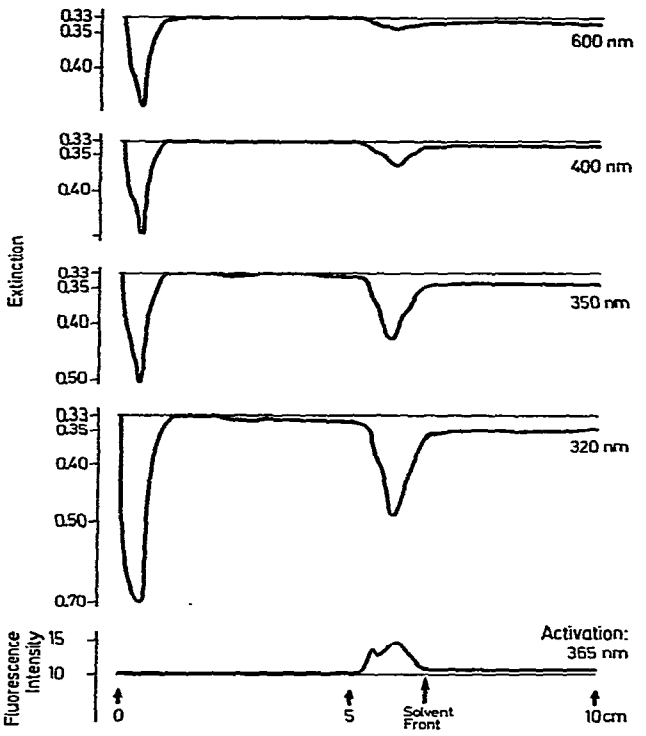

Fig. 1. Transmittance and fluorescence of a commercial HPTLC plate. A fresh plate was developed with methanol for a distance indicated by the arrow. The transmittance at different wavelengths and the total fluorescence were scanned with the device described by Seiler[31]. Absorbing and fluorescing material accumulates at the solvent front. The low transmittance near one edge of the plate is due to inhomogeneity of the layer.

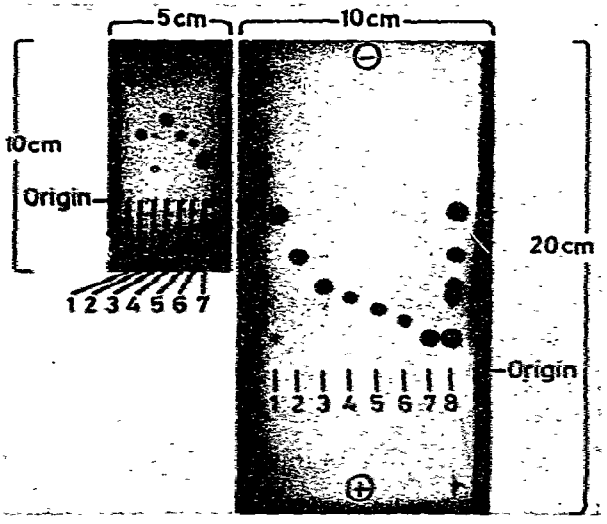

Fig. 2. Comparison of electrophoretic mobilities of some non-derivatized biogenic amines and amino acids on HPTLC and silica gel G 1500 plates (Schleicher & Schüll, Dassel, G.F.R.) with 0.5 *M* pyridinium acetate buffer of pH 4.8. HPTLC plate: 40 V/cm, 15 min; silica gel G 1500 plate: 20 V/cm, 45 min. 1 = Putrescine; 2 = spermidine; 3 = spermine; 4 = monoacetylputrescine; 5 = putreanine; 6 = homocarnosine; 7 = 4-aminobutyrate; 8 = mixture of putrescine, spermidine, spermine, monoacetylputrescine and 4-aminobutyrate. Visualization by reaction with ninhydrine.

