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IDENTIFICATION AND MEASUREMENT OF 6-HYDROXY-1-METHYL-1,2,3,4-TETRAHYDRO-β-CARBOLINE BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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Abstract—A glass capillary gas chromatographic–mass spectrometric method for the analysis of 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline in urine, plasma and blood platelets was developed. The method involves the use of a deuterated analogue as internal standard, addition of semicarbazide, extraction with methylene chloride and formation of pentafluoropropionyl derivatives. The 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline was found to occur in the urine of controls and alcoholics as both the free and conjugated compound. In the controls the mean level of free and conjugated 6OMTHBC was 75 ± 20 nmole/l. When intoxicated, the alcoholics had a statistically higher excretion rate of the conjugate(s) than the controls. The compound was not detectable in blood platelets or plasma.

The natural occurrence of tetrahydro-β-carbolines in mammalian tissue and fluids has been reported [1–3]. These compounds, which are tricyclic indole derivatives, are known to be formed under physiological conditions of temperature and pH by a Pictet–Spengler reaction involving an indole ethylamine and an aldehyde (Fig. 1) [4]. The tetrahydro-β-carbolines have been shown to possess various pharmacological activities which have included monoamine oxidase inhibition, serotonin (5HT) uptake inhibition, and stimulation of the potassium-induced release of 5HT from brain slices [5–7].

It has been suggested that 1-methyltetrahydro-β-carbolines may be formed in the body after ethanol intake and play a role in the alcoholic syndrome [8]. The significance of this hypothesis is supported by behavioural studies of Melchior and Myers [9], which have shown tetrahydro-β-carboline to increase the voluntary intake of ethanol in rats after intraventricular injection. Recently, 1-methyl-1,2,3,4-tetrahydro-β-carboline (1MTHBC, the product from tryptamine and acetaldehyde) has been identified in human urine, plasma and blood platelets [3, 10, 11].

Serotonin (5-hydroxytryptamine, 5HT) is the indole ethylamine most widely distributed in mammals and its reaction with acetaldehyde would produce 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline (6OMTHBC) (Fig. 1). In this paper, we report the first identification and quantitation of 6OMTHBC in human urine by means of gas chromatography–mass spectrometry. The method involves the use of a capillary column and a deuterated analogue as internal standard which provides high specificity and sensitivity.

MATERIALS AND METHODS

Chemicals and biological samples. 5-Benzyloxyindole, sulphatase type H1 and 5-benzyloxytryptamine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO); lithium aluminium deuteride from Fluka AG (Buchs, Switzerland); pentafluoropropionic anhydride (PFPA) from Massanalys AB (Bromma, Sweden); and dichloromethane from Merck AG (Darmstadt, F.R.G.). Other chemicals used were of analytical purity.

5-Benzyloxy-[α,α,β,β-$^2H_4$]tryptamine hydrochloride and 5-hydroxy-[α,α,β,β-$^2H_4$]tryptamine picrate (5HT-$^2H_4$) were synthesized from 5-benzyloxyindole, according to Shaw *et al.* [12]. 6OMTHBC was synthesized from 5-benzyloxytryptamine hydrochloride according to Taborsky and McIsaac [13]. Similarly, 6-hydroxy-1-methyl-(3,3,4,4-$^2H_4$)-1,2,3,4-tetrahydro-β-carboline (6OMTHBC-$^2H_4$) was synthesized from 5-benzyloxy-[α,α,β,β-$^2H_4$]tryptamine hydrochloride. The mass spectra of the dipentafluoropropionyl (PFP) derivatives of 6OMTHBC and 6OMTHBC-$^2H_4$ are shown in Fig. 2.

Urine was collected in the morning from 8 healthy male volunteers (age range 21–35 years) and 9 male alcoholic subjects (age range 38–56 years) in plastic vessels and stored immediately at −20° prior to analysis.

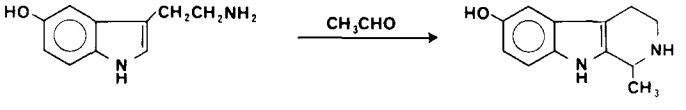

Fig. 1. Formation of 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline (6OMTHBC) from serotonin and acetaldehyde by a Pictet–Spengler reaction.

