CHROM. 3886

A SPOT TEST DIAGNOSTIC OF HYDROXYL GROUPS

J. GEORGE POMONIS, RAY F. SEVERSON* AND PARNELL J. FREEMAN**
Metabolism and Radiation Research Laboratory, Entomology Research Division, Agricultural Research Service, USDA, Fargo, N.D. 58103 (U.S.A.)
(Received November 19th, 1968)

SUMMARY

A spot test specifically diagnostic of hydroxyl groups was devised. It makes use of the reagent 4-(*p*-nitrobenzyl)-pyridine, which, on cellulose thin-layer plates, is sensitive to alcohols in the microgram range. Amino, ester, and ether groups do not interfere, but carboxylate and phenolic groups do.

INTRODUCTION

The detection of aziridines[1], alkyl halides[1,2], and methanesulfonates[3] by the use of the reagent 4-(*p*-nitrobenzyl)-pyridine (PNBP) has been a standard procedure for several years. However, the detection of alcohols on thin-layer or paper chromatograms is difficult and generally requires derivatization before chromatography with visualization of the spot under ultraviolet light after treatment with rhodamine B[4,5]. SAWICKI *et al.*[2] reported a spectrophotometric method of assaying for alkyl tosylates and suggested that PNBP might be used for the analysis of alcohols. No other investigators have suggested or used PNBP to detect alcohols.

In the preliminary investigation reported here[6], we attempted to develop a spot test specifically diagnostic of hydroxyl groups and investigated the use of PNBP. We felt that the test had to be sensitive at microgram levels and should be easily and inexpensively performed. Also, the test should be free of interference from other functional groups. Most of these requirements were met.

EXPERIMENTAL

Test chemicals

Most compounds used in the analyses were purchased from commercial sources and were of acceptable purity; any substances below standard purity were purified by known procedures. The criteria for determining purity were melting point, boiling point, index of refraction, and, when necessary, spectroscopic procedures.

* Predoctoral assistantship, Entomology Research Division, ARS, USDA, North Dakota State University, Fargo.
** Part-time student assistant from North Dakota State University.

Tosylates

The *p*-toluenesulfonate esters (tosylates) of alcohols were prepared by reaction of the alcohols with the *p*-toluenesulfonyl chloride in pyridine. Procedures for isolating and purifying these derivatives are described in the literature. The physical constants were in agreement with the values cited in the literature.

Chromatography

Thin-layer chromatographic (TLC) plates (5 × 20 cm) were prepared with 0.25-mm thick cellulose (Brinkman, MN-Cellulose powder 300 HR)*. Whatman No. 1 filter paper was used for spot tests on paper. The TLC plates were developed with hexane–ethyl acetate (4:1, v/v) by the ascending technique to a distance of 10 cm.

Reagents

Reagent A: 5 % solution of *p*-toluenesulfonyl chloride in a 1:1 (v/v) mixture of anhydrous pyridine–toluene.

Reagent B: 2 % solution of 4-(*p*-nitrobenzyl)-pyridine (PNBP) in acetone.

Reagent C: aqueous 1 *M* sodium carbonate.

Procedure

The alcohols or alcohol derivatives were spotted on the chromatographic supports in acetone or water solutions with a microliter syringe.

Method A

Solutions of the alcohols were spotted on paper strips or TLC plates that were divided into a grid pattern. The spotting solvent was evaporated with a slow stream of nitrogen, and the plates were transferred to the hood. The test sample was sprayed with reagent A, allowed to stand until nearly dry, and then sprayed with reagent B. After 1 min, the plates were heated with a heat gun for 1 min and then sprayed with a gentle, fine mist of reagent C. The development of a deep blue or purple spot indicated a positive result.