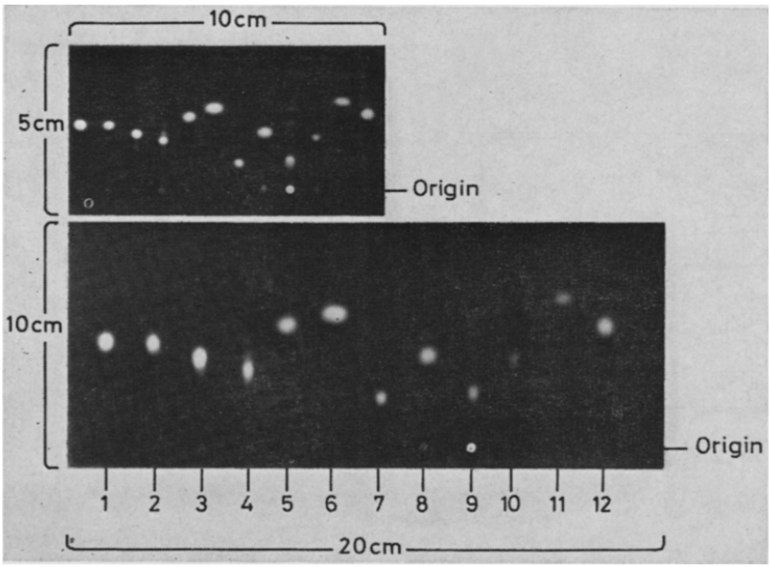

Fig. 3. Relative mobilities of Dns-amines on HPTLC and 300-μm silica gel G layers. *Solvent*: ethyl acetate–cyclohexane (3:2). Development in horizontal tanks (solvent-vapour-saturated atmosphere) for 5 and 10 cm, respectively. 1 = Ammonia; 2 = putrescine; 3 = spermidine; 4 = spermine; 5 = methylamine; 6 = dimethylamine; 7 = ethanolamine; 8 = 2-oxopyrrolidine (reaction product of 4-aminobutyrate); 9 = histamine; 10 = 5-hydroxytryptamine; 11 = phenethylamine; 12 = tyramine. Amounts: about 2 nmoles on the silica gel and 1 nmole on the HPTLC plate.

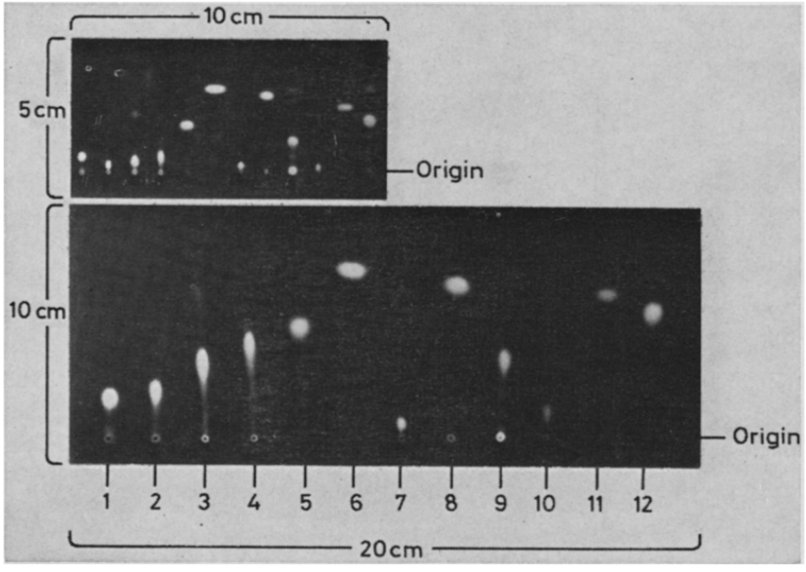

Fig. 4. Relative mobilities of Dns-amines on HPTLC and 300-μm silica gel G layers. Solvent: benzene–triethylamine (5:1). Other conditions, compounds and amounts as specified in Fig. 3.

on HPTLC plates. Fig. 5 shows a two-dimensional separation of Dns-amines with a solvent combination useful for the separation of Dns-2-oxopyrrolidine, the reaction product of 4-aminobutyric acid with Dns-Cl[15–17]; the slight differences in the distribution patterns on normal and HPTLC plates are of no practical consequence. Similarly, the polyamines spermidine and spermine are separable from Dns-treated tissue extracts on HPTLC plates (see Fig. 6) by using previously described solvents[4,8,18,19]. The high capacity of the HPTLC plates allows the separation of relatively large amounts of Dns derivatives without effect on the regular shape of the spots. It is possible to detect tissue components present at low concentration in the presence of large amounts of other tissue constituents.

