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Note

Excretion of *m*-hydroxymandelic acid in human urine

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Until recently, reports of octopamine in tissues and biological fluids have implied that either *p*-octopamine or a combination of octopamine isomers were being analyzed. In the last few years however techniques have been developed which permit the isomers of octopamine to be separated and quantitated [1—4]. The natural occurrence of *m*-octopamine in tissues suggests that its oxidatively deaminated metabolite, *m*-hydroxymandelic acid (*m*-HMA), might also occur and be excreted in urine. Very recently Midgley et al. [5] have shown that *m*-HMA is a normal constituent of human urine.

Independently we have also developed a procedure for the identification and quantitation of *m*-HMA in urine based on the heptafluorobutyryl-methyl ester derivative of *m*-HMA. We have utilized the gas chromatography—mass spectrometry—integrated ion current technique (GC—MS—IIC) to identify and quantitate *m*-HMA in human urine and so have confirmed the finding of Midgley et al. [5]. In this paper we report the use of this method to assess the longitudinal excretion of *m*-HMA in a human male and to determine its average excretion in a human population.

EXPERIMENTAL

Materials

HPLC-grade solvents were purchased from Caledon Labs. (Georgetown, Canada); heptafluorobutyric anhydride from Pierce (Rockford, IL, U.S.A.) and *m*-HMA from Sigma (St. Louis, MO, U.S.A.). *m*-HMA was ring deuterated by heating 200 mg at 80°C in 3 ml 9% deuterium chloride in deuterium oxide for 24 h. After rotary evaporation of the solvent and back-exchange of the phenolic and carboxylic deuterium atoms, the resulting pale brown crystalline

product was shown by mass spectrometry to contain 79.8% m-HMA-D_3 (ring).

Urines from a young, healthy male were collected onto 10 ml conc. HCl in polyethylene bottles over twelve consecutive 24-h periods, each 24-h collection starting with the first voiding of the day. After recording the total volume, a 250-ml aliquot was removed and stored at —16°C until analyzed. One 24-h urine sample from each of six other persons was collected and stored in a similar manner.

Methods

At the time of analysis, each urine sample was completely thawed and mixed before 3-ml aliquots were removed. To each aliquot were added 520 ng (nominal) of m-HMA-D_3 and about 0.5 g of sodium chloride. The samples were then extracted with ethyl acetate (4 × 2 ml), concentrated to about 200 μl in a stream of nitrogen at 50—60°C, transferred to a 0.3-ml Reacti-vial (Pierce), and then evaporated to dryness. A solution of HCl in methanol (about 15%) (150 μl) was added and the mixture allowed to stand at room temperature for 30 min. The solvent was then evaporated in a stream of nitrogen. The samples were dried by adding benzene (100 μl), mixing thoroughly, and evaporating in a stream of nitrogen. To the dried samples were added hexane (150 μl) and heptafluorobutyric anhydride (40 μl). The samples were then heated in a heating block at 65°C for 1 h, cooled and washed once with 100 μl phosphate buffer (pH 6.0). The hexane layer was withdrawn, the remaining aqueous layer washed once with hexane (100 μl) which was then combined with the original hexane solution. This hexane solution was then concentrated to about 30 μl and submitted for GC—MS—IIC analysis.

A blank sample (3 ml 2 N HCl) was carried through the above procedure. Some urines were analyzed in triplicate in order to determine the reproducibility of the method.

A calibration curve (which was linear at least over the range 10 ng—10 μg) was constructed by preparing and analyzing solutions of mixtures containing various amounts of the derivative of m-HMA acid and a constant amount of the derivative of m-HMA-D_3.

Instrumentation

A Hewlett-Packard 5710A gas chromatograph equipped with a flame ionization detector was coupled by means of an S.G.E. jet separator to an AEI-MS902S mass spectrometer. A support-coated open-tube (SCOT) column (S.G.E., GSC-SP2250, 57 m) was installed in the chromatograph and the helium flow-rate set at 24 cm/sec. The chromatography conditions are: oven temperature, 130°C (isothermal); injector temperature, 200°C; detector temperature, 200°C. Under these conditions, the retention time for the authentic di-(heptafluorobutyryl)methyl ester of m-HMA is 8.2 min. p-HMA has a retention time of 9.4 min and is completely separated from m-HMA. The mass spectrometer was operated at a resolution of 7000 for the IIC measurements and the temperature of the electron impact (70 eV) source was 200°C.

