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SEPARATION OF DINITROPHENYL DERIVATIVES OF AMINO SUGARS AND AMINODEOXYALDITOLS BY POLYAMIDE THIN-LAYER CHROMATOGRAPHY

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SUMMARY

The separation of glucosamine, galactosamine, muramic acid and the corresponding aminodeoxyalditols as their 2,4-dinitrophenyl (DNP) derivatives by two-dimensional thin-layer chromatography on polyamide is described. Reaction conditions are recommended that minimize artefact formation during derivatisation. Initial development with 2% (v/v) aqueous formic acid separates the DNP derivatives from salts and by-products, which otherwise interfere with separation. Solvents for the second dimension contain chlorobenzene and acetic acid, with ethylenediaminetetra-acetic acid to reduce tailing. Inclusion of benzeneboronic acid is particularly effective for separating compounds of interest.

INTRODUCTION

Several thin-layer chromatographic (TLC) systems will separate 2,4-dinitrophenyl (DNP) derivatives of amino acids on polyamide-coated sheets[1]. However, TLC separation of the DNP derivatives of amino sugars and their alcohols has not hitherto been reported. When TLC is used for quantitative analysis of amino acid derivatives, isotope-dilution methods[2,3] minimize the errors due to incomplete derivatisation and sample recovery that are inherent in colorimetric[4] or fluorimetric[5] procedures. If DNP derivatives of amino sugars could be separated by TLC, then similar microtechniques might be developed for carbohydrate-containing systems. For example, the levels of individual amino sugars might be estimated by isotope-dilution analysis of acid hydrolysates, together with chain lengths from the proportion of aminodeoxyalditols obtained when tetrahydroborate reduction precedes acid hydrolysis. For biosynthetic studies, the specific activities of both monomeric precursor and polymeric product could be readily determined following TLC separation of DNP derivatives.

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Here, we report on some of the factors influencing the derivatisation and separation of DNP derivatives of amino sugars and their alcohols on polyamide thin layers and recommend suitable solvent systems. Since amino sugars commonly occur in the presence of amino acids, systems were sought in which interference by DNP-amino acids was avoided.

MATERIALS AND METHODS

Chromatography

TLC was carried out on silica gel plates containing a fluorescent indicator [silica gel 60 F_{254} (Merck, Darmstadt, G.F.R.)] and on flexible double-sided micro-polyamide sheets [F 1700 (Schleicher and Schüll, Dassel, G.F.R.)] by ascending development in the dark. Methanol solutions of individual or mixed chromatographic markers were applied as 1 or 2-μl volumes containing about 1 nmole of each DNP derivative. Solvents were redistilled unless of reagent quality. Free amino sugars and their alcohols were detected with ninhydrin or alkaline silver nitrate, and DNP compounds by irradiation at 254 nm.

Synthesis of reference compounds

Conversion of glucosamine (GlcN), galactosamine (GalN) and muramic acid (Mur) into the corresponding aminodeoxyalditols ($GlcNH_2$, $GalNH_2$, and $MurH_2$) was monitored by TLC and required up to 4 moles of sodium tetrahydroborate for completion[6]. If too great an excess of reducing agent was used with Mur, an extra ninhydrin-positive spot was produced, which ran ahead of muramitol. It might represent formation of the Morgan–Elson chromogen from a β-elimination reaction caused by increased pH (ref. 7).

Even although reaction of equivalent amounts of 1-fluoro-2,4-dinitrobenzene (FDNB) and amino sugar in aqueous ethanol[8] does not produce quantitative conversion[6], DNP-GlcN, DNP-GalN and DNP-Mur were prepared in this way, as stronger conditions can cause by-product formation. Product stability increases and the tendency to form by-products decreases in the order Mur, $MurH_2$, GlcN, $GlcNH_2$ (ref. 6). As DNP-Mur is rather labile [*e.g.*, unstable, less hydrophilic by-products were formed on deionisation with Amberlite CG-120 (H^+) (Rohm & Haas, Philadelphia, PA, U.S.A.), or on extraction with methanol from silica gel preparative-layer chromatograms], it could never be obtained chromatographically homogeneous. Samples that appeared uniform in a TLC system used by other workers[9] gave additional spots[6] when tested with a different solvent. Hence the crude reaction mixture was used as a marker.

DNP-GlcN and DNP-GalN were extracted from the dried reaction mixtures with ethyl acetate–water (1:1, v/v). After removing 2,4-dinitrophenol (DNP-OH) by vacuum sublimation, the products were recrystallised from dry ethanol.

