THIN-LAYER CHROMATOGRAPHY IN THE STUDY OF ESTER SULPHATES

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It is generally recognised that thin-layer chromatography is superior to that on filter paper for the study of weakly polar substances. As yet, however, this rapid and sensitive technique has been relatively little used for the separation of organic molecules bearing strongly ionised groups.

In connection with studies on the biochemistry of a wide variety of sulphate esters, paper chromatography and electrophoresis are used routinely in these laboratories to establish the homogeneity of starting materials and to follow their transformations in biological systems. Frequent use is made of ^{35}S-labelled sulphate compounds which are then detected by autoradiography[1,2] or automatic strip-scanning[3].

This communication describes the application of thin-layer techniques to the separation of a variety of alkyl, aryl, and steroid sulphuric acid esters.

EXPERIMENTAL

Materials

Isomeric nitrocatechol sulphates were prepared by a modification of the method of SMITH[4] and other phenolic sulphate esters by a modification of the procedure of BURKHARDT AND LAPWORTH[5].

Sulphate esters of alcohols and hydroxylated amino acids were prepared according to LLOYD, TUDBALL AND DODGSON[6]. L-Tyrosine O-sulphate and related *para*-substituted phenolic sulphates and their parent compounds were obtained as described by FLANAGAN[7].

The N-sulphates of L-serine and bis(2-hydroxyethyl)amine were prepared by the method of WARNER AND COLEMAN[8] and L-cysteine S-sulphate by a modification of the method of CLARK[9].

Estriol sulphate and estradiol mono- and disulphates were gifts from A.B. Leo, Hälsingborg, Sweden; shark bile scymnol sulphate was provided by Professor G. A. D. HASLEWOOD whilst other steroid sulphates were either commercial preparations or were synthesised as described by ROY[10].

All sulphate esters were crystallised as sodium or potassium salts.

Solvents

Solvent A. Benzene–ethyl methyl ketone–ethanol–water, 3:3:3:1 (v/v).
Solvent B. 2-Propanol–chloroform–methanol–water, 10:10:5:2 (v/v).
Solvent C. 2-Propanol–chloroform–methanol–10 *N* ammonia, 10:10:5:2 (v/v).
Solvent D. 1-Butanol–acetic acid–water, 3:1:1 (v/v).

Spray reagents

Steroids were detected by spraying the dried plate with 50 % aqueous sulphuric acid and heating at 140° until coloured spots appeared[11]. Though the final colour obtained is characteristic for the steroid nucleus (for instance, grey-blue for cholesterol and dehydroepiandrosterone sulphates, orange for estrogen sulphates) the individual compounds listed vary in the ease with which the colours appear and in the intermediate shades obtained. In cases where identifications are not possible on the basis of R_F values alone comparisons of the colours produced by this spray with unknown and reference compounds should be of value in the characterisation of steroid sulphates.

Amino acids and their O-sulphate esters were rendered visible by a spray of 0.5 % ninhydrin in ethanol.

Para-substituted phenols related to L-tyrosine and their corresponding ester sulphates were detected by a modification of the spray for *para*-alkylated phenols on paper as described by TOMPSETT[12]. The dry plate was sprayed lightly with a 0.3 % solution of 1-nitroso-2-naphthol in acetone, followed by a solution of nitrous acid prepared by mixing equal volumes of ice-cold aqueous solutions of sulphuric acid (10 %) and sodium nitrite (5 %). Heating at 140° gives a purplish-red colour with *para*-alkylated phenols and (more slowly) their sulphate esters while most other phenol derivatives give a sandy-red colour.

Many phenolic sulphates, in particular those bearing a nitro substituent, could be located as dark areas when the plate was viewed under ultraviolet light.

Any compound not readily detected by one of the above methods was made visible by means of a spray prepared by dissolving potassium permanganate (0.5 g) in sulphuric acid (15 ml)[13]. Greater contrast was obtained, where necessary, by over-spraying with an aqueous solution of a redox indicator (barium diphenylamine sulphonate, British Drug Houses Ltd.).

