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Note

The determination of 5-methoxyindole-3-acetic acid in human urine by mass fragmentography

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Appreciable quantities of 5-methoxytryptamine occur in rat hypothalamus [1]. This has led to speculation [2] that 5-methoxytryptamine could be a transmitter within the central nervous system and could possibly be the B-type fluorophore of Bjorklund and coworkers [3]. The major metabolite of exogenous 5-methoxytryptamine in rats and rabbits is 5-methoxyindole-3-acetic acid (5-MIAA) [4]. It is likely that in man too the urinary excretion of 5-MIAA can give an indication of the overall turnover of 5-methoxytryptamine within the body.

With this in mind, we have developed a mass fragmentographic method using an internal isotopic standard to detect and quantitate 5-MIAA in urine and have shown that it is a normal urine constituent. The presence of this acid in humans has not previously been demonstrated. The levels found are probably below the limits of sensitivity of the methods used in earlier searches for this compound, e.g. paper chromatography followed by chemical visualisation [5]. We have measured the urinary output of 5-MIAA in normal subjects and have also confirmed that in man unconjugated 5-MIAA is a major metabolite of exogenous 5-methoxytryptamine.

MATERIALS AND METHODS

Preparation of 5-methoxyindole-3-acetic acid — [methylene-$^2H_2$]

5-Methoxyindole-3-acetic acid (Sigma, St. Louis, Mo., U.S.A.) (20 mg) dis-

solved in 0.75 ml ^2H_2O containing 10% NaO^2H was heated at 125° for 6 h. After acidification with 2 *M* HCl the mixture was extracted with diethyl ether (3 × 10 ml); the extract was dried (Na_2SO_4) and the ether removed by distillation under reduced pressure to leave a yellow oil which crystallised on standing. Thin-layer chromatography on cellulose (*n*-butanol—pyridine—water (4:3:3, v/v); visualisation with dimethylaminocinnamaldehyde [1% in ethanol—conc. hydrochloric acid (1:1)] showed one compound with the same properties as the starting compound. The isotopic composition of the material was calculated from the mass spectrum of the bis(trimethylsilyl) derivative to be 97% $^2H_2$ and 3% $^2H_1$.

Solutions of the compound containing approximately 1 mg per 100 ml water were prepared and standardised by mass fragmentography against solutions of the undeuterated material. These solutions were stable at —14° for several weeks.

Preparation of urine samples. The deuterated standard solution (10 ml) was added to an aliquot (500 ml) of urine which was then acidified to pH 4 with 6 *M* HCl and extracted with ether (500 ml in portions). The extract was dried (Na_2SO_4) and the ether removed by distillation under reduced pressure. The residue in pyridine formate buffer, pH 2.60 (prepared by the addition of constant boiling formic acid to 0.1 *M* aqueous pyridine) (50 ml) was applied to a 15 × 2.5 cm column of Dowex 50W X4 ion-exchange resin (Serva, Heidelberg, G.F.R.) in the pyridinium form, pretreated by washing with two bed volumes (b.v.) of pyridine formate buffer (pH 2.60). The column was washed successively with 2 b.v. of pyridine formate (pH 4.20), 2 b.v. water, and 1 b.v. of 0.5 *M* aqueous pyridine adjusted to pH 11.5 with concentrated ammonia solution. The water wash contains most of the hippuric acid and the aqueous pyridine wash contains indole-3-lactic acid. A further wash with 2 b.v. of the 0.5 *M* aqueous pyridine eluted an obvious dark band which contained indole-3-acetic acid, 5-hydroxyindole-3-acetic acid and 5-MIAA. Evaporation of this eluate under reduced pressure below 40° gave a dark residue containing the acids which were converted to their trimethylsilyl derivatives using pyridine and bis-(trimethylsilyl) trifluoroacetamide with 1% chlorotrimethylsilane. This silylated mixture was generally satisfactory for mass fragmentographic analysis but the 5-MIAA could be further purified by preparative thin-layer chromatography on cellulose Avicel F; (10 × 20 cm; 500 μm; Anachem, Luton, Great Britain) using benzene—propionic acid—water (57:40:3, v/v) of the residue from the column eluate. Indoleacetic acid and 5-MIAA run to the top third of the plate ahead of 5-hydroxyindoleacetic acid and coloured material. This procedure was adopted for some early experiments.