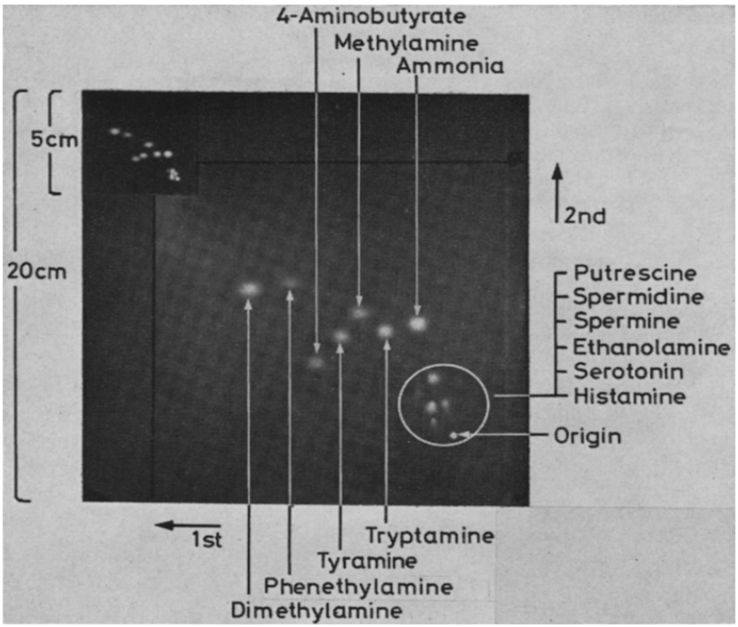

Fig. 5. Two-dimensional separation of some Dns derivatives by using solvents proposed for separating the reaction product of 4-aminobutyrate from other Dns tissue constituents[16,17]. Solvents: first dimension, benzene–cyclohexene–methanol (85:15:2) (two runs); second dimension: diethyl ether–cyclohexane (3:1). Ascending development (filter-paper-lined Camag tanks).

Separation of Dns-amino acids

Most solvents previously suggested for the separation of Dns-amino acids on silica gel or related active layers[4–6] are mixtures of polar with non-polar compounds. Because of limited mutual solubility and preferential adsorption of the polar component by the HPTLC layer, many of these solvents separated into two phases on the plate and were therefore only of limited value. By using chloroform–acetic acid–water (10:9:1) in the first dimension, and methyl acetate–propan-2-ol–25% ammonia (9:7:2) in the second (with activation of the layer for 10 min at 100° between runs), adequate separation of the most important Dns-amino acids was achieved, both on normal and on HPTLC plates (see Fig. 7).

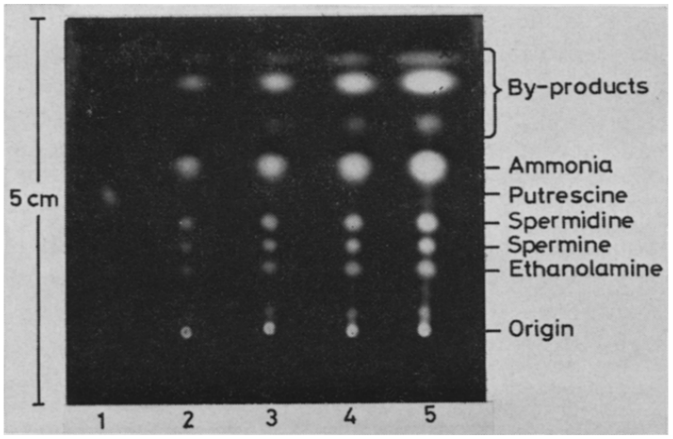

Fig. 6. One-dimensional separation of a dansylated perchloric acid extract of mouse liver on a 5 × 5 cm HPTLC plate. 1 = putrescine (reference), 2, 3, 4 and 5 = Dns derivatives corresponding to 5, 10, 50 and 100 μg of liver tissue, respectively (about 5, 10, 50 and 100 pmoles of spermidine and spermine). Solvent: cyclohexane–ethyl acetate (1:1) (two runs).