Method B

Acetone solutions (50 μl) containing 50, 20 and 10 μg of the alcohol were transferred to 250-μl test tubes with a microsyringe, and 50 μl of a 15 % solution of *p*-toluenesulfonyl chloride in anhydrous pyridine was added. After 1.5 h at 0°, aliquots of the mixture were spotted on TLC plates and developed in the solvent system described. When the mixture had developed to 10 cm, the plates were allowed to air dry, treated with a spray of reagent B, and heated to 110° for 20 min in an oven or heated with a heat gun for 1–2 min. Subsequent spray treatment of the plates with reagent C caused the development of the positive color.

RESULTS AND DISCUSSION

The mechanism of reaction of PNBP with alkyl tosylates and the resulting reaction products were previously described[2]. The mechanism requires the displace-

* Mention of a proprietary product does not necessarily imply endorsement of this product by the United States Department of Agriculture.

ment of the tosylate by the nucleophile (PNBP) to form an alkyl quaternary pyridinium salt. The product, in alkaline solution, gives a chromogenic substance that is deep blue to royal purple. In the spot test described here, the tosylate was formed *in situ* by allowing the alcohol to react with a toluene and pyridine solution of *p*-toluenesulfonyl chloride that was applied by spraying (reagent A). It was then treated with a solution of PNBP (reagent B) and then with a solution of sodium carbonate (reagent C). The result was the chromogen described.

Modifications such as the substitution of several organic bases for the sodium carbonate and changes in reaction temperature, reaction time, and reaction solvents were attempted, but only the reported conditions and reagents gave the optimum reliability and sensitivity. Several variables involving the structural nature of the alcohols and the response to the reagents were investigated and will be discussed. With more volatile alcohols, care had to be taken to avoid loss of the test alcohol by codistillation: when such substances were assayed, the alcohol spot had to be treated quickly with a gentle spray of *p*-toluenesulfonyl chloride in toluene (reagent A). Appropriate blanks gave negative color reactions.

Chromatographic supports

The common thin-layer supports were investigated, but only cellulose and paper provided a positive color. All reported tests were therefore performed on cellulose TLC plates or paper.

Monofunctional alcohols

Initially, a series of monofunctional alcohols were spotted neat and treated by Method A, and solid alcohols were applied as 10 % solutions of acetone. The reaction was always positive, as shown in Table I. Little difference was apparent in the reactivity or primary, secondary, and tertiary alcohols to PNBP at the levels of concentration used in these preliminary tests. However, a more definitive difference will probably be seen when the rates of displacement are studied.

Selected alcohols were chosen to determine the minimum quantity detectable by spotting graded concentrations of solutions in acetone. Table II shows these results. Also, the tosylates generated *in situ* (Method A) on the chromatographic supports were compared with tosylates purified before spotting (Table III) for sensitivity to the PNBP reagent. Thus, a comparison of the data in Table II with that in Table III

TABLE I

MONOFUNCTIONAL ALCOHOLS SPOTTED NEAT, REACTING WITH THE REAGENTS ACCORDING TO METHOD A

Compound	*Compound*
Cyclopentanol	Cyclopropylmethyl carbinol
1-Methylcyclopentanol	(−)-Isopulegol
1-Ethylcyclopentanol	Decahydro-2-naphthol
2-Methylcyclopentanol	1-Hexen-3-ol
Cyclohexanol	Linalool (3,7-dimethyl-1,6-octadien-3-ol)
cis-4-Methylcyclohexanol	Geraniol (*trans*-3,7-dimethyl-2,6-octadien-1-ol)
trans-4-Methylcyclohexanol	Octanol
Cycloheptanol	Phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol)

TABLE II

MINIMUM LIMITS OF DETECTABILITY OF MONOFUNCTIONAL ALCOHOLS SPOTTED ON CELLULOSE THIN-LAYER PLATES (METHOD A)

Alcohol	*Minimum quantity detected* (μg)
Octanol	0.5
Farnesol (3,7,11-trimethyl-2,6,10-dodecatrien-1-ol)	5.0
1-Tetradecanol	5.0
1-Hexadecanol	1.0
Phytol	5.0
Cholesterol	10.0
Ergosterol	1.0
Androst-5-ene-3β,17β-diol	Neg. (to 50 μg)