Preparation of plates

A slurry of Silica Gel G (Merck and Co.) (30 g) in water (60 ml) was applied to 5 × 20 cm and 20 × 20 cm glass plates using a Unoplan Leveller (Shandon & Co.) with the spreader set at 250 μ. The plates were dried at 110° for 45 min and stored over anhydrous silica gel.

Chromatographic procedure

Loads of 3–5 μg were applied in 1 μl aliquots of aqueous solution. Some classes of compounds required lower (steroid sulphates) or higher (alkyl sulphates) loads due to the differing sensitivity of the spray reagents.

Plates were developed by the ascending technique over a distance of 10 cm in a pre-equilibrated tank lined with filter paper, or by the use of a saturation chamber as described by DAVIES[14].

Though R_F values on thin-layer plates are reproducible if conditions are carefully controlled (for instance, unvarying room temperature and maintenance of a constant distance between the origin and solvent level in the reservoir—see ref. 15) it was found more convenient, for routine runs, to measure mobilities relative to those of reference compounds. Thus the mean R_F values of L-tyrosine (solvent D), phenyl sulphate (solvents A and B) and dehydroepiandrosterone-3β-sulphate (solvents

A and C) were calculated from at least five independent determinations. The mobilities of all other compounds were then corrected with reference to one of these three standards and absolute R_F values calculated accordingly.

The separation of ester sulphates of phenols, aliphatic alcohols and steroids

The results of this study are summarised in Tables I to V.

TABLE I

R_F VALUES OF L-TYROSINE, RELATED *p*-HYDROXYPHENYL DERIVATIVES, AND THEIR ESTER SULPHATES

Compound	*R_F of parent compound*		*R_F of ester sulphate*	
	Solvent D	*Solvent A*	*Solvent D*	*Solvent A*
L-Tyrosine	0.58	0.15	0.32	0.05
4-Hydroxyphenylpyruvate	0.65, 0.45	0.13	0.25	0.06
4-Hydroxyphenylacetate	1.00	0.31	0.61	0.08
4-Hydroxyphenylpropionate	1.00	0.42	0.72	0.10
4-Hydroxyphenylacrylate	1.00	0.41	0.78	0.08
4-Hydroxybenzoate	1.00	0.52	0.76	0.07
4-Hydroxybenzaldehyde	1.00	0.88	0.75	0.42
Methyl L-tyrosine	0.65	0.74	—	—
Ethyl L-tyrosine	0.66	0.80	—	—

TABLE II

R_F VALUES OF HYDROXYLATED AMINO ACIDS, THEIR ESTER SULPHATES, AND MISCELLANEOUS RELATED COMPOUNDS

Ester sulphate	*R_F in solvent D*	
	Substance	*Parent compound*
L-Serine O-sulphate	0.21	0.30
L-Serine N-sulphate	0.25	0.30
L-Threonine O-sulphate	0.23	0.34
L-Hydroxyproline O-sulphate	0.22	0.28
L-Cysteine S-sulphate	0.28	0.43
Glycollate O-sulphate	0.27	0.54
Bis(2-hydroxyethyl)amine N-sulphate	0.28	—
Singirin	0.35	—

TABLE III

R_F VALUES OF ESTER SULPHATES OF ALIPHATIC ALCOHOLS IN SOLVENT B

Compound	*R_F*	*Compound*	*R_F*
Methyl sulphate	0.31	Ethyl sulphate	0.38
Cyclopentyl sulphate	0.45	Cyclohexyl sulphate	0.53
n-Hexyl sulphate	0.56	Propanediol monosulphate	0.11

TABLE IV

R_F VALUES OF SUBSTITUTED PHENOL SULPHATES

Ester sulphate of	*R_F in solvent*	
	A	*B*
Phenol	0.46	0.45
2-Chlorophenol	0.48	0.47
3-Chlorophenol	0.50	0.49
4-Chlorophenol	0.50	0.48
2-Methylphenol	0.47	0.47
3-Methylphenol	0.47	0.51
4-Methylphenol	0.47	0.49
2-Methoxyphenol	0.40	0.41
3-Methoxyphenol	0.45	0.46
4-Methoxyphenol	0.45	0.44
4-Hydroxy-3-nitrophenol	0.46	0.48
4-Hydroxy-2-nitrophenol	0.60	0.53
2-Hydroxy-5-nitrophenol	0.51	0.41
2,3-Dichlorophenol	0.54	0.53
2,4-Dichlorophenol	0.54	0.53
3-Nitrophenol	0.53	0.50
4-Nitrophenol	0.57	0.52
2-Hydroxy-4-chlorophenol	0.56	0.47

