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TYROSINE METABOLISM: URINARY EXCRETION STUDIES IN PARKINSONISM

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SUMMARY

Vanilmandelic, homogentisic and homovanillic acid excretions were studied in parkinsonian patients on and off L-dopa.

Vanilmandelic acid excretions were quantitated both by gas-chromatographic and colorimetric methods. A marked increase in the excretion of this acid was demonstrated in the parkinsonian patient as compared to controls. Although vanilmandelic excretion rises under the influence of L-dopa, the increase is much smaller than that of free homovanillic acid. The latter consistently accounts for some 18–20% of the administered L-dopa dose. While vanilmandelic acid excretions even under the influence of L-dopa correlate well with the patients' weight, no such correlation could be obtained with homovanillic acid.

Homogentisic acid could not be detected in urine specimens of patients either on or off L-dopa. No correlation was observed between the clinical status or response to L-dopa of the patients and their excretion of acidic urinary catabolites.

INTRODUCTION

In conjunction with studies[1,2] of dopa metabolites in the cerebrospinal fluid of a series of patients with parkinsonism, urinary excretion studies of homovanillic acid (HVA), vanilmandelic acid (VMA) and homogentisic acid (HA) were undertaken.

HVA determinations utilized the method of Sato[3]; for both VMA and HA innovations and/or modifications of current methodologies were required, especially in the samples obtained from patients receiving L-dopa therapy.

No difference was observed between the 24-h urinary excretion values of HVA

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from parkinsonian patients and normal individuals drawn from the same age group as the patients. HA could not be demonstrated in the urine of any of the patients either before or after initiation of L-dopa therapy. VMA excretion on the other hand was almost doubled in parkinsonian patients as compared to controls. This paper then is concerned with HVA, VMA and HA excretion in parkinsonian patients, on and off L-dopa, as well as the methodologies employed to measure these parameters. In the latter area the comparison of gas-chromatographic and colorimetric data for the determination of VMA is, perhaps, of particular interest.

METHODS

Urine collection

Twenty-four-hour urine specimens were obtained from patients and controls in bottles which contained 20 ml of 6 *N* HCl as a preservative. While the collections were being made the bottles were kept on ice, and after the specimens were completed, aliquots were frozen immediately until use. The controls, or non-parkinson urine specimens, were obtained from individuals over 50 years old, since Parkinson's disease most commonly manifests itself in this age group. The completeness of various 24-h collections was monitored by creatinine determinations.

HVA determination

Homovanillic acid was determined by the method of Sato[3].

Homogentisic acid determination

The method of Stoner and Blivaiss[4] was the first used in an attempt to locate HA in dopa urines. It was found that such compounds as 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylpyruvic acid, norepinephrine, and dopamine produced interfering absorbance readings. Although small amounts of these compounds are of little consequence in quantitating the gram amounts of HA encountered in alcaptonuria, their presence in the dopa urines vitiated any attempt to use the method in the current study.

Six dopa urines were run by an enzymatic assay for HA[5] and no HA was detected. However, when a 4-μg spike of the acid was added, it was not recovered in 4 of these urines. An additional 10 μg had to be added before detection occurred. This suggested that levels of less than 3 μg HA/ml of urine or several mg/24 h would pass undetected.

Consequently, the following method was developed for the partial purification of urine and subsequent qualitative detection of homogentisic acid. The pH of the urine was adjusted to 4.0 with NaOH and 10 ml added to a Dowex 50W-X4 column 50–100 mesh, 8 cm × 1 cm which had previously been washed with 15 ml 1 *N* NaOH, 15 ml H_2O, 15 ml 1 *N* HCl, and 45 ml H_2O. The 10-ml eluate was collected and added to an AGl-X4 anion column (4 cm × 1 cm). The column was then rinsed with 2 ml 50% methanol, 2 ml 75% methanol, and 2 ml 90% methanol. Ten ml of 100% methanol were added, followed by 9 ml of methanol–acetic acid (98:2, v/v). The HA was then eluted from the column with 20 ml of the methanol–acetic acid mixture.

The material was evaporated to dryness at room temperature under vacuum and 1 ml of 0.01 *N* HCl added. The HA was extracted from the acid with two 1.5-ml portions of ethyl acetate. This ethyl acetate extract was then dried under vacuum at

room temperature and the residue dissolved in 1 ml methanol, which in turn was evaporated on a water bath (80°) to one or two drops. Homogentisic acid alone and urine samples spiked with 10 μg of homogentisic acid were also taken through the procedure.

