Journal of Chromatography, 94 (1974) 294–297

CHROM. 7429

Note

A versatile and sensitive method for the detection of organic acids and organic phosphates on paper chromatograms

JOHN LEONARD FIRMIN

Department of Clinical Pharmacology, Royal Postgraduate Medical School, Hammersmith Hospital, DuCane Road, London W12 (Great Britain)

and

DAVID OCTAVIUS GRAY

Department of Botany and Biochemistry, Westfield College (University of London), Kidderpore Avenue, Hampstead, London NW3 (Great Britain)

(Received January 2nd, 1974)

Reagents sensitive to pH[1] are widely used for the detection of organic acids on paper chromatograms and can be very effective after neutral solvent systems (minimum detectable quantities after chromatography (M.D.s) ~ 1 μg). However, sensitivities are lowered by up to a factor of 100 when the more useful acidic or alkaline solvents are employed; methods of eliminating acidic/alkaline residues are tedious and not always effective. Alternative reagents[2] can generally only detect a specific and limited range of compounds.

However, the iron(III)-complexing ability of organic acids and organic phosphates does form the basis of a more general test[3], capable of detecting as little as 1 μg of citric and malic acids. Unfortunately this method is only moderately sensitive towards other organic acids, M.D.s normally being 50–100 μg, and at least one of the reagents employed is a known health hazard.

Thus we have devised a test based on similar principles in which uncomplexed iron(III) is revealed by its well known reaction with soluble thiocyanates. Its chief advantages are that it will detect less than 5 μg of any one of a wide range of compounds and that its sensitivity is not impaired by most solvent residues. Moreover, the reagents used are innocuous, inexpensive and readily available while the treated chromatograms are quite stable.

The most generally useful form of this test is to dip the chromatograms through 1.1% (w/v) AR ammonium thiocyanate in acetone and, after drying at 20–30°, to dip them through 0.03% (w/v) anhydrous iron(III)chloride in acetone. Iron-complexing spots generally appear as bleached, white zones on a pink background immediately the paper dries. However, cinnamic, glycollic, lactic, oxalic and quinic acids give yellow spots, while maleic acid gives a red and *p*-aminobenzoic acid a bluish colour.

Table I shows that the test was reasonably sensitive towards all the compounds examined, except for benzoic and *p*-aminobenzoic acids. Moreover, many substances

BLE I

SITIVITY OF THE IRON(III)–THIOCYANATE REAGENT TOWARDS ORGANIC ACIDS AND GANIC PHOSPHATES

compounds were spotted cold in the form indicated in parentheses onto Whatman No. 3MM paper. The chromograms were run in the organic phase of *tert*-amyl alcohol–formic acid–water (80:10:40, v:v) for 16 h at 20–25°, d for 5 h at 20–25°, and treated with 1.1% (w/v) AR ammonium thiocyanate in acetone followed by 0.03%) anhydrous iron(III) chloride in acetone as described in the text. The papers were examined by reflected day-t. Unless otherwise stated, compounds yielded a single, white spot.

pound	*Thiocyanate only,* 15 min after dipping*			*Full treatment 30 min after dipping through $FeCl_3$*		*Full treatment 3 days after dipping through $FeCl_3$*		
	M.D. (μg)	*Spot area (cm^2)*	*Colour*	*M.D. (μg)*	*Colour*	*M.D. (μg)*	*Spot area (cm^2)*	*Colour*
anic acids								
-Aminobenzoic acid (H)	>500			100	blue	100		
is-Aconitic acid (H)	> 40			10**	blue centre	5	5.0**	
enzoic acid (H)	>500			50		50	1.5	
innamic acid (H)	> 40			5	yellow	5	2.0	yellow
itric acid (H)	1	3.0		2		5	3.5	
socitric acid (Na_3)	2	6.0		5**		5	4.5**	
umaric acid (H)	10	2.0	pink	5	pink centre	2	2.0	
lycollic acid (H)	> 40			2	yellow with pink centre	2	2.0	yellow
lyoxylic acid (H)	> 40			2		2	2.5	cream
aconic acid (H)	> 40			5		4	2.0	
actic acid (H)	> 40			2	yellow	2	2.5	yellow
aleic acid (H)	40	2.0	pink	10	pink	40	1.0	pink
alic acid (H)	1	3.5		2		3	3.0	
alonic acid (Na_2)	2	4.0		5**	yellow	2	3.0**	yellow
xalic acid (H)	1			5	***	40		***
xaloacetic acid (H)	> 40			20§		40	2.0§	
-Oxoglutaric acid (H)	> 10			5		2	3.5	
almitic acid (H)	> 40			2		10	2.5	
yruvic acid (Na)	10	3.0**		2§		5	1.5§	
uinic acid (H)	> 40			5	yellow	5	2.0	yellow
uccinic acid (H)	50			5		2	2.5	
artaric acid (Na, K)	1	3.5		2**	white with yellow centre	1	1.0	
anic phosphates								
denosine triphosphate (Na_3)	0.5	0.5		10**		10	0.5**	
ructose 1-phosphate (Ba)	2	2.0		7		5	1.5	
ructose 6-phosphate (Ba)	0.5	1.0		5**		2	0.5**	
ructose 1,6-diphosphate (Na_4)	0.5	1.0		5**		5	0.5**	
lucose 1-phosphate (K)	1	1.0		5**		5	0.5**	
lucose 6-phosphate (Na)	5	1.0		10**		10	1.0**	
lyceric acid 2-phosphate (Ba)	0.5	1.0		2		2	0.5	
lyceric acid 3-phosphate (Na)	0.25	2.5		2**		1	2.5**	
lycerol 2-phosphate (Na)	1	2.5		5**		2	3.5**	
hosphoenolpyruvate (Na)	1	2.5		2**		1	3.0**	
ibose 5-phosphate (Na)	1	3.0		1		1	1.0	
rthophosphate (K)	0.25	2.5		0.5		0.5	2.5**	

