RESEARCH PAPERS

THE DETERMINATION AND EXCRETION OF POLYHYDROXY (CATECHOLIC) PHENOLIC ACIDS IN URINE

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The Mitchell reaction (ferrous sulphate) has been applied to the determination of catecholic phenolic acids and pyrogallol in urine. A technique has been described for the determination of pyrogallol in the presence of catecholic phenolic acids. The separation of catecholic phenolic acids by paper chromatography has been examined. A study has been made of the excretion of these substances in urine.

THE present paper is concerned with the determination in urine of certain catecholic phenolic substances, namely pyrogallol and gallic, 3:4-dihydroxybenzoic and caffeic acids. Although certain aspects of the pharmacology of tannic acid have been investigated[1–5], the group has received little attention, probably because of a lack of suitable analytical techniques.

The determination is by means of the Mitchell reaction[6,7]. The purple colour so produced is specific for this group of substances and the intensity follows Beer's Law up to 500 μg. The intensity of colour is not however proportional on a molecular basis when different reacting substances are compared. As a result, 3:4-dihydroxybenzoic acid has been used as the general standard. A preliminary separation by means of ether is necessary since the reaction cannot be applied directly to urine. Hot acid-hydrolysis is also an essential preliminary since phenolic acids are mainly excreted as conjugates which may not react and are not soluble in ether.

When the reaction is applied to urine extracts, there is some development of a non-specific yellow colour—this may amount to the equivalent of 20 mg./day expressed as 3:4-dihydroxybenzoic acid.

Catechol, which is poorly soluble in ether, and which reacts in the Mitchell reaction, could not be recovered and hence is not included in the final results. The methoxy phenolic acids like ferulic, vanillic and syringic acids, which also occur in human urine, are not included since the methoxy group is stable under the conditions of hydrolysis employed.

An attempt has been made to determine the individual reacting phenolic substances. Pyrogallol has been determined by reason of its ready volatility in hot ethanol vapour. Paper chromatography has been employed to separate the acids. A complete separation of gallic acid was achieved but caffeic and 3:4-dihydroxybenzoic acids could be partially separated only.

The Determination of Total Catecholic Phenols and Phenolic Acids

Reagents. (1) Ferrous sulphate reagent (Mitchell). Ferrous sulphate ($FeSO_4 \cdot 7H_2O$) 0·1, sodium potassium tartrate 0·5 g., and water to 100 ml.

(2) 10 per cent (w/v) ammonium acetate. (3) 2N aqueous ammonia solution (approximate). The concentration of the ammonia solution should be so adjusted that the pH of the final reaction mixture is 7·8.

TABLE I

THE RECOVERY OF CATECHOLIC PHENOLS AND PHENOLIC ACIDS ADDED TO 10 ML. URINE

	Quantity added, µg.	Recovery, per cent
Initial content of urine—18 mg./l.		
A. Gallic acid	50	89
	100	109
	250	104
	500	98
Initial content of urine—9·8 mg./l.		
B. 3:4-Dihydroxybenzoic acid ..	50	87
	100	89
	250	94
	500	97
Initial content of urine—12·5 mg./l.		
C. Caffeic acid	50	85
	100	88
	250	91
	500	96
Initial content of urine—14·5 mg./l.		
D. Pyrogallol	50	89
	100	91
	250	93
	500	97

Hydrolysis. 10 ml. of urine and 1 ml. of 10N hydrochloric acid are heated in a glass tube with a ground glass stopper in a boiling water bath for 1 hour.

Extraction. The cooled mixture is extracted three times with 40 ml. quantities of ether in a glass stoppered measuring cylinder, the ether

TABLE II

THE DETERMINATION OF PYROGALLOL IN THE PRESENCE OF GALLIC AND 3:4-DIHYDROXYBENZOIC ACID—REMOVAL OF PYROGALLOL BY VOLATILISATION

Pyrogallol, µg.	Gallic acid, µg.	Gallic acid recovered, µg.
50	150	156
100	200	192
150	200	208
Pyrogallol, µg.	**3:4-Dihydroxybenzoic acid, µg.**	**3:4-Dihydroxybenzoic acid recovered, µg.**
50	150	158
100	200	210
150	200	208

extracts being separated with a teat pipette. On the addition of ether, the mixture is shaken vigorously for 2 minutes.

Removal of ether. The ether is allowed to evaporate spontaneously at room temperature.

Development of the colour. The residue is dissolved in 0·5 ml. of water and 0·5 ml. of ferrous sulphate reagent added. Then 10 ml. of 10 per cent

ammonium acetate solution are added, followed by 1 ml. of 2N ammonia solution. The mixture is allowed to stand at room temperature for 20 minutes and then read against a blank at 560 mμ (Unicam Spectrophotometer S.P.350).

Standard. A standard containing 250 μg. of 3:4-dihydroxybenzoic acid is set up at the same time.

The Determination of Pyrogallol

Pyrogallol is determined by difference. An ethereal extract is prepared as described above. This is evaporated to dryness in an all glass still *in vacuo* (water pump), a water bath heated to near boiling point being

TABLE III

THE DISTRIBUTION OF CATECHOLIC PHENOLIC ACIDS ON PAPER (WHATMAN NO. 1) AFTER CHROMATOGRAPHY (*n*-BUTANOL/ACETIC ACID/WATER)

Strip No.	Per cent acid located		
	Gallic acid	3:4-Dihydroxybenzoic acid	Caffeic acid
5	11	—	—
6	89	—	—
7	—	39	—
8	—	61	31
9	—	—	69

employed. 80 ml. of ethanol are added and the evaporation repeated. This process is repeated twice. Pyrogallol is thereby removed by volatilisation.