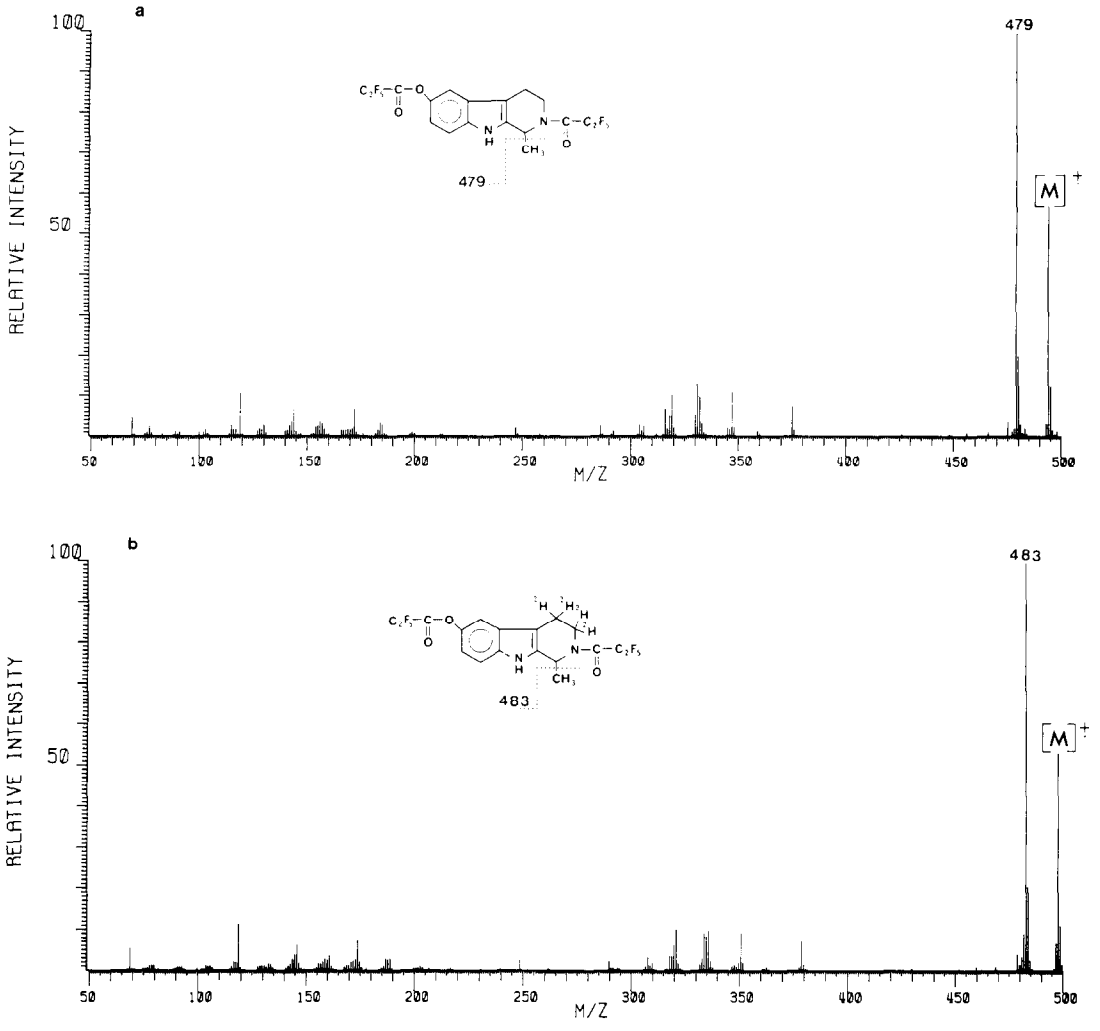

Fig. 2. Electron impact mass spectra of the pentafluoropropionyl derivatives of (a) 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline and (b) 6-hydroxy-1-methyl-(3,3,4,4-$^2H_4$)-1,2,3,4-tetrahydro-β-carboline.

Venous blood was collected in the morning from 8 healthy volunteers (4 males, 4 females—age range 25–62 years) and 8 male alcoholic subjects (age range 32–66 years) in 8.5 ml Vacutainer® tubes containing ACD buffer. The samples were immediately cooled on ice and centrifuged at 280 *g* for 15 min. Platelet-rich plasma was removed and the platelet concentration determined by counting under the microscope. Platelets were obtained as pellets by centrifuging platelet-rich plasma in plastic tubes at 4000 *g* for 15 min and decanting off the platelet-poor plasma. The samples were frozen immediately and stored at −20°.

The alcoholic subjects were inpatients and fulfilled the criteria of dependence according to the World Health Organization [14]. The subjects had been drinking (>125 *g* ethanol per day) for at least two weeks prior to admittance into hospital. Blood samples and the first urine samples were collected upon arrival at the hospital when the subjects were still intoxicated.