$GlcNH_2$ and $GalNH_2$ were allowed to react with FDNB without isolation of the aminodeoxyalditols[10]. The products were deionised with Zeo-Karb 225 (H^+) (Permutit, London, Great Britain), dried repeatedly from methanolic solution to remove borate, separated from DNP-OH by sublimation, and recrystallised from ethanol.

Milder reaction and purification conditions were required to convert $MurH_2$

into DNP-$MurH_2$ in good yield with minimal by-product formation[6]. After reaction for 2 h at room temperature with a 12-fold excess of ethanolic FDNB in carbonate buffer solution (pH 9.0), the mixture was dried at less than 20°C. The product was extracted with ethanol, dissolved in water, separated from DNP-OH by continuous extraction with ether (while maintaining the pH at 4.4 by addition of acetic acid), and dried. DNP-$MurH_2$ was separated from residual salts by ethanol extraction and recrystallised from ethanol–diethyl ether.

RESULTS

As with other applications of polyamide TLC[11], choice of the first solvent for two-dimensional chromatography was dictated by the need to remove the components of interest from areas occupied by salts and other by-products (*e.g.*, DNP-OH) present in derivatised sample hydrolysates. In 20% (v/v) aqueous formic acid (solvent I in Table I), DNP-OH and even relatively mobile, hydrophilic amino acids migrated more slowly than the DNP-amino sugars and DNP-aminodeoxyalditols, which were rather poorly separated from one another. Most of the salt, monitored by using [^{36}Cl]-chloride, travelled close to the solvent front. Although solvent I was useful in some two-dimensional separations where not all amino sugar derivatives were expected, much better (but more time-consuming) resolution could be attained by two or three

TABLE I

MOBILITIES OF SOME DNP-AMINO SUGARS, DNP-AMINODEOXYALDITOLS, DNP-AMINO ACIDS AND BY-PRODUCTS ON POLYAMIDE TLC PLATES

Solvent systems: I, formic acid–water (1:4); II, formic acid–water (1:49); III, chlorobenzene–acetic acid containing 2.9 m*M* EDTA (5:1); IV, chlorobenzene–acetic acid containing 2.9 m*M* EDTA–TEA (5:1:0.2); V, chlorobenzene–acetic acid containing 2.9 m*M* EDTA–methanol (5.1.0.2); VI, chlorobenzene–acetic acid containing 2.9 m*M* EDTA–TEA–methanol (20:1.2:1), VII, chlorobenzene–acetic acid containing 2.9 m*M* EDTA–1-pentanol–methanol (50:3:8:4); VIII, chlorobenzene–acetic acid containing 2.9 m*M* EDTA–1-pentanol–5% (w/v) benzeneboronic acid in methanol (50:3:8:4).

Compound	$R_F \times 100$							
	I	II	III	IV	V	VI	VII	VIII
DNP-GlcN	55	19	4	16	9	41	14	16
DNP-$GlcNH_2$	66	29	4	20	11	33	12	54
DNP-GalN	56	17	2	18	11	40	14	46
DNP-$GalNH_2$	61	24	2	19	10	26	15	41
DNP-Mur	40	8	3	23	9	57	12	21
DNP-Mur (minor component)	8	2	12	54	26	73	35	40
DNP-$MurH_2$	53	19	2	29	9	36	10	29
DNP-Ala	13	2	50	88	57	84	58	59
DNP-Glu	18	2	18	67	25	82	22	21
DNP-Lys	1	1	50	86	58	87	58	60
DNP-Gly	19	1	32	82	40	85	38	38
DNP-Ser	25	3	7	63	14	78	17	18
DNP-Thr	25	2	15	74	23	83	23	24
DNP-OH	27	3	90	82	85	82	54	58
DNP-NH_2	18	2	54	72	60	72	69	72

successive developments in 2% (v/v) aqueous formic acid (solvent II). Table I lists the R_F values after a single development.

Apart from the greater relative mobility differences of amino sugar derivatives, solvent II also produces more compact spots. In both instances, the advancing solvent front wets the immediate area of the starting spot more slowly than other parts of the plate; it then encircles the spot, leaving a dry central area, which is wetted more slowly. In this way, solute from the sides of the spot is carried forward before the central area starts to move, producing an elongated spot, which is often V-shaped. This is most marked if the plate is heavily loaded, or the sample contains much salt; migration is normal once the salt has moved ahead of the compounds of interest.

