URINARY PHENOLIC ACIDS ARISING FROM ENDOGENOUS METABOLISM

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Summary: The gut of four post operative intestinal surgery patients maintained on glucose saline drip, was sterilized and then blood and urinary phenolic acids were estimated by paper chromatographic technique. Blood and urinary phenolic acids were also studied in 8 normal individuals. There were no exogenous sources of phenolic acids for the patients since the antibiotics being administered to them are not known to be metabolised to phenolic acids. Comparing the phenolic acids of the normals with those of patients, it is concluded that vanillic acid, vanilloylglycine, feruoyl glycine, 3 methoxy, 4 hydroxy phenyl hydracrylic acid, the m-hydroxy phenolic acids and two spots staining yellow with p-nitraniline are all exogenous phenolic acids.

Key words: endogenous phenolic acids plasma phenolic acids

Urinary phenolic acids can arise from endogenous metabolism of aromatic amino acids, from ingested vegetables and fruits (3,4), from the metabolism of intestinal bacteria (2) and from the break down products of certain drugs (13). Endogenous phenolic acids, naturally, will be of interest as indicators of disturbed metabolism of aromatic amino acids in disease. Attempts have, therefore, been made to identify these endogenous phenolic acids from the other phenolic acids. In one such study (11) 4 normal adults were kept only on glucose diet for three days prior to the study of their urinary phenolic acids. In another study (2) 6 normals and 2 patients of alcoholic cirrhosis were given oral neomycin for the gut sterilisation before investigating urinary phenolic acids. Present study is also an attempt in the same direction.

MATERIALS AND METHODS

Four patients in the post operative period after intestinal surgery (palliative gastro jejunostomy for carcinoma of pancreas, anterior resection and anastomosis for cancer of rectum, herniotomy for strangulated hernia and exploration for stab would abdomen), and 8 normals as controls were studied to estimate their blood and urinary phenolic acids. In two of the four patients gut was sterilized pre-operatively by giving chlorostreptomycin two capsules 6 hourly (each capsule contains 125 *mg* chloromycetin and 125 *mg* streptomycin) for three days prior to operation. In post-operative period all the four patients were on glucose-saline drip for a period of 4 to 5 days and were getting 500 *mg* achromycin twice daily intravenously follwed by a dose of 250 *mg*, orally, 6 hourly. Thus the exogenous sources of phenolic acids (dietary and bacterial) in the patients were suppressed. Blood and urine samples were collected 96 hours after the

operation. In controls, fasting morning urine samples were collected for study after discarding the night urine. Blood samples were also collected while collecting the urine samples.

Extraction of urine samples (10): Urine made alkaline with sodium bicarbonate was extrated with 3 x ½ volume of peroxide free ether (ether kept over ferrous sulfate crystalls). Every time the ethereal layer was discarded. The sample was, next, made acid (pH 2.0) with 4 N sulfuric acid and saturated with ammonium sulfate. It was again extracted with 3 x ½ volumes of ether-ethyl alcohol mixture (8:2 v/v). The ether extract contained the phenolic acids.

Extraction of blood samples: This was done using the following method (9):—

Proteins aad the formed elements of blood were precipitated by mixing 10 *ml* of an oxalated sample with 5 *g* of ammonium sulfate and 10 *ml* of a mixture of ether (peroxide free)-ethanol-sulfuric acid (80 : 20 : 08 v/v) in a 30 *ml* centrifuge tube. The tube was kept under fan for half an hr to complete the precipitation of proteins and to reduce the volume of the volatile solvents, at the same time. On centrifugation, 3 layers separate out. The upper organic layer is added to a 50 *ml* beaker. Piercing through the middle layer consisting of precipitated proteins and the formed elements, the lower aqueous layer is drawn out, acidified and extrated with 3 x ½ volume of ether-ethanol mixture (8:2 v/v), organic layer each time being transferred to the 50 *ml* beaker referred above. Lastly the middle layer of precipitated proteins and the formed elements is first extracted with 7 *ml* of the above ether-ethanol mixture and then with 6 *ml* of 95% ethanol: extracts being pooled in the same 50 *ml* beaker. The solvents in the 50 *ml* beaker are evaporated off under a fan.

