THE IDENTIFICATION AND DETERMINATION OF SOME PHENOLIC ACIDS IN URINE USING TWO-DIMENSIONAL PAPER CHROMATOGRAPHY

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The identification and determination of vanillic, homovanillic, *p*-hydroxybenzoic, *p*-hydroxyphenylacetic, *m*-hydroxybenzoic and *o*-hydroxyphenylacetic acids by the use of two-dimensional paper chromatography has been investigated.

THE present investigation is concerned with the qualitative and quantitative examination of those phenolic acids that are stable to hot acid hydrolysis in the presence of 5N hydrochloric acid—vanillic, homovanillic, *p*-hydroxybenzoic, *p*-hydroxyphenylacetic, *o*-hydroxyphenylacetic and *m*-hydroxybenzoic acids. Preliminary hot acid hydrolysis does not appear to be particularly favoured by American investigators because of the lability of some phenolic acids. Hot acid hydrolysis was employed by Boscott and Cooke (1954) but the concentration of acid used was insufficient to affect the hydrolysis of glycine conjugates.

All the above phenolic acids, with the exception of *o*-hydroxyphenylacetic acid, are readily detectable on paper chromatograms prepared from normal human urine and represent different aspects of human metabolism. *m*-Hydroxybenzoic and vanillic acids are probably almost entirely of dietary origin although it has been suggested that the latter may be related to noradrenaline metabolism (Smith and Bennett, 1958) but this has been disputed (Shaw and Trevarthan, 1958). *p*-Hydroxyphenylacetic acid is an important metabolite of tyrosine and increased urinary excretion has been observed in a variety of conditions (Boscott and Cooke, 1954). It has been reported that *p*-hydroxybenzoic acid may be a metabolite of tyrosine (Booth, Masri, Robbins, Emerson, Jones and DeEds, 1960) although this substance may also be derived from *p*-cresol produced in the intestines as the result of bacterial activity (Williams, 1959). Homovanillic acid is recognised as a metabolite of 3,4-dihydroxyphenylalanine and dopamine and its determination could be of value in the study of phaeochromatocytoma. It does not appear to be derived from any dietary source. *o*-Hydroxyphenylacetic acid is derived from phenylalanine (Boscott, 1953, 1956; Armstrong, Shaw and Robinson, 1955) but appears to be only readily detectable in phenylketonuria. Booth, Masri, Robbins, Emerson, Jones and DeEds (1959) have, however, shown that in the rat and the rabbit, this substance may also be formed from coumarin.

Phenolic acids are excreted in urine mainly as conjugates with sulphate, glucuronic acid and glycine. The form of conjugation may be important in some instances but in general examinations, a mixture of conjugates may create confusion. Hydrolysis of conjugates was effected before chromatographic examination, hot acid hydrolysis in the presence of

5N hydrochloric acid being employed. Hydrolysis of sulphates, glucuronidates and glycine conjugates is effected. The methoxyl group and the parent substances—*m*-hydroxybenzoic, *p*-hydroxybenzoic, *p*-hydroxyphenylacetic, vanillic, homovanillic and *o*-hydroxyphenylacetic acids are unaffected by such treatment. Free phenolic acids are separated with ether and the extracts evaporated to dryness *in vacuo*. Volatile phenols are thus removed.

Three two-dimensional solvent systems have been used extensively for the examination of urinary phenolic acids. (1) Isopropanol : ammonia : water and benzene : propionic acid : water (Armstrong, Shaw and Robinson, 1956). (2) Benzene : acetic acid : water and 20 per cent aqueous potassium chloride (Boscott and Cooke, 1954). (3) Chloroform : acetic acid : water and 20 per cent aqueous potassium chloride (Booth, Emerson, Jones and DeEds, 1957).

The system of Armstrong and others has been used in this investigation.

EXPERIMENTAL

Hydrolysis of Conjugates and Extraction of Phenolic Acids

1 volume of urine and 1 volume of 10 N hydrochloric acid were boiled under a reflux condenser for 1½ hr. The cooled mixture was extracted three times with 4 volumes of ether. The combined ether extracts were evaporated to dryness in an all glass vacuum still. The residue was dissolved in ethanol so that 1 ml. ethanolic solution was equivalent to 50 ml. urine.

Two Dimensional Paper Chromatography

20 μl. of an ethanolic solution of the urinary phenolic acids (equivalent to 1 ml. of urine) was applied to a sheet (55 × 45 cm.) of Whatman No. 1 filter paper which was then stapled in the form of a cylinder. Development was carried out by the capillary ascent method with (i) isopropanol : ammonia 0·88 : water (8 : 1 : 1) as the first solvent mixture, and (ii) benzene : propionic acid : water (2 : 1 : 1), the organic phase, as the second solvent mixture. An all glass tank capable of holding a number of paper cylinders was used.

After development, the sheets were allowed to dry and then treated by one of the following systems.

