# 6-Hydroxy-5-Methoxyindole-2-Carboxylic Acid in Normal Human Urine

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The presence of 6-hydroxy-5-methoxyindole-2-carboxylic acid (6H5MI-2-C) in normal human urine was demonstrated by mass spectrometry. A quantitative method based on HPLC with fluorometric detection was developed for the determination of 6H5MI-2-C. In normal subjects the mean urinary concentration of 6H5MI-2-C was 38 μmol/mol of creatinine (range 6–76 μmol/mol). *Key-words: Melanin; Urine; Indole derivatives.* (Received September 9, 1983.)

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The formation of indole derivatives by oxidation of dopa in the melanocyte was postulated already by Raper (1). Much later Duchon et al. demonstrated metabolites of this metabolic pathway in urine from a melanoma patient (2) and identified the two isomeric compounds, 5-hydroxy-6-methoxyindole-2-carboxylic acid and 6-hydroxy-5-methoxyindole-2-carboxylic acid.

Further compounds belonging to the eumelanin pathway have since been presented. The main Thormählen-positive compounds were convincingly shown to be the isomeric methylated glucuronides of decarboxylated indole derivatives (VI) which were found in urine from a melanoma patient (3). An immediate precursor of eumelanin, 5,6-dihydroxyindole-2-carboxylic acid (II), was found in normal urine (4, 5) (Fig. 1).

5,6-dihydroxyindole-2-carboxylic acid is a readily oxidized compound present in low concentrations in normal urine. The most stable of its two isometric methylated metabolites, 6-hydroxy-5-methoxyindole-2-carboxylic acid, was therefore studied as a marker of the eumelanin pathway. This compound has been demonstrated in urine from a patient in the terminal stage of malignant melanoma but not in normal urine or in urine from patients with less widely spread melanoma (6).

The present study describes the identification of 6-hydroxy-5-methoxyindole-2-carboxylic acid (6H5MI-2-C) in normal human urine by means of GC-MS. An analytical method based on HPLC with fluorometric detection which permits the quantification of 6H5MI-2-C in normal urine is also presented.

## MATERIAL AND METHODS

*Chemicals.* 6-Hydroxy-5-methoxyindole2-carboxylic (6H5MI-2C) and 5-hydroxy-6-methoxyindole-2-carboxylic acid (5H6MI-2-C) were kindly provided by Prof. Duchon (2). 6-Hydroxy-5-methoxyindole-2-carboxylic acid was synthesized by debenzylation of 6-benzyloxy-5-methoxyindole-2-carboxylic acid (Sigma) as described by Benigni & Minnis (7) and compared with authentic material. N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) was purchased from Pierce Eurochemie, Rotterdam, Holland. Ethyl acetate, methanol, phosphoric acid, pyridine, benzene, and acetic acid, all of analytical grade, were obtained from E. Merck, Darmstadt, GFR. The column packing material, Nucleosil $C_{18}$, was purchased from Macherey, Nagel & Co., Düren, GFR.

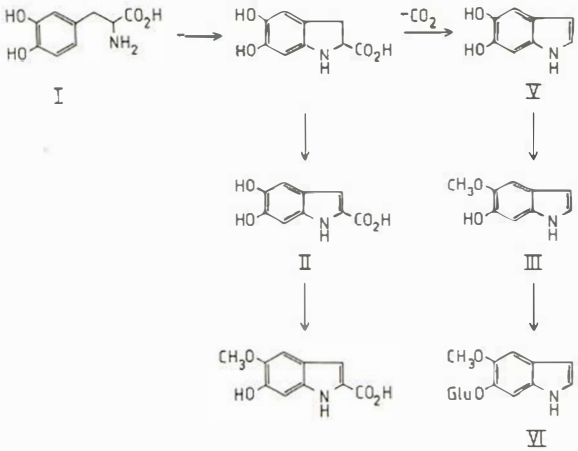

*Fig. 1.* Formation of indole metabolites.