Chromatographs were developed with benzene–chloroform–acetic acid–water (1:3:4:2) on 0.25-mm silica gel plates (Adsorbosil-1, Applied Science) and with benzene–chloroform–acetic acid–water (1:6:6:2) and benzene–methanol–acetic acid (45:8:4) on pre-coated silica gel F-254 plates (Merck). Visualization was obtained with phenol reagent (Folin–Ciocalteau, Fisher Scientific) followed by 10% Na_2CO_3. The sensitivity of the method corresponds to a detection of 0.5 μg/ml.

VMA colorimetric determination

The method of Wybenga and Pileggi[6] for VMA determination appeared suitable provided certain precautions were observed. Accurate quantitation demands that a series of standards be run together with each series of specimens. The VMA standard solution, made by dissolving 6 mg VMA (Calbiochem, Los Angeles) in 100 ml 0.01 *N* HCl, was stable for somewhat less than two weeks, even when stored at 4°. The color reagent addition, removal from ice, and subsequent absorbance readings have to be timed carefully to insure reproducibility. Finally, the reading of the urines and the urine blanks against water was omitted in favor of reading each sample against its own blank.

VMA gas-chromatographic procedure

Five ml of urine (pH adjusted to 5.5–6.1) mixed with 5 ml of 0.1 M phosphate buffer (pH 6.1) were passed through a column packed with AG1-X4 anion resin (6 cm × 1 cm, Bio-Rad Laboratories, California) previously washed with 1 *N* NaOH, H_2O, 1 *N* acetic acid, and finally with 0.2 M sodium acetate until equilibrium at pH 6.1 was reached. The effluent was discarded and VMA eluted from the column with 12 ml of 3.0 *N* NaCl. The material was then heated in a boiling water bath in order to avoid inhibition of VMA oxidation by other urinary constituents. After the eluate cooled to room temperature, 8 ml of 1 M K_2CO_3 were added. VMA standards were taken through the same procedure, which thus far is essentially the same procedure used in the colorimetric determination of VMA.

The eluates were divided into two 10-ml portions, one of which was treated as a blank. To one 10-ml aliquot was added 0.4 ml of 4% $NaIO_4$. To prevent oxidation, 0.6 ml of 10% $Na_2S_2O_5$ was added to the blanks. The solutions were then heated at 50° for 30 min to oxidize VMA to vanillin. At the end of this period, $Na_2S_2O_5$ is added to the standards and urine samples and $NaIO_4$ to the blanks.

The pH was adjusted to 7.5–7.6 and the vanillin twice extracted with reagent grade CH_2Cl_2 (8 ml, 8 ml). To the organic layer were added 5 g Na_2SO_4 (anhydrous) and the solution mixed and centrifuged. The supernate was dried under N_2 to 1 ml and 0.040 ml of chlorodifluoroacetic anhydride (K and K Laboratories, Inc., Plainview, New York) added to both samples and blanks. The solutions were left at room temperature for at least 20 h after which time they were completely dried under N_2. Two ml of distilled ethyl acetate were added and 1 μl thereof injected into a gas chromatograph equipped with an EC detection unit (Hewlett Packard Model 402 with a ^{63}Ni cell). Conditions used for the chromatography were: a 4′ U-shaped column, I.D. 4 mm,

packed with 80/100 Gas Chrom Q coated with 1% XE-60; carrier gas, 95% Argon, 5% methane at a flow rate of 60 ml/min; oven temperature 120°, flash heater 140°, and EC detector 280°.

A peak which corresponds to the chlorodifluoroacetic derivative of vanillin appeared at 7 min. When VMA itself is chromatographed, before and after treatment with chlorodifluoroacetic anhydride, no such peak is observed. Nor does underivatized vanillin give a peak of the appropriate retention time. The peak height is proportional to the amount of material injected and such a standard curve obtained from VMA standards taken through the entire procedure is illustrated in Fig. 1. Urines spiked with VMA and taken through the procedure show a proportional increase in peak