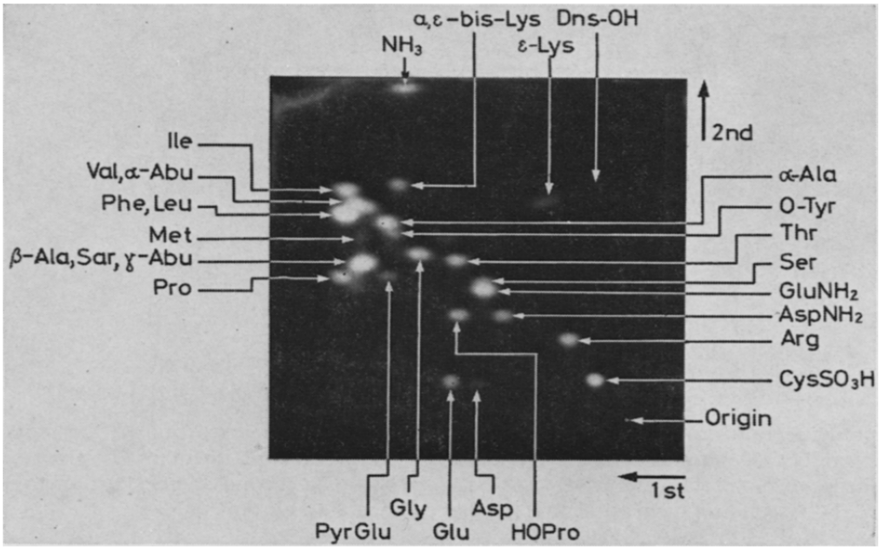

Fig. 7. Two-dimensional separation of Dns derivatives of some common amino acids on a 5 × 5 cm HPTLC plate. Solvent: first dimension, chloroform–acetic acid–water (10:9:1); second dimension, methyl acetate–propan-2-ol–25% ammonia (9:7:2) (two runs); solvent-vapour-saturated tank; activation of the layer at 100° for 10 min after each run.

Preparation of four two-dimensional chromatograms on a single chromatographic plate

A method has been suggested[20], and applied to the determination of 4-aminobutyrate[17,19], 5-hydroxytryptamine and bufotenin[21] in tissues, which allows two-dimensional separations of several samples at the same time on a single chromatographic plate. In the previous work, porous polyethylene supports were used for solvent transfer. A considerable improvement in the method was the construction of

supports from stainless-steel, the supports being covered by a sheet of filter paper (fibre direction vertical to the direction of solvent movement); details of the application of such a support can be seen in Fig. 8, and Fig. 9 shows separations of dansylated tissue samples. The channels scored in the layer divide the plate area into, *e.g.*, four 5-cm × 5-cm areas, and samples are applied at an appropriate distance (*e.g.*, 1 cm) from the plate corners. The plates are developed by placing them (layer downward) on a support of appropriate size, until the solvent fronts reach the scored channels from two opposite plate edges. After drying (or activation at elevated temperature), separation can be performed in the second direction.