Instrumentation. Gas chromatography—mass spectrometry (GC—MS) was carried out using a Finnigan 3200 GC—MS system (Finnigan, Sunnyvale, Calif., U.S.A.) under the control of a Finnigan 6110 data system. A 5 ft. × 2 mm I.D. glass GC column packed with 3% OV-17 on 100—120 mesh Supelcoport (Supelco, Bellefonte, Pa., U.S.A.) and a 4 ft. × 2 mm I.D. column packed with 3% OV-1 on 80—100 mesh Supelcoport were used, programmed from 150° to 280° at 4°/min respectively. The injection temperature was 280°, and the separator oven and transfer line were at 270°. The mass spectrometer was run with 0.30-mA emission at 70-eV ionising energy. Quantitative analysis

was made by selected ion monitoring using the peaks at *m/e* 232 and 234 (M—COOTMS) and the molecular ion peaks at *m/e* 349 and 351 for the 5-MIAA and its deuterated analogue, respectively.

Subjects. Ten normal adult subjects on a free diet collected 24-h urine samples, the urine being placed in a deep freeze immediately after each voiding. An oral load of 5-methoxytryptamine (1 mg) in water (100 ml) was taken in divided dose over 1 h by two normal adults (1 male, 1 female). No mental or systematic effects were noticed. Urine was collected for 24 h from the start of the load.

RESULTS AND DISCUSSION

The mass spectrum of the bis(trimethylsilyl) derivative of 5-MIAA (Fig. 1) is relatively simple with the major peaks being the molecular ion at *m/e* 349, the M—COOTMS ion at *m/e* 232 and low mass silyl fragments. The presence of 5-MIAA in urine without internal standard was shown by the simultaneous monitoring of fragments at *m/e* 202, 232, 306 and 349 in an extract. For quantitation the fragments at *m/e* 232 and 349 were used. The contributions at *m/e* 234 and 351 from the natural compound and the contribution at *m/e* 232 from the deuterated compound (corresponding to the fragment at *m/e* 230 in the spectrum of the unlabelled compound) were corrected for in the calculations.

In the two subjects who took 5-methoxytryptamine orally the urinary unconjugated 5-MIAA excretion over 24 h accounted for 53% and 71% of the administered dose. Thus 5-MIAA is a major metabolite of 5-methoxytryptamine in man and should give some indication of the turnover of this compound.

Although the 5-MIAA was always readily detectable (see Fig. 2), quantitative analysis of those extracts from urines containing the lowest concentrations of the compound (below about 10 ng/ml) presented some difficulties.

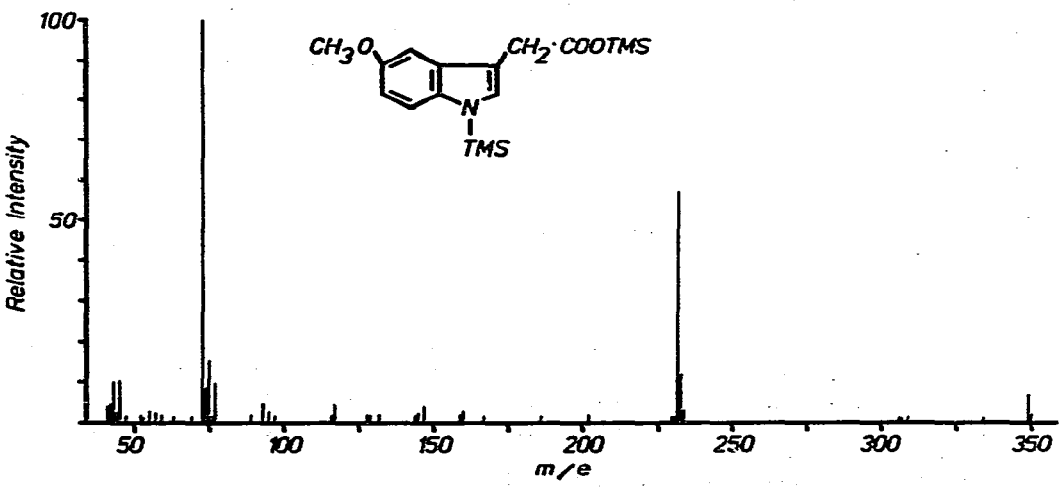

Fig. 1. Mass spectrum of the bis(trimethylsilyl) derivative of 5-methoxyindoleacetic acid.