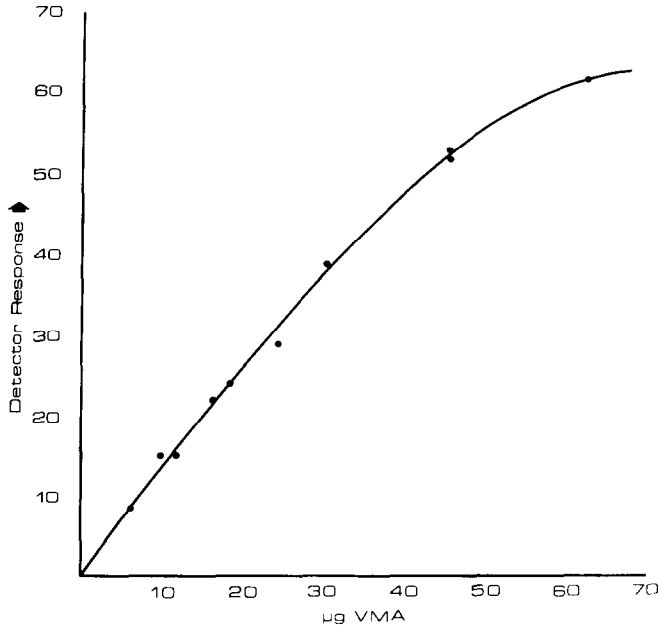

Fig. 1. Standard curve for the GLC determination of vanilmandelic acid. Points on this curve correspond to standards taken through the entire analytical procedure described in the text.

height at 7 min and urines treated as blanks (VMA not oxidized to vanillin) show little or no peaking at the 7-min retention time. Since the sensitivity of the gas chromatograph is subject to change, standards have to be run with every set of urines. Precision for the gas-chromatographic method is of the order of 3% if the initial sample contains approximately 30 μg of VMA. Accuracy calculated on the recovery of 10- and 24-μg quantities of VMA added to the urine samples was of the order of 98.1 $\pm$ 6.6%.

RESULTS

HVA

No difference was observed in baseline excretions (*i.e.*, pre-dopa) of HVA as compared to normal controls, which were 4.0 $\pm$ 1.4 mg/24 h and 3.8 $\pm$ 1.7 mg/24 h respectively. As reported by others[7,13] HVA excretion rose sharply with the dose of

ingested L-dopa. Approximately 18–20% of a given dose of the chemotherapeutic agent was recoverable as free HVA in the urine (Table I).

TABLE I

AVERAGE HVA EXCRETION OF L-DOPA PATIENTS

L-Dopa (grams/day)	*Number tested*	*HVA average (mg/24 h ± SD)*
0	15	4.0 ± 1.4
2	5	427 ± 223
4	2	645
5	14	906 ± 169
6	13	1100 ± 338
7.5	6	1401 ± 374

HA

As pointed out in the introduction, no HA was recovered from any of the urines tested either pre or post dopa. The methodology was adequate to pick up readily as little as 0.5 mg HA/l of urine. This finding was in accord with that of Cotzias[8] and in contrast to that reported by Arras and Bailey[9].

VMA

Both the colorimetric and the gas-chromatographic procedures show an increased VMA excretion in the parkinsonian patient as compared to normals (Table II).

TABLE II

AVERAGE VMA EXCRETION OF CONTROLS AND PARKINSON PATIENTS

Subject	*Method*	*Number tested*	*VMA average (mg/24 h ± SD)*
Controls*	Colorimetric	10	5.4 ± 1.6
Parkinsonian*	Colorimetric	32	6.8 ± 2.4
Controls+	Colorimetric	9	5.7 ± 2.2
Parkinsonian+	Colorimetric	6	7.3 ± 2.0
Controls+	Gas chromatography	9	2.9 ± 1.6
Parkinsonian+	Gas chromatography	11	5.2 ± 2.1

Subjects fell into two groups. Group I urines* were analyzed colorimetrically only. Group II+ represents a different group of controls and patients whose urines were analyzed both colorimetrically and by GLC.

The preliminary work was done colorimetrically; with recognition of the fact that gas-chromatographic procedures for urinary VMA[10–12] in general yield lower values than colorimetric ones, the question arose whether the observed increases (6.8 mg/24 h for 27 parkinsonian patients as compared to 5.2 mg/24 h for 5 controls[13]) were in fact due to VMA or to whatever substance contributes to the color of the spectrophotometric method. The gas-chromatographic procedure which was then developed not only confirmed the colorimetric data but sharply accentuated the difference between controls and parkinsonian patients. The implication is that the increase noted in the latter group by the colorimetric method was de facto due to VMA so that on a percentage basis the error incurred by the colorimetric method is much greater for the controls than for the patients.