The exact masses of the molecular ions and [M — COOCH₃]⁺ ions of the derivatives of m-HMA and its D_3 analogue are located in the mass spectrometer by means of the reference gas, heptacosafluorotri-n-butylamine, and the decade

box ratio is set so that the instrument alternately records the signals from the endogenous acid and the internal standard, as described previously for the integrated ion current analyses of trace amines [6]. For the IIC analyses the $[M - COOCH_3]^+$ ions were used; the exact masses for these are 514.9964 (endogenous acid) and 518.0152 (internal standard).

RESULTS AND DISCUSSION

m-HMA in its unconjugated form has been identified and quantitated in the twelve urine samples obtained from a single individual (Table I) and in the seven different urine samples from different individuals (Table II).

TABLE I

LONGITUDINAL URINARY EXCRETION OF UNCONJUGATED m-HYDROXYMAN-DELIC ACID IN A HUMAN

Samples 1—3 were analyzed in triplicate, while samples 4—12 represent single estimations.

Sample No.	m-HMA (µg/24 h)
1	58.6 ± 5.7
2	64.6 ± 1.1
3	46.9 ± 1.8
4	55.3
5	82.5
6	53.5
7	40.4
8	70.4
9	68.6
10	70.7
11	100.3
12	48.5

TABLE II

URINARY EXCRETION OF UNCONJUGATED m-HYDROXYMANDELIC ACID IN A HUMAN POPULATION

Sample No.	m-HMA (µg/24 h)
1	58.6
2	127.6
3	53.4
4	65.5
5	68.5
6	24.4
7	15.0

The unambiguity of the identification rests on three factors. First, the *meta*- and *para*-isomers of the acid are separated completely (by 1.2 min) on the SCOT capillary column, so that overlapping of signals does not occur; this is an important point because p-HMA is present in quantities 40—50 times larger than the *meta*-isomer. Second, the retention times of authentic m-HMA and

the endogenous acid are identical. Finally, at the correct retention time, the correct high-resolution masses of the M^+ and $[M - COOCH_3]^+$ ions of the derivative of the endogenous acid were recorded by the mass spectrometer and in the same intensity ratio as for authentic acid.

The ion chosen for the IIC analyses was the $[M - COOCH_3]^+$ ion since its relative intensity is seven times greater than that of the molecular ion (6.7% as opposed to 0.9%), and yet it is still unique to m-HMA.

The reproducibility of the method is indicated by the small standard deviations obtained (see Table I) for those samples analyzed in triplicate.

In a recent paper, Fell et al. [7] have claimed that m-HMA is not normally present in urine in detectable amounts, although they did not provide any data, or references, to substantiate their claim. They did however show that quite large amounts of labelled m-HMA were excreted in urine following ingestion of labelled m-tyrosine. Maruyama et al. [8] identified, but did not quantitate, m-HMA in rat, guinea pig and rabbit urine following injection of tritiated m-octopamine. Karnassiotis and Kramer [9] found 173 $\mu g/100$ ml of m-HMA in the serum of human patients suffering with terminal renal insufficiency, a level that reduced to 114 $\mu g/ml$ following dialysis. Karoum and Sandler [10] achieved, using a SCOT column, a good separation of m-HMA from other phenolic acids using standard solutions of their methyl ester trimethylsilyl ether derivatives. A chromatogram from a derivatized urine extract as presented by Karoum and Sandler in their paper, however, did not include a peak at the retention time for m-HMA. This is perhaps not particularly surprising since we have also observed that if the attenuation for the chromatograph is set so as to permit the major peaks to be on scale, then substances present in smaller amounts do not show up.

Midgley et al. [5] have reported values for urinary m-HMA in the range of 11—71 ng/mg creatinine. Our values (see Table I), converted to these units, are similar (28—53 ng/mg creatinine) for the longitudinal study; we did not measure creatinine values in the other study.

This identification of m-HMA in the unconjugated form and its relatively consistent excretion both in a single individual and across individuals argues in favour of an endogenous origin for m-octopamine and m-HMA and of course confirms our earlier identification of m-octopamine [1].

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