TABLE III

TOSYLATES GIVING POSITIVE RESULTS WITH PNBP

Alcohol tosylate	*Minimum quantity detected* (μg)
1-Hexadecanol	0.1
Farnesol	0.2
1-Tetradecanol	1.0
Octanol	0.2
Phytol	2.0
Cholesterol	0.1
Ergosterol	0.2
Androst-5-ene-3β,17β-diol	0.2

TABLE IV

R_F VALUES OF ALCOHOLS REACTED WITH *p*-TsOCl AND SPOTTED ON CELLULOSE THIN-LAYER PLATES (METHOD B)

Minimum sensitivity: 0.75 μg.

Compound	R_F *values*
1-Methylcyclohexanol	0.46
cis-4-Methylcyclohexanol	0.46
trans-4-Methylcyclohexanol	0.46
Cyclohexanol	0.52
Cycloheptanol	0.52
(−)-Isopulegol	0.52
Borneol	0.60
1-Ethylcyclopentanol	0.62
Geraniol	0.62
D,L-Isoborneol	0.62
1-Cyclopropylethanol	0.64
1-Hexen-3-ol	0.64
(+)-Linalool	0.64
Decahydro-2-naphthol	0.66
1-Methylcyclopentanol	0.70
Cyclopentanol	0.72

indicates that purification of the tosylate increases the sensitivity of the test by a factor of 10. These data also suggest that the source of variation is incomplete tosylation of the alcohol *in situ* and/or partial codistillation of the volatile alcohols with the solvent.

Method B was developed to assure completeness of reaction when very low concentrations of alcohol were assayed. The reaction mixture resulting from Method B was spotted on TLC plates and developed to 10 cm in hexane–ethyl acetate (4:1, v/v). However, chromatography was necessary to separate the ester from the remaining reaction products since these substances interfered with the assay. The method was always sensitive to 0.75 μg of alcohol. The alcohols and the R_F values are listed in Table IV.

Polyhydroxy compounds

The reaction of six polyols (Table V) with the reagents was conducted, and acceptable results were obtained in all tests. We feel that the reaction of 4-(*p*-nitrobenzyl)-pyridine with polyol tosylates would present an interesting study and should be investigated further.

TABLE V

DETECTION AND SENSITIVITY OF POLYHYDROXY COMPOUNDS TO PNBP (METHOD A)

Compound	*Minimum quantity detected* (μg)
D-Glucose	5.0
D-Galactose	5.0
D-Xylose	5.0
D-Sorbitol	5.0
Glycerol	1.0
1,2-Ethanediol	1.0

Reaction in the presence of other functional groups

The influence of other functional groups on the reactivity of the hydroxyl group to the reagent was studied by using a series of amino alcohols, amino acids, phenolic compounds, hydroxylated carboxylic acids, ethers, and esters (Table VI). Tentative conclusions were drawn from the results:

The presence of an amino group does not interfere with the test since a series of five amino alcohols (1–6, Table VI) yielded a positive assay. Reaction of *p*-toluenesulfonyl chloride with amino alcohols yields the bis-derivative (tosylamide and tosylate), but only the tosylate groups are displaced by PNBP[6], a conclusion that is also supported by the negative results observed with phenethylamine and 3,4-dimethoxyphenethylamine (9–10, Table VI), both of which lack a hydroxyl group but carry an amino group that would form a tosylamide.

Phenolic compounds (11, 12, Table VI) gave negative results. In addition, the phenolic group appears to interfere with the assay of an aliphatic hydroxyl group in the side chain since noradrenaline (7, Table VI) did not give the typical blue of the alcohols. In fact, dopamine (8, Table VI), which lacks an aliphatic hydroxyl group, gave similar atypical results.