(I) Pauly's Reagent (Bolling, Block and Sober, 1949; Block, 1951).

Reagents: (*a*) 1 per cent sulphanilamide in N hydrochloric acid. (*b*) 5 per cent sodium nitrite (w/v). (*c*) 10 per cent sodium carbonate (w/v).

Procedure: 10 ml. of (*a*) and 10 ml. of (*b*) were mixed in a 100 ml. glass stoppered measuring cylinder. After standing for 1 min., 80 ml. of butanol was added and the mixture shaken. Papers were sprayed with the aqueous phase of this mixture, and when dry with 10 per cent sodium carbonate solution.

(II) Diazotised *p*-nitraniline followed by 20 per cent aqueous sodium carbonate solution (Bray, Thorpe and White, 1950).

(III) Diazotised diethylaminoethyl *p*-aminophenylsulphone (Boscott and Cooke, 1954).

Standard solutions. (1) ethanolic solutions of *p*-hydroxybenzoic, *p*-hydroxyphenylacetic, *o*-hydroxyphenylacetic, vanillic, homovanillic and *m*-hydroxybenzoic acids containing 1 mg. acid/ml. (20 μl. is equivalent to 20 μg. acid). (2) an ethanolic solution containing all the above acids at 1 mg. acid/ml. (20 μl. is equivalent to 20 μg. of each acid). 20 μl. of a standard solution was applied to a paper. Development was carried out at the same time as the unknown.

In an examination, four paper chromatograms were developed simultaneously. (1) blank, (2) urine extract applied for spraying, (3)

TABLE I

THE REACTION (COLOUR DEVELOPMENT) OF PHENOLIC ACIDS ON PAPER CHROMATOGRAMS WHEN TREATED WITH 3 SPRAY REAGENTS

Substance	Spray Reagent		
	1	2	3
m-Hydroxybenzoic Acid	Yellow	Dark red	Yellow
p-Hydroxybenzoic Acid	Yellow	Red	Purple
o-Hydroxyphenylacetic Acid ..	Orange	Purple	Purple
p-Hydroxyphenylacetic Acid ..	Purple	Purple	Purple
Vanillic Acid	Brick red	Purple	Purple
Homovanillic Acid	Purple	Light brown	Purple

NOTE: aqueous sodium carbonate added subsequently or with the reagent.

Spray Reagent

1. Diazotised sulphanilic acid (Bolling and others, 1949; Block, 1951.
2. Diazotised *p*-nitroaniline (Bray and others, 1950).
3. Diazotised diethylaminoethyl *p*-aminophenylsulphone (Boscott and Cooke, 1954).

urine extract applied, unsprayed, for the colorimetric determination of *m*-hydroxybenzoic and *p*-hydroxyphenylacetic acid, (4) standard quantities of the 6 phenolic acids applied for spraying.

Quantitative Evaluation of Coloured Spots

A section of paper containing the coloured spot was separated and extracted for 2 hr. with 10 ml. of 50 per cent aqueous methanol. The extinction of the coloured solution was then read against a blank at an appropriate wavelength. A measured area of paper containing the coloured spot was always separated from the paper. A "blank" paper was prepared at the same time. The "blank" solution was prepared from this paper, using a section of identical area and R_F value.

RESULTS AND DISCUSSION

The six phenolic acids could be readily identified on paper chromatograms and although *p*-hydroxyphenylacetic acid and *m*-hydroxybenzoic acid showed some overlapping, the other phenolic acids showed good separation.

Diazotised sulphanilic acid appeared to be the most suitable spray reagent since it showed a greater differentiation in colour of spots of individual phenolic acids (see Table I). For quantitative examinations this spray reagent has the lowest blank values.

Examination of urinary extracts showed the invariable presence of homovanillic, vanillic, *m*-hydroxybenzoic, *p*-hydroxybenzoic and *p*-hydroxyphenylacetic acid. *o*-Hydroxyphenylacetic acid was just detectable (less than 1 mg./day) in conditions other than phenylketonuria. In two cases of phenylketonuria, values of 24·0 and 8·2 mg./day were found.

Values obtained from the examination of 12 normal urines are recorded in Table III. The excretion of *p*-hydroxybenzoic acid was found to be

TABLE II

WAVELENGTH AT WHICH AQUEOUS METHANOLIC EXTRACTS OF THE COLOURED COMPLEXES OF THE FOLLOWING PHENOLIC ACIDS FROM PAPER CHROMATOGRAMS ARE MEASURED COLORIMETRICALLY

Substance	Wavelength mμ
m-Hydroxybenzoic acid	460
p-Hydroxybenzoic acid	470
o-Hydroxyphenylacetic acid	470
p-Hydroxyphenylacetic acid	500
Vanillic acid	480
Homovanillic acid	500

increased by about 45 per cent after the ingestion of 10 g. of tyrosine, suggesting that this acid might be derived in part from the aromatic amino-acid. The oral ingestion of 6 g. of benzoic acid produced no appreciable effect upon the urinary excretion of *p*-hydroxybenzoic acid. It does not appear that *p*-hydroxylation occurs in the metabolism of benzoic acid.