Preparation of samples. An ice-cold solution of 6OMTHBC-$^2H_4$ (92.7 pmole), semicarbazide (3.6 μmole), ascorbic acid (0.5 nmole) and EDTA (0.05 nmole) in 1.0 ml of 0.4 M $HClO_4$ was added to plastic tubes containing platelet-pellets. The samples were homogenized using an Ultra-Turrax homogenizer and centrifuged (4°) at 70,000 *g* for 30 min. The supernatant was transferred to clean acid-washed (dichromate–sulphuric acid), silanized, glass tubes containing NaCl (0.4 *g*) and 0.1 ml of 5.8 M K_2CO_3.

Aliquots (1.0 ml) of platelet-poor plasma or urine were added to glass tubes containing 6OMTHBC-$^2H_4$, semicarbazide, NaCl and K_2CO_3 (same amounts as above).

Five millilitres of CH_2Cl_2 was then added and the tubes were shaken for 10 min and centrifuged at 1000 *g* for 5 min. The organic layers were transferred to new tubes and evaporated to dryness under nitrogen. The residues were derivatized with 50 μl of PFPA for 20 min at 60°. After cooling, the excess

reagent was evaporated under a stream of nitrogen and the extracts were dissolved in 25 μl of EtOAc.

Enzymatic hydrolysis of urine. Aliquots of 1.0 ml of urine were pipetted into 15 ml glass tubes containing 6OMTHBC-$^2H_4$ (92.7 pmole), semicarbazide 3.6 μmole), 0.5 ml of an 0.5M citrate buffer (pH 6.0) and 2.5 mg of sulphatase. The samples were incubated at 37° for 16 hr and prepared for 6OMTHBC analysis as described above.

Gas chromatography–mass spectrometry. A computer controlled LKB 2091 gas chromatograph–mass spectrometer was used for the multiple ion detection and recording of mass spectra.

The gas chromatographic separations were achieved with a 20 m SE52 WCOT glass capillary column (i.d. 0.30 mm) using helium as a carrier and make-up gas. Splitless injections were carried out using a 'moving needle' device. The gas chromatographic conditions were: injector heater 280°, column temperature 250°, column flow rate 2 ml/min, and make-up gas flow rate 12 ml/min. Aliquots of 2 μl of the samples were injected and an initial delay of ~2.0 min in opening the separator valve was used to avoid contamination of the ion source. Under these conditions, the retention time of 6OMTHBC-PFP and the deuterated analogue was approximately 2.5 min. The mass spectrometric conditions were: separator temperature 260°, ion source temperature 240°, electron energy 70 eV and trap current 50 μA.

Quantitation. Calibration curves were constructed by plotting the peak height ratios (*m/z* 494/498) of standard samples against the 6OMTHBC concentration. The 6OMTHBC levels were determined from the peak height ratios of each sample by reference to the calibration curve.

RESULTS

Mass spectra of 6OMTHBC-(PFP)$_2$ and 6OMTHBC-$^2H_4$-(PFP)$_2$

The electron impact mass spectra of the PFP derivatives of 6OMTHBC (Fig. 2a) and 6OMTHBC-$^2H_4$ (Fig. 2b) revealed the presence of two PFP groups per molecule in the predominant derivative. Derivatization most likely occurs at the 6-hydroxyl and the pyridyl nitrogen positions, since the indole ring nitrogen is less reactive and sterically hindered. The compounds showed abundant molecular ions at *m/z* 494 and 498, respectively. The base peaks at *m/z* 479 and 483, are formed by loss of the 1-methyl group as indicated in Fig. 2. No evidence for the formation of a tri-PFP derivative was obtained.

Identification of 6OMTHBC in urine

Multiple ion detection was performed by monitoring the ion intensities at *m/z* 494, 498 and *m/z* 479, 483. One ion pair was monitored in a single injection. The chromatograms obtained from the analysis of extracts from urine (Fig. 3a and b) showed that a compound was present possessing both the same retention time and relative ion intensity as authentic 6OMTHBC. This represents the first identification of 6OMTHBC in a human sample.