*Gas chromatography-mass spectrometry.* 24-hour specimens of urine were collected in plastic bottles containing 50 ml acetic acid and 1 g sodium metabisulfite. One litre of the pooled urine was extracted 3 times with 250 ml ethyl acetate, and the organic phase was evaporated under reduced pressure (water bath temperature 25°C). When 15 ml remained a further 25 ml of ethyl acetate was added to form an aqueous salt phase, which was discarded. Three ml $H_2O$ was added and all ethyl acetate was evaporated. The aqueous residue was chromatographed on a semipreparative HPLC-system in 600 $\mu$l portions using methanol (10%) in water as mobile phase (4). The pH of the mobile phase was adjusted to 4.0 with concentratred acetic acid. The fractions with the same retention time as synthetic 6H5MI-2-C were collected. The pooled fractions were evaporated to about 1 ml on a Rotavapor at +25°C. The sample was then transferred to a reaction vial and evaporated to dryness under a stream of nitrogen. Benzene (100 $\mu$l) was added twice and evaporated to complete azeotropic removal of water. 200 $\mu$l of BSTFA and 100 $\mu$l freshly distilled dry pyridine were then added. Trimethylsilylation of the phenolic and the carboxylic groups took place immediately at room temperature, and trimethylsilylation of the indole nitrogen was done by heating the vial for 15 min at 100°C.

The mass spectra were obtained with the use of a Finnigan instrument operated at 70 eV; the samples were introduced by gas chromatography using 3% OV 17 on Chromosorb packed in a glass column 1 m long and 2 mm i.d. The column temperature was programmed from 150 to 250° at 4°C/min.

*HPLC analysis.* A Waters Model 6000 A liquid chromatography pump equipped with a Rheodyne 7125 loop injector (10 $\mu$l) and a Perkin-Elemer MPF-2A fluorescence detector with a 37 $\mu$l home-made flow cell were used. All separations were performed on a Nucleosil ODS column (5 $\mu$m, 250×5 mm) using aqueous methanol (30%) with phosphoric acid (25 mM) as mobile phase. The pH was adjusted to pH 4.0 with NaOH. All runs were performed at room temperature at a flow rate of 1.3 ml/min.

The fluorescence detector was set at excitation/emission wavelengths of 325/405 nm. Crude fresh urine was injected within 4 hours of collection, and the concentration of 6H5MI-2-C in the urine was determined by comparison with external 6H5MI-2-C standard solution of about the same concentration.

## RESULTS

### *Identification of 6H5MI-2-C in normal urine*

HPLC-analysis of fresh urine showed a peak with the same retention time as synthetic 6H5MI-2-C. On close examination the chromatographic properties of the urinary compound proved to be identical to those of synthetic 6H5MI-2-C. Large amounts of pooled urine were extracted with ethyl acetate and after concentration extracted back to an aqueous phase. Separation on a semipreparative HPLC column was done, and the fraction with the same retention time as synthetic 6H5MI-2-C was collected and evaporated to dryness. Trimethylsilylation was performed by N,O-bis-(trimethylsilyl)-trifluoroacetamide in dry pyridine.