Because the DNP-amino sugars are much less mobile in solvent II than in solvent I, use of the former solvent for the initial development produces much less elongation. The difference between the rate of solvent permeation of the starting spot compared with that for the surrounding area is greatly reduced if the solvent flow-rate is decreased. This can be exploited to reduce spot elongation by feeding the plate with solvent through a paper wick. Spot elongation is also reduced when penetration of the starting zone is facilitated by pre-equilibration with solvent vapour. An attempt to assist penetration by including 0.01 to 1% (v/v) of Triton X-100 (Rohm & Haas) in the solvent as wetting agent was unsuccessful.

Solvents for the second development were derived from chlorobenzene–glacial acetic acid (9:1, v/v)[12] by raising the solvent polarity to increase the mobilities of amino sugar derivatives. Streaking was eliminated and sharper spots were obtained with little change in R_F values when the acetic acid was saturated (approximately 2.9 m*M* at 20°C) with ethylenediaminetetra-acetic acid (EDTA). Amelioration of paper chromatograms with EDTA has been attributed (at least in part) to chelation with traces of metal ions in the support medium[13]. The improvement was less noticeable with a mixture of standards than with a test mixture (containing most of the DNP derivatives listed in Table I) produced by lysozyme digestion of bacterial peptidoglycan, reduction with sodium tetrahydroborate, acid hydrolysis and reaction with FDNB.

The effects of increasing the polarity of the mobile phase were tested by comparison with solvent III [chlorobenzene–2.9 m*M* EDTA in glacial acetic acid (5:1, v/v)], in which DNP-amino sugars and their alcohols moved slowly and were poorly separated from each other, but well separated from most DNP-amino acids and by-products. Addition of triethylamine (TEA) increased the separation of DNP-amino acids from DNP-amino sugars and their alcohols more substantially than did addition of the same volume of methanol (compare solvents IV and V). This agrees with the predicted effects of TEA on the partition coefficients between mobile and stationary phases when an ion-pair mechanism is assumed. With mixtures based on chlorobenzene–acetic acid ratios of only 5:1, R_F values were somewhat sensitive to small changes in TEA concentration. Alternatively, mixtures with higher chlorobenzene contents often produced tailed spots if the methanol content was too low, suggesting that the compounds of interest were poorly soluble in the mobile phase. From this was developed solvent VI [chlorobenzene–2.9 m*M* EDTA in acetic acid–TEA–methanol (20:1:2:1, v/v)], which has found some use in two-dimensional separations.

Resolution of closely related DNP-amino sugar derivatives as their borate

complexes has long been known[14]. Addition of boric acid to some of the more promising chlorobenzene-based solvents produced mobility changes that were too small to be useful. Selectivity due to borate may be lost in acidic systems[15]. Raising the TEA–acetic acid ratio to avoid this produced unacceptably high R_F values. In contrast, by using benzeneboronic acid, which forms cyclic borate esters of increased hydrophobicity, very satisfactory separations were obtained, especially when acetic acid was present. As in other separations involving polyol–benzeneboronic acid complexes[16], success was achieved by somewhat arbitrary adjustments to solvent composition rather than by accurate prediction. Comparison of solvents VII and VIII suggests that DNP-GalN, DNP-Mur and the three DNP-amino sugar alcohols all form complexes rather readily with this reagent.

Combinations of these solvent mixtures produce satisfactory two-dimensional separations without undue cost. Spots can deviate slightly from the positions predicted from one-dimensional work, and it is wise to include standards when new solvent pairs are tested. The best method (essential when all three DNP-amino sugars and their alcohols are present, and usually the best for simpler mixtures) involves three developments in solvent II, followed, in the second dimension, by one or two developments in solvent VIII (Fig. 1). Unfortunately, multiple developments in solvent II cannot be replaced by a single development in solvent I, which, although good enough for two dimensional separation of simpler mixtures, does not resolve the complete