The possible artifactual formation of 6OMTHBC during the sample preparation was considered and studied in two ways. One test consisted of analysing a sample of water (1 ml) containing 5.4 nmoles of 5HT creatinine sulphate. Using this test artifactual formation was observed on one occasion and found to be due to an impure sample of dichloromethane, which was replaced. In another test, 7.0 nmoles of

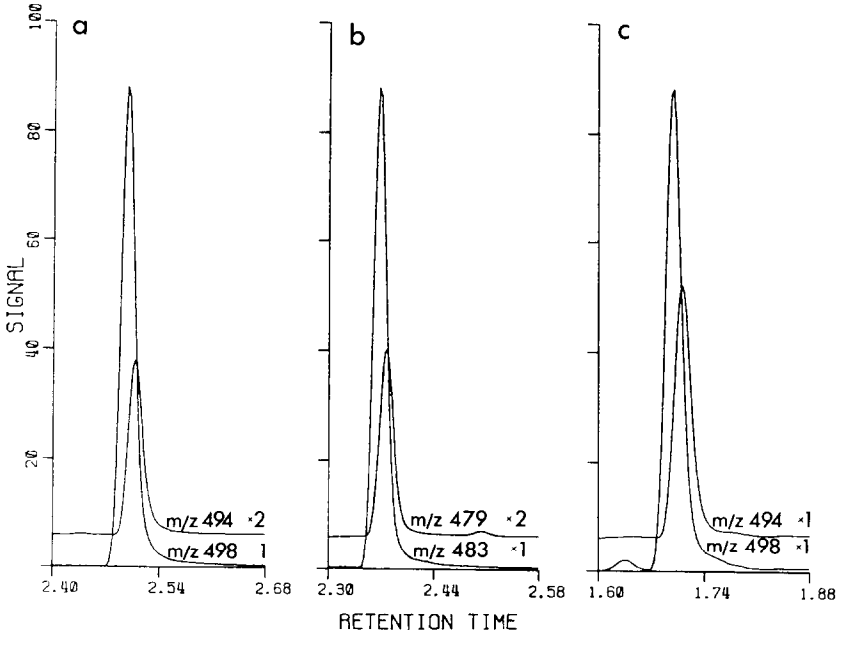

Fig. 3. Chromatograms obtained in the analysis of a urine before hydrolysis monitoring (a) *m/z* 494/498 and (b) *m/z* 479/483, and after enzyme hydrolysis monitoring (c) *m/z* 494/498. Relative amplification factors are indicated in the figure.

Table 1. Reproducibility of 6OMTHBC analysis

Treatment of urine	pmole/ml ± S.D.	n
Direct work-up	65.4 ± 1.5 (2.3%)	10
Enzyme hydrolysis	148.0 ± 7.0 (4.7%)	10
Incubation without enzyme	64.0 ± 0.9	4

5HT-$^2H_4$ was added to 1.0 ml aliquots of urine from 8 individuals prior to workup. No formation of 6OMTHBC-$^2H_4$ was observed. Since the added amounts of 5HT and 5HT-$^2H_4$ was higher than the endogenous level of 5HT in urine [14], this demonstrates that 6OMTHBC was not formed during the workup. The storage of urine at −20° for 6 months did not affect the levels of 6OMTHBC.

When urine was treated with sulphatase containing β-glucuronidase activity the level of 6OMTHBC was increased (Fig. 3c). When urine was incubated without enzyme no change in the level of 6OMTHBC (Table 1) was observed. Nor was 6OMTHBC present in the enzyme preparation. Thus, the result demonstrates the presence of 6OMTHBC in urine both in a free and conjugated form.

Quantitation of 6OMTHBC in urine

The quantitation of 6OMTHBC utilized the ion intensities monitored at *m/z* 494/498. The calibration curve demonstrated a linear relationship of the peak height ratio (*m/z* 494/498) to the concentration of 6OMTHBC, in the range of 0–400 pmole/sample. The reproducibility of individual 6OMTHBC analysis was determined and showed a precision better than 10% (Table 1).

The mean level of total 6OMTHBC in the urine from 8 male healthy volunteers was 75 pmole/ml (Table 2). Most of the 6OMTHBC (74%) occurred in a free form. The excretion rate expressed in terms of the creatinine concentration was 3.3 pmole/μmole creatinine. There was considerable variation in the excretion pattern between the subjects.

The analysis of 6OMTHBC in urine from alcoholics revealed its presence both in a free form and as a conjugate. The results demonstrated higher values in the urine from intoxicated alcoholics (day 1) than after recovery (day 8) or in the controls (Table 2). The only observed significant ($P < 0.05$) difference, however, was between alcoholics day 1 and controls, when the excretion of the conjugated form was expressed in terms of the creatinine concentration. The results showed that a few alcoholics excreted higher amounts of 6OMTHBC when intoxicated but returned to normal following recovery and hospitalization for one week (Fig. 4).