(Continued on p. 296)

TABLE I *(continued)*

Compound	Thiocyanate only,* 15 min after dipping			Full treatment 30 min after dipping through $FeCl_3$		Full treatment 3 days after dipping through $FeCl_3$		
	M.D. (μg)	Spot area (cm^2)	Colour	M.D. (μg)	Colour	M.D. (μg)	Spot area (cm^2)	Colour
Amino acids								
Aspartic acid (H)	100			2	cream	5	1.0	cream
Glutamic acid (H)	100			5	cream	5	1.0	cream
Glycine (H)	100	2.0		5	cream	10	1.5	cream
Leucine (H)	100			5	yellow	5	1.5	yellow
Arginine (H)	100			5	yellow	5	1.0	yellow

* The R_F values of the components located by thiocyanate alone were identical with those revealed by the full form of the test except in the case of arginine. Moreover, on the basis of position and relative intensity the test revealed the same components in the organic phosphate preparations as the molybdenum blue reaction[4].

** Gave two spots at the 40-μg level.

*** At low concentrations (*ca.* 5 μg) oxalic acid runs as a small compact spot close to the origin which bleaches the reagent maximally after 24 h, and acquires a brown centre after about 7 days. At higher concentrations (*ca.* 40 μg) a red streak is formed in addition.

§ Gave three spots at the 40-μg level.

were visible as bleached spots against a very pale background after the chromatogram had been dipped through thiocyanate alone, and this probably provides the most sensitive known chemical method of detecting organic phosphates.

Iron-complexing compounds originally present in the paper and/or solvent become localised into zones during chromatography and this will cause an uneven background if the detecting reagents are insufficiently concentrated. The effect is worse for two-dimensional chromatograms and those run in phenol-containing solvents: such chromatograms may have to be dipped through 2.2% ammonium thiocyanate and 0.06% iron(III)chloride even though this halves the mean sensitivity according to tests on citric, fumaric, 2-oxoglutaric, and succinic acids. On the other hand, under favourable conditions, it is possible to obtain double the normal sensitivity by using 0.4% ammonium thiocyanate and 0.015% iron(III)chloride.

The test has given completely satisfactory results on chromatograms run in combinations of phenol, propanol, butanol, amyl alcohol, benzyl alcohol, formic acid, acetic acid, propionic acid, water, ammonia, chloroform and diethyl ether. However, its sensitivity is much impaired by phenol–water (100:39, w/v) in equilibrium with 2 *N* ammonia or methyl ethyl ketone–acetone–formic acid–water (80:6:2:12, v/v).

Stock solutions of the reagents in acetone are stable for at least thirty days at 20° if kept in darkness. However, solutions which have already been used to dip chromatograms should not be stored. Neither should unused solutions be exposed to intense light for more than a few hours as this decomposes the thiocyanate. Dipped chromatograms should not be allowed to lie in contact with each other for long periods but otherwise may be stored for at least one year in darkness or 36 h in daylight without significant loss of spot visibility: light does partially bleach the background however.

Chromatograms treated with these reagents fog X-ray film by contact within

two weeks. Thus, where appropriate, radioautograms should be prepared before the papers are dipped.

The iron(III)–hexacyanoferrate(II) reaction can also be used to detect organic acids and sugar phosphates but the best mean sensitivity obtained with 0.03% (w/v) anhydrous iron(III) chloride in acetone followed by 0.04% (w/v) potassium hexacyanoferrate(II) (potassium ferrocyanide) in 50% (v/v) aqueous ethanol, was only one quarter of that of the test described here for the compounds given in Table I.

NOTE BY THE EDITOR

This reaction has already been employed for detecting fluoride on paper chromatograms and it should be kept in mind that inorganic anions which complex iron(III) can interfere.

REFERENCES

1 R. M. C. Dawson, D. C. Elliot, W. H. Elliot and K. M. Jones (Editors), *Data for Biochemical Research*, Oxford Univ. Press, Oxford, 2nd. ed., 1969, Ch. 21, pp. 512–515.
2 R. M. C. Dawson, D. C. Elliot, W. H. Elliot and K. M. Jones (Editors), *Data for Biochemical Research*, Oxford Univ. Press, Oxford, 2nd ed., 1969, Ch. 21, pp. 515–517.
3 H. T. Gordon, L. N. Werum and W. W. Thornburg, *J. Chromatogr.*, 13 (1964) 272.
4 F. Leuthardt and E. Testa, *Helv. Chim. Acta.*, 34 (1951) 931.