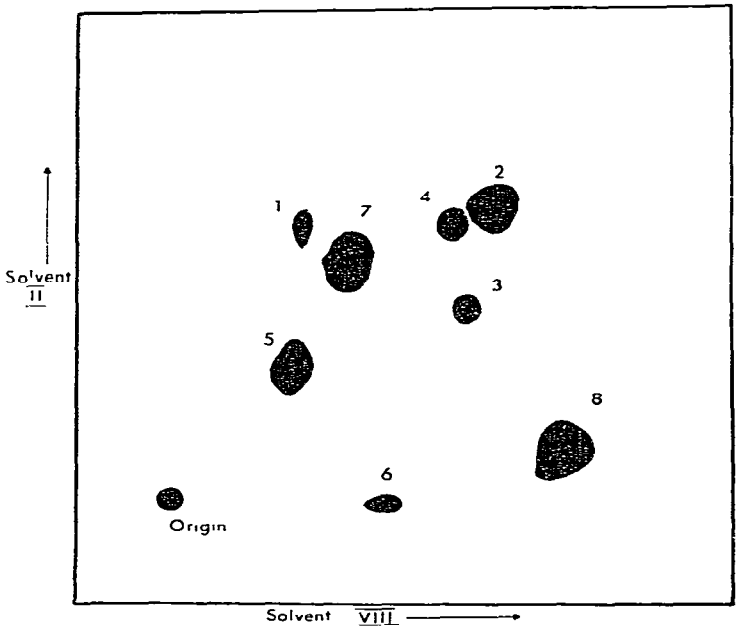

Fig. 1. Separation of DNP-amino sugars and DNP-aminodeoxyalditols by TLC on polyamide. With a plate 5 cm square, separation was achieved by three developments in solvent II followed by one development in solvent VIII. 1 = DNP-GlcN; 2 = DNP-GlcNH$_2$; 3 = DNP-GalN; 4 = DNP-GalNH$_2$; 5 = DNP-Mur; 6 = DNP-Mur (minor component); 7 = DNP-MurH$_2$; 8 = DNP-OH.

mixture satisfactorily. If no DNP-aminodeoxyalditols are present, or if both DNP-GalN and DNP-$GalNH_2$ are absent, a single development in solvent I, followed by one or more developments in solvent VIII will suffice. If only DNP-GlcN, DNP-$GlcNH_2$, DNP-Mur and DNP-$MurH_2$ are present, two-dimensional separation is possible by using solvent I or II followed by solvent VI. This combination can also be used to separate the three DNP-amino sugar alcohols. Perhaps because of its rather poor solubility in solvent VI, DNP-$GalNH_2$ can move rather unpredictably. In this instance, it is more than usually important to include standard markers in parallel with the test mixture.

The excellent separations obtainable on polyamide are affected adversely if large amounts of salts are present. Hence, the smallest possible amounts of sodium tetrahydroborate should be used to reduce the amino sugars. After derivatisation with FDNB, any reagent remaining unreacted can interfere with subsequent chromatography. Following development in solvent I or II, surplus FDNB can spread out from the origin during drying, invading the area containing the amino sugar derivatives and ruining the chromatogram. This can be avoided completely if a strip containing the starting zone is cut off and discarded immediately after development.

DISCUSSION

A reasonably successful separation of the DNP derivatives of both amino sugars and aminodeoxyalditols from each other and from the bulk of the corresponding amino acid derivatives has been achieved. It is likely that the separation systems used could, with minor modifications, be extended to the chracterisation and measurement of other, less common, amino sugars.

Experiments were directed towards separations involving weak acid solvents on polyamide layers for several reasons. First, both alkali and strong base anion exchangers cause DNP-amino sugar instability[17], especially with DNP-Mur[6], and are best avoided.

Secondly, because of the high resolving power of polyamide, spot diameters are generally considerably smaller than on silica gel chromatograms of comparable sizes. This allows detection, in most instances of approximately tenfold lower sample levels than on silica gel. Although the recent introduction of high-resolving-power silica gel has reduced the development times and running distances required about threefold[18], satisfactory separations can still be carried out more rapidly on polyamide and on an even smaller scale (*e.g.*, 3 × 3 cm plates for some applications[19]).

Thirdly, polyamide appears to be a more inert support than silica gel, since spots of DNP derivatives absorbed to polyamide appear to fade more slowly when stored in the dark. Silica gel is known to catalyse decomposition of absorbed compounds, especially when it is dry and activated[20].

Fourthly, unlike silica gel, the polyamide layer (together with the sample) can be dissolved in formic acid and mixed with scintillation fluid to give a single-phase system. This avoids the disadvantages[21] of heterogeneous counting systems.