Analysis of 6OMTHBC in platelets

The analysis of 6OMTHBC in platelets and platelet-poor plasma from controls and intoxicated alcoholics demonstrated that the compound was not detectable at a level of sensitivity of 1–4 pmole/10^9 platelets or 0.5 pmole/ml of platelet-poor plasma.

DISCUSSION

The results obtained here demonstrate that 6OMTHBC occurs normally in the urine of man. The level is about 10–20 times lower than that of serotonin [15]. Similar to serotonin, 6OMTHBC occurs partly in a conjugated form [16]. The conjugate(s) was not further characterized. The structurally related 5-hydroxyindoles are known to be substrates for both sulpho and glucuronyl transferases [16]. A related compound to 6OMTHBC, 1MTHBC, was recently identified as a normal constituent of human urine [3]. In that case the level in urine was about 250 times lower than that of the possible precursor, tryptamine.

It has been suggested that the formation of 6OMTHBC occurs during ethanol intoxication by reaction of acetaldehyde with 5HT. The present work was aimed to test if analyses of blood and urine could support evidence for this. The results were inconsistent with this idea, since no evidence of abnormal blood levels or urinary excretion of 6OMTHBC in alcoholics was obtained. However, in three of the nine alcoholic subjects an elevated urinary excretion was evident upon arrival at the hospital. The excretion decreased after a week in hospital, which indicates a link of the elevated excretion to their ethanol abuse. These results are similar to those obtained for the related compound IMTHBC in a previous study [3]. Subsequently, the presence of IMTHBC in alcoholic beverages was reported [17]. Preliminary experiments in this laboratory have shown that 6OMTHBC is also present in alcoholic beverages. This makes the interpretation of the cause of the elevated urinary excretion in a few alcoholic subjects difficult. The fact that controls have similar excretion rates without having consumed alcoholic beverages, and that most alcoholic subjects have similar excretion rates at the end of a drinking period as after one week of abstinence, implies that something other than previous ethanol consumption determines the presence of 6OMTHBC in urine. It cannot be excluded, however, that

Table 2. Levels of 6OMTHBC in urine of healthy volunteers and alcoholics*

		Free 6OMTHBC		Conjugated 6OMTHBC		Total 6OMTHBC	
	n	pmole/ml	pmole/μmole creatinine	pmole/ml	pmole/μmole creatinine	pmole/ml	pmole/μmole creatinine
Controls	9	56 ± 17	3.3 ± 0.8	19 ± 10	1.7 ± 0.9	75 ± 20	5.1 ± 1.3
Alcoholics, day 1	9	52 ± 30	6.5 ± 2.6	59 ± 23	7.7 ± 2.2†	111 ± 48	14.2 ± 4.6
Alcoholics, day 8	9	17 ± 6	2.5 ± 0.5	24 ± 6	3.8 ± 0.6	41 ± 12	6.3 ± 1.0

* Values are mean ± S.E.M.
† Differs from controls $P < 0.05$.

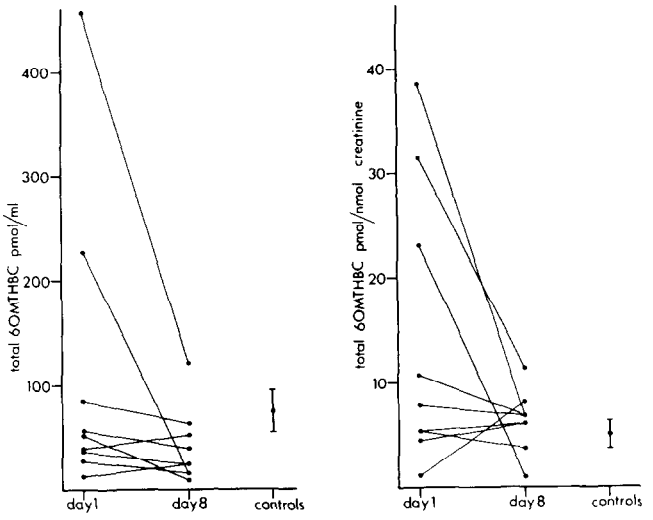

Fig. 4. Levels of total 6OMTHBC in urine from alcoholics and controls. On day 1 the subjects were intoxicated and hospitalized and day 8 is one week after hospitalization.

6OMTHBC is formed during ethanol intoxication but, if so, then it is of minor importance, at least from a quantitative point of view.

Further investigations are required to determine the origin and possible significance of 6OMTHBC.

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