TABLE V

R_F VALUES OF STEROID SULPHATES

Substance	*R_F in solvent*	
	A	*C*
Cholesterol-3β-sulphate	0.58	0.67
Cortisone-21-sulphate	0.44	0.58
Dehydroepiandrosterone-3β-sulphate	0.49	0.66
Estradiol-3-sulphate	0.48	0.60
Estradiol-17-sulphate	0.46	0.60
Estradiol-3,17-disulphate	0.16	0.27
Estriol-3-sulphate	0.35	0.48
Estrone-3-sulphate	0.54	0.71
Parent steroids	0.85–0.95	1.00
Scymnol sulphate	0.29	0.35

In general ester sulphates of weakly polar compounds can be resolved in solvent A (solvents B and C give closely similar results) while those with other polar groups in the molecule require a conventional 1-butanol–acetic acid–water mixture of which solvent D is a typical example.

DISCUSSION

Though the R_F values of only a selection of alkyl, aryl and steroid ester sulphates are quoted here it is clear that silica gel can usefully replace filter paper as the supporting medium for chromatographic studies on compounds of this type. It also seems likely

that the same can be said for S- and N-sulphates since the examples studied here [those of L-cysteine, L-serine and bis(2-hydroxyethyl)amine] had mobilities of the same order as those of similar O-sulphates.

Since it is often desirable to locate the ester sulphate and its parent compound on the same chromatogram, the spray reagents chosen were ones which either split-off the sulphate group instantaneously or reacted with another part of the molecule. The introduction of an ester sulphate group has a profound effect on the chromatographic mobility of all compounds except those (such as sugars) which are highly polar themselves. This means that, in solvents which resolve ester sulphates, the parent unsulphated compounds usually move at, or near, the solvent front.

Isomeric monosubstituted phenolic sulphates are poorly resolved while the parent phenols[16] can be separated readily on thin-layers of Silica Gel G. This can be attributed to the effect on R_F values of interactions between the hydroxyl group and other substituents on the phenolic nucleus, particularly those in the *ortho* position[16]. Such interactions are impossible for the highly ionised, chemically-saturated sulphate group. Though mixtures of such isomers are unlikely to be met in practice, they can be resolved using specific colour reagents (for instance, the nitrosonaphthol spray for various substituted phenol sulphates) or by removing the ester sulphate group by acid hydrolysis and identifying the parent compound. Many workers have already studied the resolution of free steroids[17] and amino acids[18] by means of thin-layer chromatography.

Apart from the greater speed and sensitivity common to all thin-layer work, the systems described here possess several advantages not found in those currently used for the separation of sulphuric acid esters. As already mentioned, the inert support allows the use of vigorous sprays which detect both sulphate esters and parent compounds at the same time. The spots can also be scraped off and are easily eluted for isolation or spectral examination of the compound. Since ascending development takes between 35 min (solvent A) and 150 min (solvent D) and the plates are easily handled, routine two-dimensional separations are quite feasible, so allowing detailed resolution of complex mixtures. Chromatography can be combined with electrophoresis in a second dimension[19], a technique which is particularly valuable in the case of strongly acidic compounds like ester sulphates since the electrophoretic mobility of compounds bearing weaker acid groups can be reduced or completely suppressed by working at a low pH. Electrophoresis and chromatography are complementary in this respect since inorganic sulphate has negligible mobility in the solvent systems quoted here.

The use of an inorganic supporting medium promises to be of particular value in studies where spots labelled with (^{35}S) are to be located since such spots are better defined than on paper and can be located either by autoradiography or by automatic scanning of the glass plate[19].

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SUMMARY

Thin-layer chromatography on silica gel has been applied to the separation of sulphate

esters of alkyl, aryl and steroid hydroxy compounds from each other and from their parent unsulphated compounds. The value of this technique in studies on the biochemistry of sulphate esters is discussed.

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