The levels of DNP-amino sugars separated on polyamide plates are some three to four orders of magnitude lower than the amounts used in early column analysis[10,14] and one to three orders of magnitude less than the amounts of amino sugars analysed in test-tube scale wet-chemistry reactions. Although the sensitivity of the present

method might be reduced by at least another order of magnitude if the DNP-group were replaced by a fluorescent label, the chemistry of those methods that have been reported is often far less well understood or involves additional pitfalls. For example, labelling with 5-dimethylaminonaphthalene-1-sulphonyl chloride (Dns-Cl) is subject to side reactions[22], and results in unstable sugar derivatives[23]. The only sugar TLC method of comparable sensitivity involves reaction with Dns-hydrazine[24]. Unfortunately, unlike neutral aldoses, amino sugars react very poorly, and, since aminodeoxyalditols will not react, this method cannot be adapted for chain-length determinations.

By using polyamide TLC for amino acid analysis, the reliability of the FDNB reagent has been extended to quantitative analysis at the sub-nanomole levels attainable with Dns-Cl by using radioactive FDNB[3]. A similar approach could probably be used with DNP-amino sugars. Although derivative formation should preferably convert each compound into a single product, it is not absolutely essential when isotope-dilution analysis is used for quantitation. For example, when amino acids are measured by isotope-dilution analysis of their DNP derivatives, accurate determination of cystine and lysine is possible from the specific activities of the DNP derivatives, even though substantial amounts of mono-substituted lysine and DNP-cysteic acid are also formed[3]. This is an important consideration with Mur, which, even when reaction conditions are carefully controlled, always gives at least one minor product when it reacts with FDNB.

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REFERENCES

1 A. Niederwieser, *Methods Enzymol.*, 25 (1972) 60–99.
2 J. P. Brown and R. N. Perham, *Eur. J. Biochem.*, 39 (1973) 69–73.
3 M. Humphries and J. S. Thompson, *Anal. Biochem.*, 105 (1980) 319–327.
4 J.-M. Ghuysen, D. J. Tipper and J. L. Strominger, *Methods Enzymol.*, 8 (1966) 685–699.
5 J. Airhart, S. Sibiga, H. Sanders and E. A. Khairallah, *Anal. Biochem.*, 53 (1973) 132–140.
6 M. Talieri and J. S. Thompson, *Carbohyd. Res.*, 86 (1980) 1–6.
7 D. J. Tipper, *Biochemistry*, 7 (1968) 1441–1449.
8 P. W. Kent, *Research (London)*, 3 (1950) 427–428.
9 D. Mirelman and N. Sharon, *J. Biol. Chem.*, 242 (1967) 3414–3427.
10 S. Leskowitz and E. A. Kabat, *J. Amer. Chem. Soc.*, 76 (1954) 4887–4892.
11 J. M. Varga and F. F. Richards, *Anal. Biochem.*, 53 (1973) 397–406.
12 K. R. Woods and K.-T. Wang, *Biochim. Biophys. Acta*, 133 (1967) 369–370.
13 G. Sheppard, *The Radiochromatography of Labelled Compounds*, Radiochemical Centre, Amersham, 1972, p. 12.
14 E. F. Annison, A. T. James and W. T. J. Morgan, *Biochem. J.*, 48 (1951) 477–482.
15 M. Ghebregzabher, S. Rufini, B. Monaldi and M. Lato, *J. Chromatogr.*, 127 (1976) 133–162.
16 E. J. Bourne, E. M. Lees and H. Weigel, *J. Chromatogr.*, 11 (1963) 253–257.
17 P. F. Lloyd and M. Stacey, *Chem. Ind., (London)*, (1955) 917–918.
18 N. Seiler and B. Knödgen, *J. Chromatogr.*, 131 (1977) 109–119.
19 G. Briel and V. Neuhoff, *Hoppe-Seyler's Z. Physiol. Chem.*, 353 (1972) 540–553.

20 E. Stahl, *Thin-Layer Chromatography, A Laboratory Handbook*, Academic Press Inc., New York 1965, p. 10.
21 C. T. Peng, *Sample Preparation in Liquid Scintillation Counting*, The Radiochemical Centre, Amersham, 1977, pp. 56–68.
22 M. Wiechmann, *Hoppe-Seyler's Z. Physiol. Chem.*, 358 (1977) 967–980.
23 A. A. Galoyan, B. K. Mesrob and V. Hcleyšovský, *J. Chromatogr.*, 24 (1966) 440–442.
24 G. Avigad, *J. Chromatogr.*, 139 (1977) 343–347.