TABLE III

AVERAGE VMA EXCRETION OF L-DOPA PATIENTS

L-dopa (grams/day)	*Number tested*	*VMA average (mg/24 h ± SD)*
0	32	6.8 ± 2.4
2	5	14.8 ± 1.5
4	2	19.8
5	14	22.4 ± 8.9
6	12	25.9 ± 7.6
7.5	6	28.6 ± 9.1

Urinary VMA, as did HVA, rose as the L-dopa dose increased (Table III). However the absolute increase is of a far smaller order of magnitude than the HVA increase nor is the percentage of L-dopa converted to VMA constant. Rather one finds that as the dose increases the percent metabolized by this route decreases (Table III).

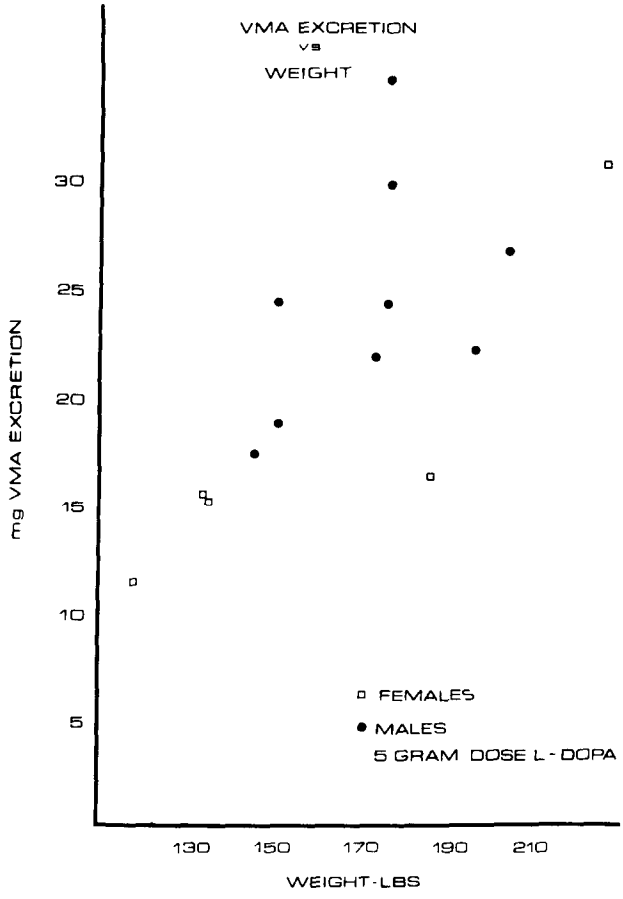

Fig. 2. Twenty-four-hour urinary excretion of vanilmandelic acid *vs.* weight of patients on 5 grams daily dose of L-dopa.

Another major difference between the pattern of metabolism of HVA and VMA is that the amount of a given dose of L-dopa metabolized to VMA correlates strikingly with the weight of the patient (Fig. 2), while no such correlation exists for HVA (Fig. 3).

