POLYHYDROXY (CATECHOLIC) PHENOLIC ACIDS—THE FORMATION OF *m*-HYDROXY- AND METHOXY-DERIVATIVES IN MAN

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A method has been described for the determination of the methoxy group in phenolic compounds. Tannic and 3:4-dihydroxybenzoic acids when administered orally to man are excreted in appreciable quantities as 4-hydroxy 3-methoxybenzoic and *m*-hydroxybenzoic acids.

THE present communication is an extension of the studies reported previously[1]. Human urine contains appreciable quantities of *m*-hydroxy- and 4-hydroxy-3-methoxyphenolic acids[2–8]. The origin of the *m*-hydroxy acids has been obscure since they do not occur in nature nor is their source dietary. Paper chromatographic studies suggest that 3:4-dihydroxyphenolic derivatives may be dehydroxylated to produce *m*-hydroxyphenyl compounds and also methylated to produce 4-hydroxy 3-methoxyphenyl[5,6,9,10].

The present paper is concerned with quantitative studies involving the determination of the methoxy group and the colorimetric determination of *m*-hydroxybenzoic and vanillic acids.

METHODS

Preparation of Urine Extracts

10 ml. of urine is placed in a test tube together with 1 ml. of 10 N hydrochloric acid and the whole placed in a boiling water bath for 1 hour. After cooling the hydrolysed urine is extracted three times with 40 ml. quantities of re-distilled ether in a measuring cylinder[1]. The combined ether extracts are evaporated to dryness. This process does not appear to have any effect upon the parent substances under investigation.

The Colorimetric Determination of m-*Hydroxybenzoic Acid and Vanillic Acid* (4-*Hydroxy*-3-*methoxybenzoic Acid*

The technique in principle has been described previously[1,8]. Initially a separation is made using paper chromatography and the Kawerau Unit with benzene : acetic acid : water as the solvent.

An ethanolic solution of the urinary extract (≡ 2·5 ml. urine) is applied to the paper. After development, *m*-hydroxybenzoic acid is eluted with ethanol from strips 1 to 4 and vanillic acid from strips 6 to 8. After removal of the ethanol, the two phenolic acids are determined by means of 2:6-dichloroquinone chloroimide (the Gibbs Reaction). The colour is allowed to develop for 30 minutes after which the mixture is shaken with *n*-butanol.

With *m*-hydroxybenzoic acid, the aqueous phase is used for colorimetric determination while with vanillic acid, the *n*-butanol extract is used. Standards of 10, 20 and 40 μg., are set up at the same time.

The Determination of the Methoxy (Phenolic) Group

The procedure consists of hydrolysis by acid and the collection of the liberated methanol, oxidation of the methanol to formaldehyde, and the determination of the formaldehyde with chromotropic acid.

Hydrolysis. Hydrolysis and distillation is carried out in all glass equipment consisting of a 100 ml. round bottomed flask attached to a water cooled condenser. The material under examination is contained in the flask and to this are added 10 ml. of water and 5 ml. of concentrated sulphuric acid. The mixture is heated and the distillate collected. The heating is continued until the sulphuric acid reaches the fuming stage. The mixture is allowed to cool, when the heating is continued after the addition of 5 ml. of water, the sulphuric acid being again allowed to reach the fuming stage. This part of the procedure is repeated again so that three distillates in all are collected.

About $\frac{1}{2}$ to 1 g. of sodium bicarbonate is added to the combined distillate which is heated in apparatus similar to that used above; 10 ml. of distillate containing the methanol is collected. Less drastic forms of hydrolysis have been found to be quite ineffective.

Oxidation of methanol to formaldehyde[11]. *Reagents.* 5 per cent v/v ethanol in water; 1 per cent w/v potassium permanganate; 25 per cent v/v phosphoric acid; hydrogen peroxide, 5 volumes.

Technique. To the 10 ml. of distillate are added 1 ml. of 5 per cent ethanol, 5 ml. of 1 per cent potassium permanganate and 1 ml. of 25 per cent phosphoric acid. The mixture is allowed to stand at room temperature with frequent shaking for 1 hour, after which excess of permanganate is removed by the addition of hydrogen peroxide. The mixture is transferred to an all-glass still and heated to boiling; 10 ml. of distillate is collected.

Colorimetric determination of formaldehyde[12]. *Reagents.* Chromotropic acid reagent (prepared fresh before use. 0·2 g. of purified chromotropic acid is dissolved in 2 ml. of water to which 48 ml. of 13 M sulphuric acid is added). 9 M sulphuric acid.