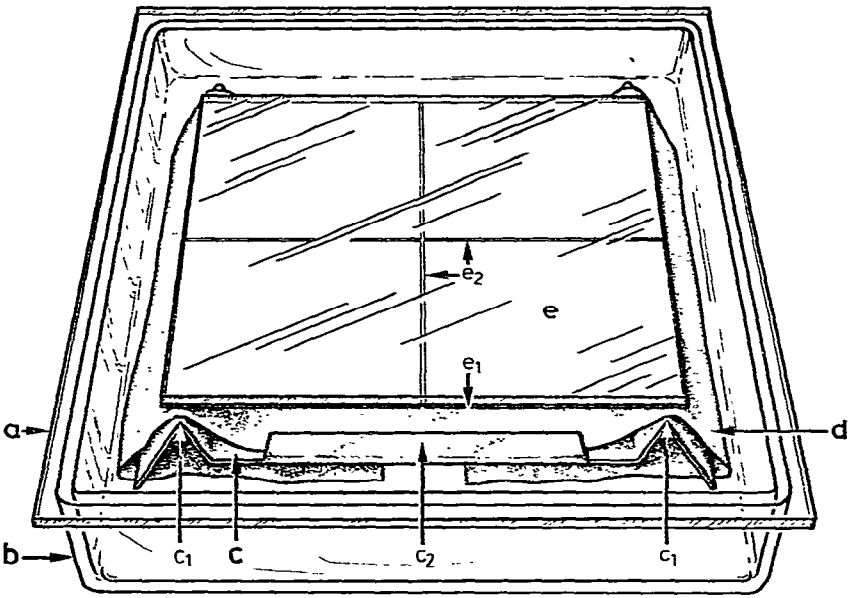

Fig. 8. Use of the horizontal tank with stainless-steel support: (a) tank lid; (b) tank containing solvent; (c) stainless-steel support, bent to hold a sheet of filter paper (d) in place at c_2, and to support the chromatographic plate (e) at each end (c_1); (e_1) thin layer; (e_2) narrow channels scored in the layer.

One of the obvious advantages of this technique, besides its rapidity, is that saturation of the layer with solvent vapour is ensured, even with mixtures containing solvents differing greatly in density. Moreover, many samples can be separated unidimensionally at the same time, if they are applied at two opposite plate edges. Evidently, the entire length of the plate can be utilized if the layer is removed from one plate edge (or if the filter paper sheet is removed from one end of the stainless-steel support).

Quantitative evaluation

The principles of quantitative measurement of Dns derivatives separated by TLC have been described in detail[4,8,22,23], and advantages of *in situ* methods for the quantitative evaluation of HPTLC plates have been discussed[24–26].

Under certain circumstances, extraction procedures are desirable, or even a

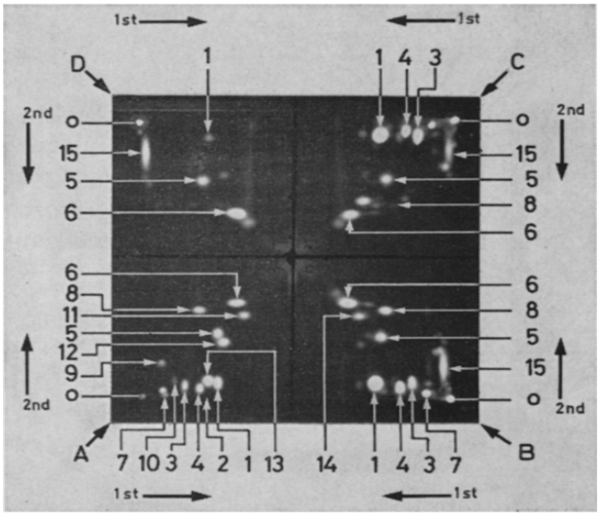

Fig. 9. Four two-dimensional separations on a single 10-cm × 10-cm HPTLC plate prepared with a stainless-steel support in a horizontal tank. A: Reference compounds (Dns derivatives), 1–12 as in Fig. 3; 13 = tryptamine; 14 = 4-aminovaleraldehyde (reaction product of lysine); 15 = side-reaction product (structure unknown) of proline with Dns-Cl. B: Dns derivatives of mouse-brain tissue; C: Dns derivatization products of mouse-liver tissue; D: Side-products of the derivatization reaction (blank). Solvents: first dimension, cyclohexane–ethyl acetate (1:1); second dimension, benzene–triethylamine (5:1).

pre-requisite, *e.g.*, if a fluorescence-scanning device is not accessible, or if such a method as mass spectrometry[27,28] is used for the determination of Dns derivatives.

