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Metabolic Hydroxylation of 1-Methyl-1,2,3,4-Tetrahydro-β-Carboline in Humans

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TSUCHIYA, H., H. TODORIKI AND T. HAYASHI. *Metabolic hydroxylation of 1-methyl-1,2,3,4-tetrahydro-β-carboline in humans.* PHARMACOL BIOCHEM BEHAV **52**(4) 677–682, 1995—We characterized the metabolites of 1-methyl-1,2,3,4-tetrahydro-β-carboline (MTBC) in human urine by gas chromatography–negative ion chemical ionization mass spectrometry (GC-NICIMS) and developed an analytical method using GC-NICIMS for their quantitative determination. When tetradeuterated MTBC was orally administered to a human subject, two peaks of the deuterated metabolites appeared on mass fragmentograms of the urine samples after administration. They were identified as tetradeuterated 6-hydroxy-MTBC (6-OH-MTBC) and 7-hydroxy-MTBC (7-OH-MTBC), indicating that MTBC was metabolically hydroxylated in humans. The proposed GC-NICIMS method could sensitively and selectively determine urinary 6-OH-MTBC and 7-OH-MTBC without interference from their artifactual formation during analysis. Its application to urine analysis has revealed that MTBC is excreted in human urine predominantly as the two hydroxylated metabolites, in which 6-OH-MTBC is present in both free and conjugated forms, whereas the 7-OH-MTBC of a conjugated form is much more than the 7-OH-MTBC of a free form.

1-Methyl-1,2,3,4-tetrahydro-β-carboline Metabolism in humans Hydroxylation Urinary excretion GC-NICIMS

NEUROACTIVE 1-methyl-1,2,3,4-tetrahydro-β-carboline (MTBC) and its related compounds are naturally distributed in various plants, including those used in foods and beverages; therefore, their considerable amounts are exogenously supplied to mammalian systems via the dietary source (1,2,9,25). MTBC is also endogenously formed by the in vivo condensation of indoleamines with either acetaldehyde or pyruvate (2,26,30,38). Because MTBC and its analogues show various neuropharmacologic effects, they have been speculated to function as false neuromodulators, to influence behaviors, and to participate in alcoholism (16,18,27,29,37).

Although a potent biologic significance is considered for MTBC, the in vivo metabolism of MTBC has not been conclusive in mammals, especially in humans. When radiolabelled 1,2,3,4-tetrahydro-β-carboline (TBC) was administered to rats, the radioactive hydroxylated metabolites of TBC were excreted in urine (20). In analogy with TBC, enzymatic hydroxylation was suggested for MTBC in rat and cat based on the fact that the enantiomers of 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline (6-OH-MTBC) were excreted in their urine in unequal abundance (6,8). The presence of hydroxylated MTBCs was also reported in mammalian body fluids and tissues (2,3,5,11,12,27). However, interspecies differences are known for the hydroxylation of tetrahydro-β-carbolines, and the in vivo metabolism of MTBC has not been studied systematically in humans.

We recently found that (R)-(+)-MTBC was predominantly excreted in human urine over (S)-(−)-MTBC, and suggested that a certain stereoselective or enzymatic reaction might occur in the metabolism of MTBC in humans (36). In the present study, after we administered MTBC labelled with deuterium (d) to a human subject, the deuterated metabolites excreted in urine were characterized by gas chromatography–negative ion chemical ionization mass spectrometry (GC-NICIMS). Uri-

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nary concentrations of the identified MTBC metabolites were also determined by a GC-NICIMS method developed for their analysis.

METHODS

Chemicals

MTBC and [3,3,4,4-$^2H_4$]-1-methyl-1,2,3,4-tetrahydro-β-carboline (MTBC-d_4) were prepared from either tryptamine or [α,α,β,β-$^2H_4$]-tryptamine (tryptamine-d_4) and acetaldehyde as reported previously (21). According to the method of Taborsky and McIsaac (32), 6-OH-MTBC and [3,3,4,4-$^2H_4$]-6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline (6-OH-MTBC-d_4) were prepared from 5-benzyloxytryptamine and 5-benzyloxy-[α,α,β,β-$^2H_4$]-tryptamine synthesized by the method of Shaw et al. (31). 7-Hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline (7-OH-MTBC) was synthesized by the catalytic reduction of 7-hydroxy-1-methyl-β-carboline in the presence of 10% palladium on charcoal based on the method of Beck et al. (8). We obtained [1-^2H]-7-hydroxy-1-methyl[$^2H_3$]-1,2,3,4-tetrahydro-β-carboline (7-OH-MTBC-d_4) by heating 10 mg of [1-^2H]-1-methyl[$^2H_3$]-7-methoxy-1,2,3,4-tetrahydro-β-carboline, which was synthesized from 6-methoxytryptamine and acetaldehyde-d_4 (32), with 0.2 ml acetic acid, 0.2 ml hydrochloric acid, and 0.8 ml water at 120°C for 1 h, and then purified it by high-performance liquid chromatography.