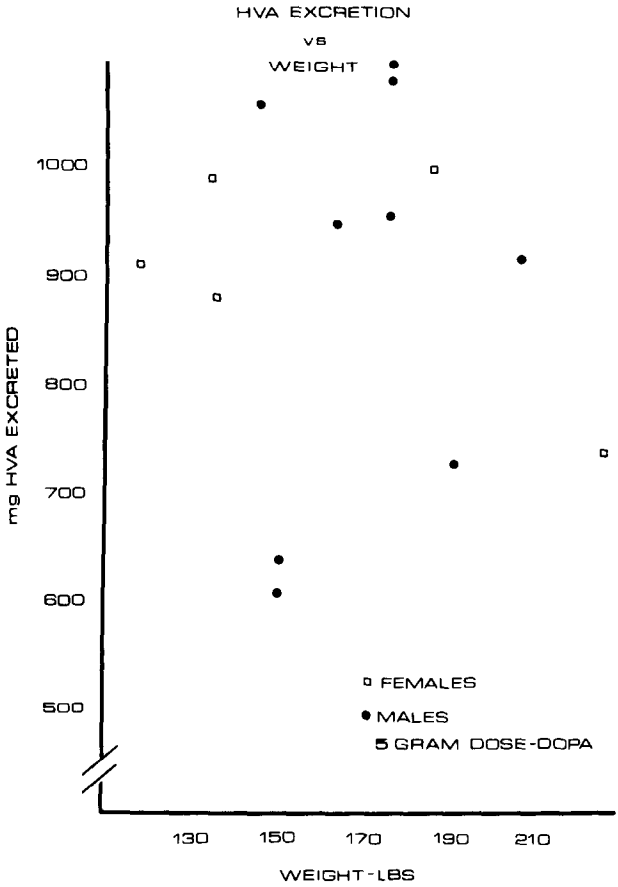

Fig. 3. Twenty-four-hour urinary excretion of homovanillic acid *vs.* weight of patients on 5 grams daily dose of L-dopa.

DISCUSSION

A defect in the handling of basal ganglia dopamine by the parkinsonian patient has been amply documented and is in fact reflected in cerebrospinal fluid HVA levels[1,2]. However the total amount of dopamine in the involved areas represents such a small percentage of the total dopamine metabolism of the body that the alterations remain undetectable in urinary excretion studies. Parallel findings were recently reported by Gruendig and Gerstenbrand[14] who discovered abnormalities in specific amino acid and transaminase levels in the cerebrospinal fluid of parkinsonian patients which were not demonstrable in blood serum obtained simultaneously from the same patients.

It is then correspondingly surprising to find VMA excretions increased markedly in the parkinsonian patients. On the other hand it is the third tyrosine catabolite

showing sufficiently extensive aberrations in metabolism to be documentable by urinary analysis. The excretion of methoxytyramine[15] and of *p*-hydroxyphenylpyruvic acid[16] were previously reported as showing marked changes in the parkinsonian patient.

As a matter of fact it was this increase in *p*-hydroxyphenylpyruvic acid which prompted the HA work reported in the current manuscript, since *p*-hydroxyphenylpyruvic is the immediate precursor of HA. From the lack of HA in the parkinsonian urine it is evident that conversion of HA to fumarylacetoacetate is not affected by the disease. It also supports the hypothesis raised in the earlier paper that the elevation of *p*-hydroxyphenylpyruvic acid is due to increases in liver tyrosine transaminase levels rather than to a block in the metabolism of the keto acid. Regulation of hepatic tyrosine transaminase activity *in vivo* by norepinephrine and pyridoxal phosphate has been demonstrated[17,18].

The significance of the elevated VMA levels of the parkinsonian patient is difficult to asses. In concert with the above arguments it is probably a symptom of systemic involvement in the disease rather than a finding which can be directly attributed to the altered central nervous system metabolism of dopamine. Goodall and Alton[19] infused small doses (ca. 1 mg) of radioactively labelled dopamine into three parkinsonian patients and six normal subjects and monitored ^{14}C excretions. They were most impressed by a decrease in the percent of the dose excreted as free norepinephrine. However a scrutiny of the percentage of the ^{14}C excreted as VMA plus its precursor dihydroxymandelic acid supports the concept of an increased norepinephrine (and/or epinephrine) metabolism. Finally, Tissot[15] reported 24-h urinary VMA excretion values for five parkinson patients (6.02 $\pm$ 4.03 mg) and 20 normal subjects (3.56 $\pm$ 1.1 mg) which closely parallel our own data.

Under the influence of orally administered L-dopa both urinary HVA and VMA rose. Free HVA rather consistently accounted for 18–20% of the administered dose of L-dopa. While VMA rose with the administered dose the percent of the dose excreted in this form fell progressively from 0.74% for a 2-g/day to 0.38% for a 7.5-g/day dose. Also there is the striking direct correlation between body weight and the amount of administered L-dopa excreted as VMA, a relationship not detectable for HVA. These relationships appear to emphasize that the catabolite VMA cannot be obtained except through either of the physiologically active compounds norepinephrine and epinephrine.

As described in the earlier paper[2] some correlations exist between the clinical status of parkinsonian patients and the measurement of some of the dopa catabolites in cerebrospinal fluid. Urines from the Group I patients (see Table II) were drawn from the same population as that used for the cerebrospinal fluid studies. In the case of the urinary catabolites of L-dopa, free HVA and free VMA, no correlation was obtained between their excretion and the clinical status or response to L-dopa of the patients.

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