The stability of Dns derivatives adsorbed on active surfaces is limited. Increase in fluorescence intensity and stabilization can be achieved by spraying with triethanolamine–propan-2-ol (1:4)[22]. Fig. 10 shows two chromatograms with nanomole amounts of various Dns-amines. Plate A was sprayed with the triethanolamine reagent im-

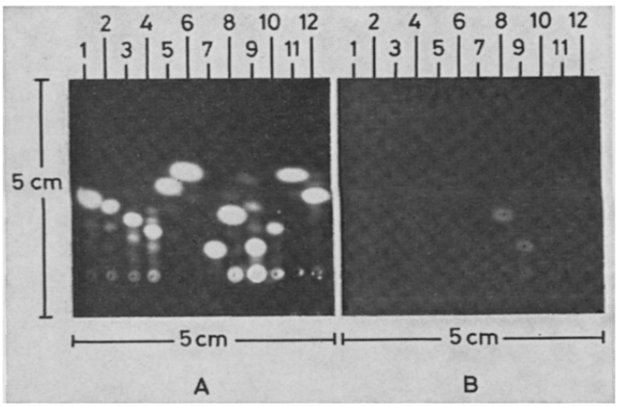

Fig. 10. Effect of spraying with triethanolamine on the stability of Dns derivatives. A: layer sprayed with 5 ml of a solution of triethanolamine–propan-2-ol (1:4); B: dried at room temperature and exposed, together with plate A, for 3 days to laboratory atmosphere. Developing solvent and compounds (about 2 nmoles each) as in Fig. 3.

mediately after development, then exposed for 3 days to the laboratory atmosphere. The Dns derivatives on plate B were irreversibly destroyed, regardless of their structure, whereas no significant losses were noticed on plate A. (Hydrolysis of Dns-esters and splitting of γ-lactam rings occurs frequently under these conditions.)

For quantitative measurement, the plates are sprayed with triethanolamine–propan-2-ol (1:4) (5 ml per 10 × 10 cm plate) and dried at room temperature. The layers of HPTLC plates are brittle, and in order to avoid losses by spirting, the marked spots are moistened with a drop of water. The extraction procedure is essentially the same as that of Seiler and Wiechmann[16], but the glass capillaries can be replaced by PTFE tubes of 1 mm I.D. and 7 cm in length. A cotton-wool plug is positioned between two constrictions (made with a pair of tweezers) at a distance of 3 cm from one end (see Fig. 11). The tubes are carefully rinsed and stored in methanol. The adsorbent is collected in one compartment of the tube by suction. A spatula may be used for scraping, but the PTFE tube is sharp enough to allow the sorbent to be scraped off.

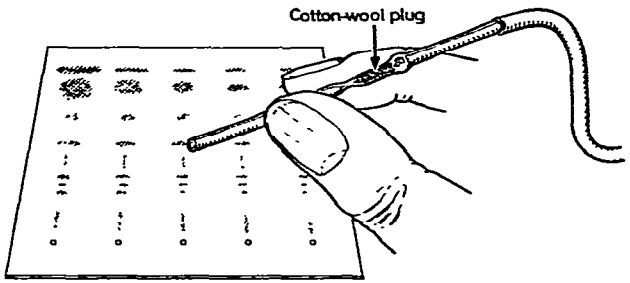

Fig. 11. Use of the PTFE capillary for removing spots from the HPTLC plate.

For elution, the end of the tube that was connected to the tubing of the vacuum pump is cut off (with a razor blade) immediately behind the constriction (in order to keep the void volume as low as possible). Then the tube is connected to a motor-driven syringe (B. Braun, Melsungen, G.F.R.), which, together with its connecting tube, is filled with a suitable solvent (usually ethyl acetate when determining amine derivatives, and methanol–25% ammonia solution (19:1) for most Dns-amino acids[4,22]). The adsorbed Dns derivative is eluted through the cotton-wool plug at a flow-rate of 0.075 ml/min, the eluent volume being measured by time control, beginning when the first drop appears at the open end of the PTFE tube; since elution requires 1 min, the eluent volume is 0.075 ml. After mixing the eluted solution, it is stored in small stoppered tubes at 4° in order to minimize evaporation.