Besides the phenolic compounds the dry residue in the beaker contains fats and acid haematin. It is washed with 2 to 2.5 *ml* of one third saturated solution of calcium hydroxide, adding small amounts each time. The calcium hydroxide extract is washed with 4 x ½ volume of ether in a 1x10 *cm* tube to remove fats, acid haematin and neutral phenols. If a ring of insoluble soaps forms at the top of the aqueous layer, it is bodily removed. Sodium carbonate solution cannot be used in this step as the removal of soaps and acid haematin becomes difficult. Stability of phenolic acids in the above calcium hydroxide solution is comparable with that in 2% sodium bicarbonate solution generally employed in phenolic acid extraction.

Paper chromatographic separation of phenolic acids: This was done using isopropanol-tert-butanol-n-butanol-ammonia (4 : 2 : 2 : 1 v/v) in the first direction and ether-xylene-formic acid-water (80:30:10 :3), in the second direction (7). Quantitative evaluation was done by eluting the coloured spots (8). The faint spots, however, were evaluated by visually comparing them with serial standard spots of p-hydroxy phenyl acetic acid (p-HPAA) and p-hydroxy benzoic acid (p-HBA). All purple spots were evaluated in terms of p-PHAA and all red spots in terms of p-HBA.

Urinary creatinine was estimated using the alkaline picrate method (12). This was done to express concentration of urinary phenolic acids in terms of urinary creatinine.

RESULTS AND DISCUSSION

Frequency of occurence of different phenolic acids in urinary samples of the patient and the normals is shown in Table I. Some of the commoner phenolic acids are quantitated and their amounts in blood and urine samples are presented in Table II.

In the present study, exogenous sources (dietary and bacterial) of phenolic acids have been suppressed in patients since their guts have been sterilized and they are only on glucose saline drip. The phenolic acids in blood and urine of these patients, therefore, should be ones arising from the endogenous metabolism. From Table I, it is apparent, therefore, that vinillic acid, vanilloyl glycine, feruoyl glycine, 3-methoxy, 4-hydroxy phenyl hydracrylic acid and the yellow spots, probably arise from exogenous sources. These phenolic acids are present in normal urine but are absent from the samples of the patients. All these compounds (except the yellow spots) have been attributed by Asatoor *et al.* (2) to arise from the metabolism of intestinal bacteria, since in their study on normal adult subjects, the excretion of all these compounds fell to almost zero level when guts of the subjects were sterilized. In their study, the excretion of 3-methoxy 4-hydroxy mandelic acid (VMA) was also reduced drastically indicating that VMA of normal urine is mostly contributed by bacterial metabolism. In the present study, however, VMA is seen in urinary chromatograms of all the patients, thereby suggesting that endogenous metabolism is also an important source of urinary VMA. The yellow spots of the present study (see above) are probably the same yellow spots which Robinson *et al.* (6) consider to arise from the metabolism of intestinal bacteria.

One spot staing purple and having Rf values 0.45 and 0.35 in the two solvents needs special mention. This spot is not seen in normal urinary chromatograms but is consistantly present in urinary chromatograms of the patients. In normal chromatograms the spot is probably, submerged in the red spot of m-hydroxy phenyl hydracrylic acid (which is very prominent in these chromatograms). This purple spot gets exposed in the chromatograms of the patients where the spot of m-hydroxy phenyl hydracrylic acid is very weak. Rf values of the purple spot (0.45, 0.35) and m-hydroxy phenyl hydracrylic acid (0.46, 0.35) are almost similar. The identity of the purple spot is not known.

Urinary levels of p-hydroxy phenolic acids are higher and those of meta hydroxy phenolic acids lower in patients as compared to normals. Very low concentrations of m-hydroxy phenolic acids in urinary samples of the patients indicates that endogenous metabolism is not an important source of these compounds. The view of Armstrong and Shaw (1) about the exogenous origin of m-hydroxy phenolic acids is, therefore, supported by the present findings. Similar were the observations made by Studnitz *et al.* (11). According to Hicks *et al.* (5) small amounts of meta and ortho hydroxy phenolic acids can arise from endogenous phenyl alanine metabolism. Excretions of m-hydroxy hippuric acid and m-hydroxy phenyl hydracrylic acid are not completely abolished in the patients of the present study (Table II). Levels of o-hydroxy hippuric acid are almost similar for the two groups. This means that endogenous source of the compound

TABLE I: Frequency of occurrence of phenolic acids in urinary samples of the patients and normals.