Paper chromatograms of urinary extracts often showed a number of coloured spots close to the benzene:propionic acid base line. These were presumably due to phenolic lactic acids which were not considered in this investigation.

Substituted cinnamic acids, for example, ferulic, *o*- and *p*-hydroxycinnamic acids produce typically coloured spots but as the result of the

TABLE III

THE NORMAL URINARY EXCRETION IN MG./DAY OF SOME PHENOLIC ACIDS

Substance	Minimum	Maximum	Average
m-Hydroxybenzoic acid	8·3	38·6	23·2
p-Hydroxybenzoic acid	2·1	12·5	6·3
o-Hydroxyphenylacetic acid		less than 0·5	
p-Hydroxyphenylacetic acid	12	28	14
Vanillic acid	4·6	36·8	14·8
Homovanillic acid	0·8	1·6	1·2

hydrolytic treatment employed, these acids are converted into forms which do not react with any of the three spray reagents.

Substituted phenolic lactic acids show instability when subjected to the hydrolytic treatment. When examined by the paper chromatographic technique, 4-hydroxy-3-methoxymandelic acid showed losses of about 50 per cent. The residues obtained from this acid had a strong smell of vanillin indicating chemical changes that may have occurred

during decomposition. Vanillin itself gave little colour on chromatograms. Phenolic pyruvic acids are unstable in the presence of the first solvent mixture. Thus the methods adopted are unsuitable for the examination of substituted cinnamic acids and phenolic lactic and pyruvic acids.

Standard solutions of the phenolic acids applied to paper could be readily eluted as the coloured complexes. Extinctions were read at the wavelengths shown in Table II and were found to be linear within the range 0 to 80 μg. Whatman No. 1 filter paper contains "phenolic" material which reacts with all three spray reagents. Much of this material follows the solvent fronts in both systems. Therefore a blank sheet of Whatman No. 1 paper was developed along with the sheets to which urine extracts or standard solutions of the phenolic acids had been applied.

The zones occupied by *m*-hydroxybenzoic and *p*-hydroxyphenylacetic acids exhibit much overlapping. Although the two acids may be distinguished visually, quantitative determination by the elution of the coloured complexes is not practical. These acids were determined in residues of alcohol eluates obtained from an unsprayed paper chromatogram which was developed at the same time. *m*-Hydroxybenzoic acid was determined by the chloroimide reaction (Tompsett, 1958) and *p*-hydroxyphenylacetic acid with 1-nitroso-2-napthol (Tompsett, 1958). Neither acid interferes with the colorimetric determination of the other.

It is very difficult to distinguish between *m*-hydroxybenzoic and *m*-hydroxyphenylacetic acids since both acids possess similar R_F values in the solvent systems employed and have similar reactions towards spray reagents.

References

Armstrong, M. D., Shaw, K. N. F. and Robinson, K. S. (1955). *J. biol. Chem.*, **213**, 797–811.

Armstrong, M. D., Shaw, K. N. F. and Hall, P. E. (1956). *Ibid.*, **218**, 293–303.

Block, R. J. (1951). *Arch. Biochem. Biophys.*, **31**, 266–272.

Bolling, D., Block, R. J. and Sober, H. A. (1949). *Fed. Proc.*, **8**, 185.

Booth, A. N., Emerson, O. H., Jones, F. T. and DeEds, F. (1957). *J. biol. Chem.*, **229**, 51–59.

Booth, A. N., Masri, M. S., Robbins, D. J., Emerson, O. H., Jones, F. T. and DeEds, F. (1959). *Ibid.*, **234**, 946–948.

Booth, A. N., Masri, M. S., Robbins, D. J., Emerson, O. H., Jones, F. T. and DeEds, F. (1960). *Ibid.*, **235**, 2649–2652.

Boscott, R. J. and Bickel, H. (1953). *Scand. J. clin. Lab. Invest.*, **5**, 380–382.

Boscott, R. J. and Cooke, W. J. (1954). *Quart. J. Med.*, **47**, 307–322.

Boscott, R. J. and Kerman (1955). *Biochem. J.*, **60**, iv.

Bray, H. G., Thorpe, W. V. and White, K. (1950). *Ibid.*, **46**, 271–275.

Shaw, K. N. F. and Trevarthen (1958). *Nature, Lond.*, **182**, 797–798.

Smith, P. and Bennett, A. M. H. (1958). *Ibid.*, **181**, 709.

Tompsett, S. L. (1958). *Clin. Chem. Acta*, **3**, 149–159.

Williams, R. T. (1959). *Detoxication Mechanisms*, 2nd ed., p. 298, London: Chapman and Hall, Ltd.