TABLE VI

INFLUENCE OF OTHER FUNCTIONAL GROUPS ON THE PNBP TEST FOR HYDROXYL GROUPS

No.	*Compound*	*Minimum quantity detected (μg)*	*Color*
1	2-Aminoethanol	positive (1.0)	blue
2	2-(Methylamino)-ethanol	positive (0.75)	blue
3	2-Amino-1-propanol HCl	positive (0.75)	blue
4	2-Amino-1-butanol	positive (0.75)	blue
5	3-Amino-1,2-propanediol	positive (0.75)	blue
6	N,N-Bis(β-hydroxyethyl)-*p*-anisidine	positive (0.5)	blue
7	Noradrenaline HCl [(α-(aminomethyl)-3,4-dihydroxy-benzyl alcohol HCl]	positive (5.0)	grey-brown
8	Dopamine HCl [4-(2-aminoethylpyrocatechol) HCl]	positive (5.0)	grey-brown
9	3,4-Dimethoxyphenethylamine	negative	
10	Phenethylamine	negative	
11	Resorcinol	negative	
12	2-Naphthol	negative	
13	Piperonyl alcohol	negative	
14	4-Aminobutyric acid	negative	
15	Glutamic acid	negative	
16	Proline	negative	
17	Serine	negative	
18	Malonic acid	negative	
19	Succinic acid	negative	
20	Tartaric acid	negative	
21	Citric acid	negative	
22	Phenylacetic acid	negative	
23	Benzoic acid	negative	
24	Diethyl tartrate	positive (5.0)	blue
25	Diethyl citrate	positive (5.0)	blue
26	Butyrobetaine (3-carboxypropyl)-trimethyl-ammonium hydroxide inner salt)	positive (10.0)	blue-grey
27	Choline bromide	positive (30.0)	purple
28	[3-(Ethoxycarbonyl)-2-hydroxy-propyl]-trimethyl-ammonium chloride	positive (10.0)	blue-grey
29	(3-Carboxy-2-hydroxypropyl)-trimethylammonium chloride acetate	positive (10.0)	blue-grey
30	Tristearin	negative	
31	1,2-Dimethoxyethane	negative	
32	Cineole (1,8-epoxy-*p*-menthane)	negative	

The several amino acids tested (14–17, Table VI) failed to give positive results. Serine, which contains a hydroxyl group in addition to an amino and carboxyl group, was also negative. Also, the carboxyl group appeared to interfere with the assay since the hydroxy acids (20, 21, Table VI) failed to give positive results though the ethyl esters (24, 25, Table VI) of these hydroxy acids did. Esters and ethers having no other functional groups did not react with the reagents (30–32, Table VI).

The quaternary ammonium compounds (27–29, Table VI) did not require pre-treatment with reagent A (tosylation) to give a positive result because these groups are easily displaced[7] by nucleophiles such as PNBP. Thus, it is possible to differentiate between alcohols (which require the 3-step procedure for assay) and substances carrying such functional groups as aziridine, oxirane, sulfonate, alkyl halides, and quaternary amino as substituents which require an alternative method of assay.

REFERENCES

1 J. EPSTEIN, R. W. ROSENTHAL AND R. J. ESS, *Anal. Chem.*, 27 (1955) 1435.
2 E. SAWICKI, D. F. BENDER, T. R. HAUSER, R. M. WILSON AND J. E. MEEKER, *Anal. Chem.*, 35 (1963) 1479.
3 T. A. CONNORS, L. A. ELSON AND C. L. LEESE, *Biochem. Pharmacol.*, 13 (1964) 963.
4 KURT RANDERATH, *Thin-Layer Chromatography*, translated by D. D. LIBMAN, 2nd rev. and enlarged ed., Academic Press, New York, 1966, pp. 252–253.
5 DIETER JÄNCHEN, *Thin-Layer Chromatography, Cumulative Bibliography I, 1965–1967*, CAMAG, Muttenz, Switzerland, 1967, pp. 32–33.
6 Results from the thesis work of one of the authors (R. F. SEVERSON) that will be reported in detail at a later date.
7 E. S. GOULD, *Mechanism and Structure in Organic Chemistry*, Holt, Rinehart, and Winston, New York, 1959, pp. 258–263.