Technique. Into a test tube is measured 1 to 3 ml. of distillate. Water is added to 3 ml. 5 ml. of chromotropic acid reagent is then added and after mixing, the tube is placed in a boiling water bath for 30 minutes. After cooling the mixture is diluted to 10 ml. with 9 M sulphuric acid. Readings are made against a blank at 570 mμ.

Blank. With each set of determinations a blank is set up using 10 ml. of water, the complete procedure being carried out except for the initial acid hydrolysis.

Standards. Initially standards of methanol (0·2, 0·5 and 1·0 mg.) were set up, the complete procedure being carried out except for the initial acid hydrolysis. Later standards of vanillic acid (0·5, 1·0 and 2·0 mg.) were set up, the complete procedure being carried out.

The Determination of Polyhydroxy (Catecholic) Phenolic Acids

The method has been described in detail elsewhere[1].

Results and Discussion

A number of compounds possessing the methoxy group have been examined using the complete procedure. Methanol was used as the standard. The results are shown in Table I. The methyl group of such

TABLE I

The determination of the methoxy group
(Quantities employed—0·5, 1·0, 2·5, 5 and 10 mg.)

Compound	Recovery expressed as per cent of theoretical
4-Hydroxy-3-methoxybenzoic acid	101 to 109
4-Hydroxy-3-methoxy cinnamic acid	98 to 108
Codeine	89 to 99
4-Hydroxy-3-methoxy phenylacetic acid	84 to 97
p-Methoxyphenylacetic acid	81 to 93
m-Methoxyphenylacetic acid	83 to 95

compounds as methionine, *N*-methylnicotinamide, and choline is not determined by the procedure. In the case of codeine the procedure measures the methoxyl but not the *N*-methyl group.

The urinary excretion of phenolic methoxy compounds after the oral ingestion of 1 g. of tannic or 3:4-dihydroxybenzoic acids has been studied in man. Catecholic phenolic acids were measured at the same

TABLE II

The urinary excretion by man of phenolic methoxy compounds after the oral ingestion of 1 g. of tannic or 3:4-dihydroxybenzoic acids

	3:4-Dihydroxy-phenolic compounds	Phenolic methoxy compounds	
	(as 3:4-dihydroxy-benzoic acid) (mg.)	(as methanol) (mg.)	(as vanillic acid) (mg.)
Tannic acid			
1. A.	18·5	4·5	22·5
B.	49·5	20·5	102·5
2. A.	16·5	3·2	16·0
B.	46·5	25·6	128·0
3. A.	14·5	3·8	19·0
B.	38·5	23·8	119·0
3:4-Dihydroxybenzoic acid			
1. A.	16·5	3·8	19·0
B.	198·0	33·3	167·0
2. A.	18·5	4·0	20·0
B.	185·0	36·8	184·0
3. A.	14·5	3·2	16·0
B.	210·0	38·0	190·0

A—control. B—after administration

time. In these experiments the night urine (10 p.m. to 7 a.m.) was used to reduce the effect of any changes in dietary intake. The results are shown in Table II and it will be seen that a marked proportion of the ingested acids are excreted in a methylated condition.

The experiments with 3:4-dihydroxybenzoic acid were repeated but "vanillic" and "*m*-hydroxybenzoic" acids were estimated. The results are shown in Table III. It will be noted that a marked increase in the

TABLE III

THE URINARY EXCRETION BY MAN OF "*m*-HYDROXYBENZOIC ACID" AND "VANILLIC ACID" AFTER THE ORAL INGESTION OF 1 G. OF 3:4-DIHYDROXYBENZOIC ACID

	3:4-Dihydroxybenzoic acid (mg.)	*m*-Hydroxybenzoic acid (mg.)	Vanillic acid (mg.)
1. A.	14·5	25·5	4·8
B.	185·0	145·0	96·5
2. A.	21·5	14·5	6·8
B.	165·0	125·0	135·0
3. A.	18·5	18·5	14·5
B.	175·0	165·0	145·0

A—control. B—after administration.

Total accountable recovery per cent of 3:4-dihydroxybenzoic acid

1.	43
2.	53
3.	49

excretion of 4-hydroxy-3-methoxybenzoic (vanillic) acid is accompanied by a marked increase in the excretion of *m*-hydroxybenzoic acid. It would appear that the metabolism of 3:4-dihydroxyphenolic acids includes methylation to produce a 4-hydroxy 3-methoxy compound and dehydroxylation to produce a *m*-hydroxy compound.

For 3:4-dihydroxybenzoic acid, known metabolites account for approximately 50 per cent of the intake during the short period of observation.

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