Tryptamine-d_4 and [α,α,β,β-$^2H_4$]-serotonin (serotonin-d_4) were purchased from Merck Frosst Canada (Montreal, Canada). Fluorescamine and sulfatase (type H-1) were obtained from Fluka (Buchs, Switzerland) and Sigma (St. Louis, MO), respectively. The other reagents were of the highest grade available. We redistilled water using an all-glass apparatus.

Urine Sample

MTBC-d_4 (10 or 20 μg/kg), suspended in 100 ml of orange juice, was orally administered to one of the authors, a male subject aged 45 years, according to the guidelines of the Japanese Pharmacological Society. Urine was collected just before administration and at time intervals of 1.5 h up to 7.5 h after administration. He freely got only water during the collection. These urine samples were used for screening urinary constituents and characterizing MTBC metabolites excreted in urine. Different urine samples, defined as urine A and urine B (Table 1), were also collected from two male age-matched subjects without administration and were used for the identification of MTBC metabolites. To determine the distribution of the urinary concentrations of MTBC and its metabolites, we collected urine from male subjects (n = 47) aged 18–22 years. All subjects freely ate food and drank water, but did not consume any alcoholic beverages for 24 h before the collection; no subject had a history of metabolic disease. Informed consent was obtained from all subjects after the nature and consequences of their participation were explained.

The urine samples were analysed immediately after collection, or were stored at −80°C and analysed within 1 week. No change was detected in the concentrations of MTBC and its metabolites under these conditions. All samples were filtered through a pore size of 0.45 μm, followed by analysis.

TABLE 1

CHROMATOGRAPHIC AND MASS SPECTROMETRIC COMPARISON BETWEEN AUTHENTIC AND URINARY COMPOUNDS

Sample	Retention time (sec)	Relative peak intensity m/z 296	m/z 393	m/z 490
Authentic				
7-OH-MTBC	185	18	100	44
6-OH-MTBC	191	23	100	56
Urine A				
Peak-1	185	17	100	44
Peak-2	191	22	100	56
Urine B				
Peak-1	185	16	100	43
Peak-2	191	21	100	56

Sample Preparation

Characterization of metabolites. A 1.0-ml aliquot of the urine filtrates collected before and after MTBC-d_4 administration (10 μg/kg, orally) was incubated at 37°C for 16 h with 1.0 ml of 0.05 M citrate buffer (pH 6.0) containing sulfatase (2.5 mg/ml) in the presence of 1.0 ml hydrazide gel (23). After the reaction mixture was centrifuged at 10,000 × g for 5 min, 0.2 ml of the supernatant was mixed with 0.5 ml of 0.1 M phosphate buffer (pH 8.5), and then 0.5 ml of fluorescamine in acetone (8 mg/ml) was added to the mixture under vortex-mixing for 30 s. The sample solution was subjected to three-step extractions with ethyl acetate and derivatization with trifluoroacetic anhydride as reported previously (21,22). The trifluoroacetyl (TFA) derivatives of the metabolites were dissolved with 50 μl of ethyl acetate, followed by GC-NICIMS analysis.

Determination of MTBC and its metabolites. The urine filtrates were enzymatically hydrolyzed for the quantitative determination of total (free plus conjugated) metabolites in urine as described before. For the free metabolites, the filtrates were directly analysed without the enzymatic reaction. The internal standards, MTBC-d_4, 6-OH-MTBC-d_4, and 7-OH-MTBC-d_4 (50.0 ng of each), were added to the supernatant or filtrate, and the solutions were similarly treated as described earlier.

Urinary creatinine was determined quantitatively by a creatinine assay kit (Sanko-Junyaku, Tokyo, Japan). All glassware used was silanized to avoid the adsorption of analytes to its surface (34).

GC-NICIMS Analysis

We performed the mass fragmentography and mass spectral recording using a Finnigan 4000 gas chromatograph-mass spectrometer (Finnigan, San Jose, CA) equipped with a pulsed positive-ion negative-ion chemical ionization accessory and a moving needle device for splitless sample injections in the negative ion chemical ionization and selected ion-monitoring mode. An RTX-1 Crossbonded SE-30 fused silica capillary column (30 m × 0.25 mm internal diam., 0.25 μm film thickness; Restek, Bellefonte, PA) was used for chromatographic separation. Head pressure of the column was kept at 0.7 kg/cm^2, giving a 1-ml/min flow rate of methane used as a carrier gas. To characterize the MTBC metabolites, we programmed the column temperature to be at 150°C for 1 min, increase to 270°C at 10°C/min, and then stay at 270°C for 2 min. The monitoring was performed at m/z 397 for the MTBC-d_4 metabolites. When MTBC, 6-OH-MTBC, and 7-OH-MTBC were quantitatively determined, the GC separation was carried out isothermally at 210°C. MTBC-d_0, MTBC-d_4, both OH-

MTBCs-d_0, and both OH-MTBCs-d_4 were monitored at m/z 378, 382, 393, and 397, respectively. The ion-source pressure, emission current, and electron energy were 0.15 mm Hg, 300 μA, and 90 eV, respectively. The other operating conditions are described elsewhere (21,36). The urinary concentrations of MTBC, 6-OH-MTBC, and 7-OH-MTBC were determined based on the peak height ratios to the internal standards by reference to the calibration graphs.