Reacting phenolic acids are then determined in the residue as described above. The difference between this result and that obtained above is taken to be representative of the pyrogallol content.

Separation of Catecholic Phenolic Acids by Paper Chromatography

Whatman No. 1 filter paper (width 15 cm.) is used and development is with the organic phase of *n*-butanol/acetic acid/water (40/10/50) by the descending technique. A beaker containing the aqueous phase is placed

TABLE IV

THE RECOVERY OF GALLIC ACID AND 3:4-DIHYDROXYBENZOIC ACID FROM 10 ML. URINE AFTER SEPARATION ON PAPER

Quantity added, μg.	Quantity recovered, μg.	Recovery, per cent
Gallic acid		
1000	910	91
500	440	88
200	185	93
3:4-*Dihydroxybenzoic acid*		
500	450	90
250	210	84
100	85	85

at the bottom of the tank. Two parallel lines, 3 cm. apart, are drawn horizontally across the paper and just below the level of the trough. The extract obtained from at least 10 ml. of urine is dissolved in 10 ml. of ethanol and applied to the paper between the parallel lines. Development

is allowed to proceed until the solvent front has advanced 30 cm. from the upper line (overnight). After drying, the paper is divided into 10 equal parts between the point of origin and the limit of the solvent front. Each strip is then extracted with cold ethanol overnight. The alcoholic extracts are evaporated to dryness and the Mitchell reaction applied to the residues.

TABLE V

CATECHOLIC PHENOLS AND PHENOLIC ACIDS IN HUMAN URINE

A, Daily exretion—as 3:4-dihydroxybenzoic acid
46 to 116 mg./day (8 cases)
B, "pyrogallol" excretion

	"Pyrogallol" (mg./day)	Total (mg./day)
1.	8·2	46
2.	10·6	84
3.	8·4	108
4.	7·6	92

C. Variations in excretion throughout 24 hours

Time of excretion	Total excreted (mg.)	Excretion (mg./hr.)
2 a.m. to 7 a.m.	14·4	2·8
7 a.m. to 10 a.m.	17·6	5·9
10 a.m. to 2 p.m.	20·0	5·0
2 p.m. to 10 p.m.	41·6	5·2
10 p.m. to 2 a.m.	12·9	3·2

D. The distribution of individual phenolic acids

	Per cent		
	A	B	C
Gallic acid	28	31	22
3:4-Dihydroxybenzoic acid ..	56	55	58
Caffeic acid	16	14	20

RESULTS

The data in Table I indicate that gallic, caffeic and 3:4-dihydroxybenzoic acids and pyrogallol added to urine can be determined quantitatively by the procedure described.

From Table II it can be seen that pyrogallol can be determined accurately in the presence of the reacting phenolic acids by the use of the difference technique.

The data in Table III show the zones occupied by the three reacting phenolic acids after separation by paper chromatography. Material occupying strips 5 and 6 and reacting to the Mitchell reaction is assumed to represent gallic acid. In the case of 3:4-dihydroxybenzoic and caffeic acids, there is some overlapping. For the purposes of the present experimental work, it will be assumed that reacting material located on strips 7 and 8 represents 3:4-dihydroxybenzoic acid whereas that located on strip 9 represents caffeic acid. It is quite possible that a complete separation could be achieved by using a longer time of development.

As shown in Table IV gallic and 3:4-dihydroxybenzoic acids added to urine can be determined quantitatively after separation by paper chromatography. Pyrogallol is not detectable after such treatment.

Excretion in Urine

Data are recorded in Table V from the examination of urine. The daily excretion appears to be about 100 mg./day (V*A*) and the greater part appears to be excreted during the day, suggesting that these substances are of dietary origin (V*C*). Dietary examinations would suggest that tea

TABLE VI

THE EXCRETION OF CATECHOLIC PHENOLIC ACIDS IN URINE AFTER THE ADMINISTRATION OF TANNIC ACID IN MG./DAY

A. Oral route (1·5 *g.**)

Day 1	Day 2	Day 3 (administered)	Day 4
38	51	149	70
106	95	185	90
34	78	168	85

B. Barium enema (1200 *ml.*) *containing* 2 *per cent tannic acid*

Day 1	Day 2	Day 3 (enema)	Day 4	Day 5
47	43	—	275	98
—	34	160	25	—
40	54	102	43	56
43	38	49	43	—
37	36	39	50	51

* 1·5 g. tannic acid was the total oral dose. Larger doses were difficult to tolerate.

infusions are responsible for most of the reacting material and that the intake is about 1 g./day. This would suggest that absorption rates are not particularly high.

More direct data on absorptive rates are recorded in Table VI. Administration of tannic acid by mouth (VI*A*) resulted in demonstrable increases in excretion but absolute absorptions are quite small. The data recorded in VI*B* are from patients receiving barium enema containing 2 per cent tannic acid for the X-ray examination of the colon. Absorptions are generally low.

The separation of phenolic acids by paper chromatography would suggest that 3:4-dihydroxybenzoic acid is the predominant reacting substance.

The presence of pyrogallol (V*B*), a relatively more toxic substance, is of some interest. It is probably derived from gallic acid by decarboxylation in the alimentary tract.

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