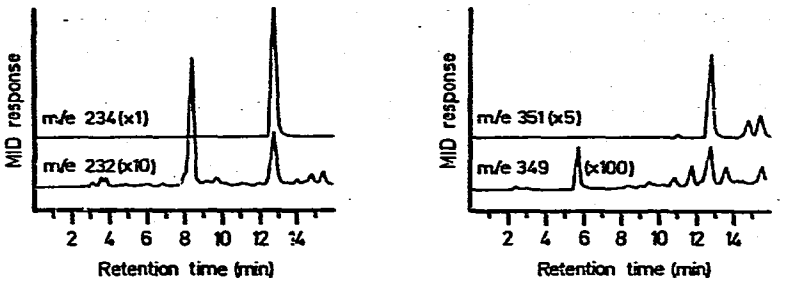

Fig. 2. Mass fragmentogram of a urine extract containing 5-($^2H_2$)-MIAA as an internal standard. The original urine contained the lowest concentration of 5-MIAA found (4.1 ng/ml; 7 μg per 24 h volume of 1700 ml). Amplifications of the MID response relative to *m/e* 234 = 1 are shown in parentheses.

There appeared to be no interference from other components of the extract with the internal standard fragments at *m/e* 351 and 234 and the ratio of these two fragments was fairly constant. However, the single ion profiles of the ions with *m/e* 232 and 349 appeared increasingly complex at the high amplifications necessary to detect the lowest concentrations of 5-MIAA. Interference from other components principally affected the ion at *m/e* 349 rather than the major fragment ion at *m/e* 232 largely because of the difference in their relative intensities. For this reason the concentration of 5-MIAA was generally calculated from the ratio of the ions at *m/e* 232 and 234. The situation was not significantly improved when a 20-m OV-1 glass capillary column was used for the chromatographic separation. The probability of interference also led us to prefer peak heights rather than peak areas for quantitation. Reproducibility of analyses on the GC phase, OV-17, was better than 1% but these results were lower by about 10% from those obtained using an OV-1 column: on this latter phase the peak due to 5-MIAA was often incompletely resolved from other product ions particularly at *m/e* 349. The results in the table were obtained using an OV-17 column.

The very wide range of 5-MIAA excretion in normal individuals suggests that at least in part this compound is of dietary origin. The lower part of this range may represent mainly endogenous production: six of the subjects excreted amounts of 5-MIAA in the range 7—18 μg per 24 h. This quantity of

TABLE I

5-METHOXYINDOLE-3-ACETIC ACID CONTENT OF URINE FROM NORMAL ADULTS (μg per 24 h)

Parameter	Value									
Sex	M	M	M	F	M	F	M	M	F	M
Age (y)	29	29	43	29	24	22	28	35	26	40
5-MIAA (μg)	56	151	9	11	13	11	7	34	18*	65*

*Approximate values calculated from *m/e* 349/351 only.

5-MIAA is about 0.003 that of 5-hydroxyindoleacetic acid excretion in normals, though allowing for some degree of conjugation, by analogy with indoleacetic acid itself [6], the overall turnover of 5-methoxytryptamine could be higher by a factor of up to two. In the rat hypothalamus the 5-methoxytryptamine content is 0.16 that of 5-hydroxytryptamine [1, 2] and a similar concentration would give the human hypothalamus a content of about 0.1 μg of 5-methoxytryptamine. 5-Methoxytryptamine is also present in the rat pineal [7] and by analogy, on a weight for weight basis, the human pineal might contain about 0.5 μg of this amine. Given a turnover time of an hour, this source alone could account for the majority of the urinary output of 5-MIAA in man. 5-MIAA is also a minor metabolite of melatonin [4, 8] and a major metabolite of 5-methoxytryptophol [9] in rats. In this context the demonstration of 5-methoxytryptophol in human cerebrospinal fluid [10] and the very ready methylation of 5-hydroxytryptophol by enzymes in the human pineal [11] are of interest. The lack of precise analytical data for the concentration and distribution of these compounds in human tissue precludes any very definite conclusions being drawn from these figures. However, it would appear that if the analogy with the rat is valid the endogenous production of 5-MIAA in man can be accounted for without involving 5-methoxytryptamine as a neurotransmitter except perhaps in minor and rather specialised roles.

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