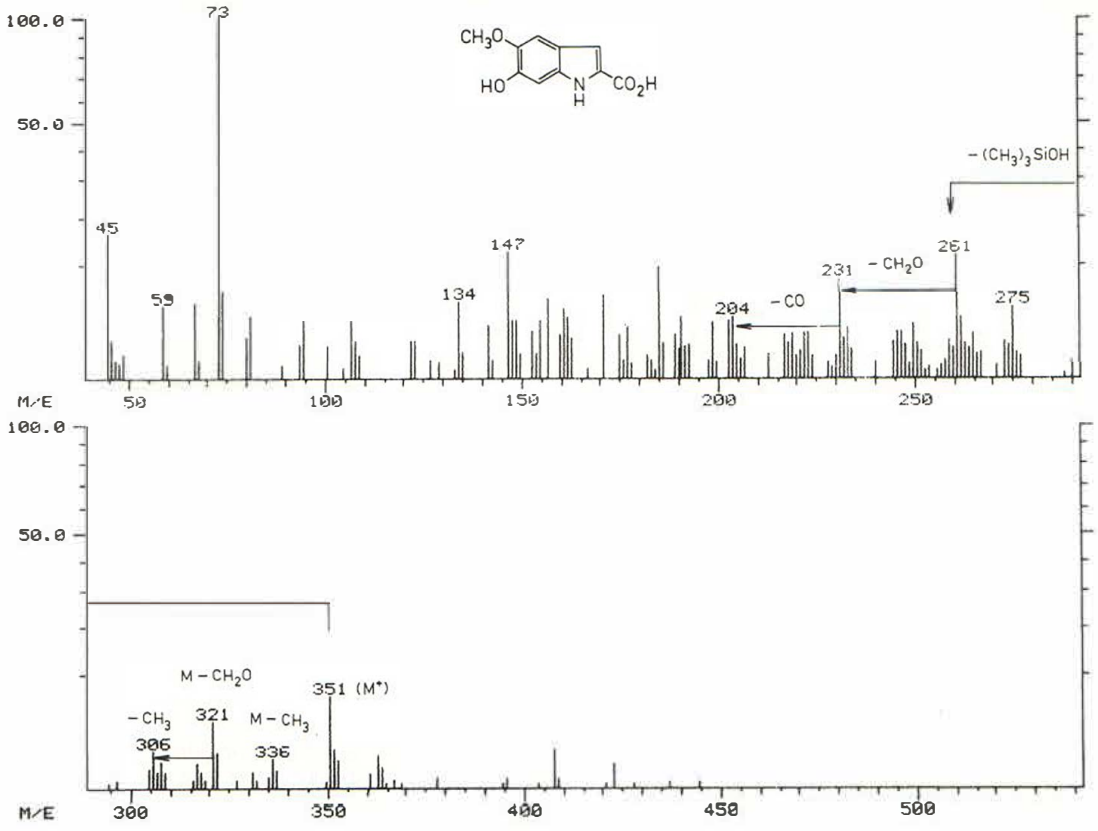


*Fig. 2.* Mass spectrum of the bis-TMS-derivative of 6-hydroxy-5-methoxyindole-2-carboxylic acid obtained from normal human urine.

Mass spectroscopy of the trimethylsilyl derivative of the purified urinary compound yielded a mass spectrum that was consistent with the mass spectrum of synthetic trimethylsilylated 6H5MI-2-C. When the trimethylsilylation was done at room temperature and analysis performed at once, trimethylsilylation of the phenolic and the carboxylic groups yielded a derivative with a molecular ion at $m/e=351$.

Trimethylsilylation of the indole nitrogen was not pronounced at room temperature, but could be performed at 100° for 15 min; the derivative formed showed a molecular ion at $m/e=423$. The electron impact mass spectra of derivatized 6H5MI-2-C extracted form normal urine is shown in Fig. 2.

### *Quantitation of 6H5MI-2-C in human urine*

Quantitative analysis was run on a reversed-phase column using an aqueous eluent with a high concentration of methanol. This performance permitted injection of small amounts of crude urine without destructive contamination of the column (Fig. 3). Although 6H5MI-2-C can be detected both by UV and electrochemical detector, we found the fluorescence detector more selective. The isomer, 5H6MI-2-C, is also detected by the fluorescence detector, but it is eluted much earlier on the chromatographic system used here. The retention time of 6H5MI-2-C proved to be readily affected by moderate changes in the methanol concentration.


*Fig. 3.* Chromatogram of a normal urine. Eluent: Aqueous methanol (30%) containing phosphoric acid (25 mM) at pH 4.0. Column: Nucleosil $C_{18}$ (5 $\mu$m) 250×5 mm. Flowrate: 1.3 ml/min.