For quantitative fluorimetry, any spectrofluorimeter with an 8–10 μl flow-through cell can be used, for example one that is suitable as a fluorescence detector in high-performance liquid chromatography. In our experiments, Perkin-Elmer 204 and Baird Fluorispec SF-100 spectrofluorimeters have been used. In order to minimize exciting light at the multiplier site, 400-nm cut-off filters were sited in the emitted beam. Excitation was usually at 365 nm, a mercury arc lamp being a suitable source, and the fluorescence was measured at 515 nm for amine derivatives (for details of the fluorescence characteristics of Dns derivatives see refs. 4, 8 and 22).

The flow-through cells were made from quartz tubes (1 mm I.D.) with black PTFE tubes (0.5 mm I.D.) attached. The upper end of the vertically arranged cell was connected by means of the PTFE tube to a water pump, and a small drop-counter was placed between the flow-through cell and the pump (allowing the flow to be controlled) and a needle valve permitting the flow-rate to be adjusted. Fluorescence measurement was achieved by inserting the inlet tube into the sample vessel, and the fluorescence intensity was recorded continuously during passage of the sample through the cell. The signal was independent of small changes in flow-rate and depedent mainly on the concentration of the fluorescent compound. After each sample, solvent was run through the cell so that changes in the baseline were immediately recognisable; sample volumes of 0.075 ml were adequate, but, as the method is simple, rapid and reliable, it can also be used with large sample volumes.

Although it is not necessary, it is advantageous to use a device for automatic signal compensation (*e.g.*, Automatische Gegenspannung AG 110, Kisch Messgeräte, Albachten, G.F.R.). Such a device allows signals of varying magnitude to be recorded with great sensitivity. When we used the extraction procedure for determining bis-Dns-putrescine, rectilinear relationships were observed between sample amount and signal in the range 1–10 pmoles ($r = 0.990$) and 10–100 pmoles ($r = 0.997$) with a variance of $s_{y.x} = \pm 4.4$ and ± 3.4, respectively (20 samples measured in each series).

DISCUSSION

Experiences with the TLC of Dns derivatives on conventional silica gel plates are not entirely applicable to HPTLC plates. Particular complications arise with solvent mixtures consisting of polar and non-polar components with limited mutual solubility, and known separation methods may be of only limited usefulness, because solvent separation is much more frequent with HPTLC plates.

The increase in signal-to-noise ratio, *i.e.*, improvement of detection sensitivity, is proportional to the decrease in spot size. As pointed out by Kaiser[29,30], the spot size of chromatographically separated compounds, and the separations attainable, are dependent on the size of the initial spot among other things. In the practical analysis of biological samples, it is difficult to keep solution volumes in the nanolitre range. By using conventional application techniques [microsyringe with micrometer gear, or Oxford samplers of different sizes (Kontron Technik, Düsseldorf, G.F.R.)], we relinquished the higher separation quality of the HPTLC plates for the sake of simplicity and rapidity of spot application. However, with toluene as solvent for sample application ($R_F < 0.05$ of the significant Dns derivatives), it is possible to apply even 0.2 ml of solvent within a few minutes without increasing the spot size beyond 1 mm in diameter. This results in separations, on 5 × 5 cm HPTLC plates, comparable with those attained with a chromatographic-path length of 15 cm on conventional plates, but in about one-third (or less) of the time.

The sensitivity of the method allows the determination of picomole quantities with good reproducibility under routine conditions if fluorimetric methods are used. The use of labelled Dns-Cl may improve the sensitivity, especially if autoradiographs are prepared. The mechanical stability of the HPTLC plates favours the adoption of methods used in combination with polyamide sheets[11,12]. However, great care has to be taken when the labelled reagent is used to avoid erroneous interpretation; in ad-

dition to fluorescent side-products of the Dns derivatization, non-fluorescent, radioactive contaminants may simulate the presence of certain compounds.

HPTLC plates are, without doubt, a major breakthrough in the development of TLC, especially as far as rapidity and sensitivity are concerned. Even though not all known separations can be carried out on HPTLC plates, they can be used in the conventional manner and applied, with appropriate modifications, to all techniques. However, in order to profit from their improved separation qualities, it is necessary to use sophisticated methods of sample application and chromatogram development.

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