The groups	Phenolic acids (Their brief names or colour with p-nitraniline reagent and Rf values in the two solvents of this study. The figures under each phenolic acid show the % of individuals investigated showing the presence of the phenolic acid).															
	p-HHA	*p-HBA*	*p-HPAA*	*m-HHA*	*m-HPHA*	*o-HHA*	*Purple* (0.45, 0.35)	*VMA*	*5HIAA*	*VA*	*VG*	*FG*	*HV*	*HMPHA*	*Yellow* (0.27), 0.40)	*Yellow* (0.37, 0.36)
Normals (8 cases)	100	87	100	100	100	100	—	100	20	50	75	75	50	100	100	100
Patients (4 cases)	100	100	100	100	100	75	100	100	75	—	—	—	50	—	—	—

Abbreviations signify phenolic acids as follows:—
p-HHA: p-Hydroxy hippuric acid; p-HBA: p-hydroxy benzoic acid; p-HPAA: p-hydroxy phenyl acetic acid; m-HHA: m-hydroxy hippuric acid; m-HPHA: m-hydroxy phenyl hydracrylic acid; o-HHA: o-hydroxy hippuric acid; VMA: 3-methoxy 4-hydroxy mandellic acid; 5HIAA: 5-hydroxy indole acetic acid; VA: vanillic acid; VG: vanilloyl glycine; FG: Feruoyl glycine; HV: homovanillic acid; HMPHA: 4-hydroxy 3-methoxy phenyl hydracrylic acid.

TABLE II: Levels of some phenolic acid in blood and urine of normals and patients.

	Blood phenolic acids (μgm/100 *ml.*)						*Urinary phenolic acids* (μgm/400 μgm *creatinine*)					
	m-HHA	*p-HPAA*	*m-HPHA*	*o-HHA*	*p-HBA*	*Purple* (0.45, 0.35)	*m-HHA*	*p-HPAA*	*m-HPHA*	*p-HHA*	*o-HHA*	*p-HBA*
Normal subjects												
1	—	10.2	—	—	—	10.6	3.5	8.6	3.8	0.4	1.5	0.7
2	0.3	5.6	0.3	—	3.5	10.2	6.8	4.8	5.2	0.7	1.1	1.5
3	0.3	5.4	0.3	—	0.4	10.2	5.8	5.2	6.2	0.4	0.5	1.0
4	0.3	4.6	0.3	6.3	3.2	8.6	5.0	4.6	5.4	0.5	5.7	1.4
5	—	5.2	—	3.7	—	5.6	2.1	4.4	2.2	0.4	3.8	—
6	—	5.7	—	—	0.5	10.6	2.5	5.8	2.1	0.5	0.3	1.0
7	0.3	5.4	—	—	0.4	10.2	4.6	5.0	2.8	0.5	0.4	1.1
8	—	3.1	—	—	0.4	5.6	2.1	3.1	1.8	0.4	0.5	0.8
Mean* values	0.3	5.6	0.3	4.5	1.4	9.6	4.0	5.1	3.7	0.5	1.7	0.9
Patients												
1	—	30.0	—	—	5.0	20.0	1.1	6.6	1.2	3.1	0.8	3.2
2	—	8.0	—	3.0	5.0	10.0	3.2	8.5	3.1	2.1	0.6	2.4
3	—	6.0	—	—	5.0	8.0	0.8	2.4	1.2	1.2	3.0	4.4
4	—	7.0	—	—	6.7	20.0	0.7	8.2	0.8	—	—	1.3
Mean* values	—	12.7	—	3.0	5.4	14.5	1.4	6.4	1.6	2.1	1.4	2.8

For significance of abbreviations of phenolic acid refer to the legend to Table I.

Mean* value for the cases in which the spot is seen.

is not insignificant. Armstrong and Shaw (1), however, believe that exogenous sources of this phenolic acid are more important while Asatoor *et al.* (2) do not find this phenolic acid to arise from the metabolism of intestinal bacteria.

Higher urinary levels of p-hydroxy phenolic acids in patients as compared to controls and may be because of higher rates of endogenous protein metabolism likely to be present in the patients.

The observations noted above with respect of meta, para and ortho hydroxy phenolic acids are also verified when plasma phenolic acids are compared in the two groups (Table II).

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