The fluorescence detector used permitted free variation of the wavelengths, and optimum wavelengths of fluorescence were determined by a stop-flow technique where the peak was held in the flow cell and the fluorescence spectrum recorded. Chromatographic studies of crude urine and of urine samples spiked with synthetic 6H5MI-2-C confirmed the homogeneity and the identity of the peak analysed. 6H5MI-2-C was determined in 10 urine samples collected from apparently healthy persons ranging from 16–62 years of age. The concentration of 6H5MI-2-C ranged from 6 to 76 $\mu$mol/mol of cratinine with a mean value of 38 $\mu$mol/mol of cratinine. The samples were always analysed fresh without addition of any reducing agents. However, storage in a refrigerator for 5 days gave only 3% decreased value when acetic acid and sodium metabisulfite were added to the urine.

## DISCUSSION

Phenolic indoles are compounds sensitive to both acid and base. The analytical HPLC method described is therefore most appropriate because it is not necessary to purify the urinary sample before injection onto the chromatographic system. The sensitivity of the method permits analysis of normal urines, and has shown the value in normal urine to be about 1/1000 of the value earlier reported in urine from a patient in the terminal stage of malignant melanoma (6).

Although 6H5MI-2-C is oxidized at a potential adequate for detection with an electro-

chemical detector, the fluorescence detector proved more specific under the chromatographic conditions used. At the excitation/emission wavelengths used the carboxylic acid group must be in the 2-position in order to achieve fluorescence. Consequently, decarboxylated indoles such as III and V are not detected, and neither are dopa nor the cysteinyldopas. The isomeric compound 5-hydroxy-6-methoxyindole-2-carboxylic acid has a shorter retention time on the HPLC-system used, and therefore cannot contribute to the chromatographic peak analysed.

The identification of the urinary compound was performed mainly by mass spectrometric analysis. This technique shows high sensitivity and selectivity if only a volatile derivative of the studied compound is obtained. The trimethylsilylation reaction used in this study has the advantage of being conducted at neutral pH and at room temperature. This procedure proved more suitable for the analysed indolic compound than did the acid-catalysed esterification followed by perfluoroacetylation usually used for amino acids such as tryptophan. The facts that the GC-peak derived from the urine sample was observed at the correct retention time and that the expected molecular ion was registered constitute strong evidence for the presence of 6H5MI-2-C as a normal constituent of human urine. The fragmentation pattern is also in accordance with that of the synthetic 6H5MI-2-C and with expected fragmentation of a trimethylsilyl derivative.

The amount of 6H5MI-2-C found in normal urine is more than 10 × greater than the amount of the non-methylated indolic carboxylic acid (4). In most urines the concentration of the indole metabolite is as high as the concentration of 5-S-cysteinyldopa (8). Among the products that have been identified wtih certainty and quantified, 5-S-cysteinyldopa is up to now the melanocyte metabolite with the highest concentration in the urine (9). The presence of appreciable amounts of 6H5MI-2-C in urine indicates that oxidation of dopa also occurs in tissues under sulfhydryl-poor conditions. Otherwise the rapid addition of cysteine should exclusively yield cysteinyldopas.

An O-methylation is an essential pathway for biotransformation of phenolic compounds. This has been demonstrated for the catecholamines (10) and catecholic amino acids (11, 12). Catechol-O-methyltransferase is widely distributed in the body, but the concentration is particularly high in the liver and kidneys. High concentrations of O-methylated dopamine have been found in an insulin-producing pancreatic tumour (13), and enzymes catalysing the O-methylation of catecholic compounds have been found in hamster melanoma (14). Methoxylated dihydroxyindoles were recently found in the supernatant of melanoma cell culture (15). The site of the methylation yielding 6H5MI-2-C under normal and pathological condition remains to be established.

## ACKNOWLEDGEMENTS

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