RESULTS

When we monitored the urine samples collected after administration of MTBC-d_4 (10 μg/kg, orally) at m/z 397, two different peaks (peak 1 and peak 2 in retention order) of the tetradeuterated metabolites appeared on selected ion-monitoring chromatograms as shown in Fig. 1. Both peak 1 and peak 2 were obtained from all urine collections (up to 7.5 h after administration). The two peaks at m/z 397 were not detected in the urine samples collected before MTBC-d_4 administration, nor in the control urine.

Two peaks at m/z 393 were also obtained from urine at the retention times identical to those of the deuterated peaks. Methane negative ion chemical ionization mass spectra of the TFA derivatives of authentic 6-OH-MTBC and 7-OH-MTBC provided major peaks at m/z 296, 393 (base peak), and 490. The peaks at m/z 490 were assigned to the molecular ions, $[M]^-$, for both. The fragment peaks at m/z 393 and 296 were also assigned to $[M - TFA]^-$ and $[M - 2\ TFA]^-$, respectively. The MTBC metabolites excreted in urine were identified as 7-OH-MTBC (peak 1) and 6-OH-MTBC (peak 2) based on the presence of peaks at m/z 296, 393, and 490 at the same retention times as authentic ones and with the same ion intensity ratios as shown in Table 1. The two tetradeuterated metabolites were similarly identified as 7-OH-MTBC-d_4 and 6-OH-MTBC-d_4.

Representative selected ion monitoring chromatograms of urine samples are shown in Figs. 2 and 3, together with the procedural blank. When the urine sample spiked with serotonin-d_4 (0.5 μg/ml) was analysed without the fluorescamine treatment, the peak of 6-OH-MTBC-d_4 appeared (Fig. 2B),

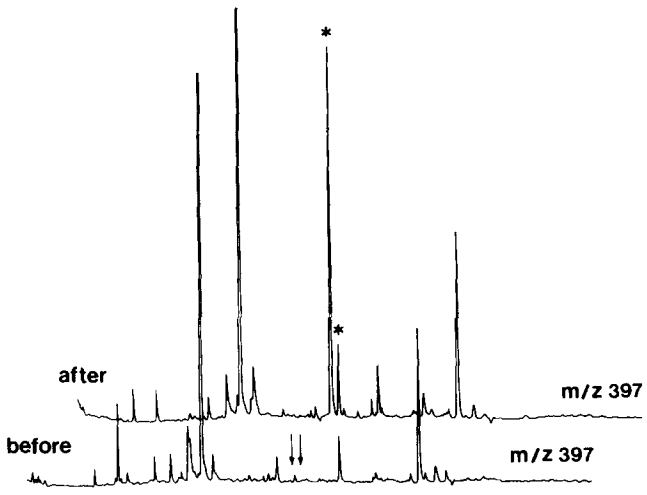

FIG. 1. Mass fragmentograms obtained from the urine samples collected before and 1.5 h after MTBC-d_4 was administered (10 μg/kg, orally). Arrows indicate the retention times of the two peaks (asterisks) that were detected only after MTBC-d_4 administration.

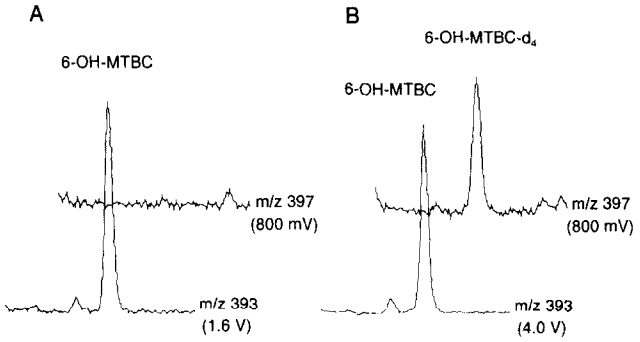

FIG. 2. Mass fragmentograms obtained from the urine samples spiked with serotonin-d_4 (0.5 μg/ml) with (A) and without (B) fluorescamine treatment.

indicating that it was artifactually formed by analytical procedures. Such a peak was never detected after the fluorescamine treatment (Fig. 2A), which completely inhibited the artifactual formation during analysis.

By the proposed GC-NICIMS method, urinary 6-OH-MTBC and 7-OH-MTBC could be quantitatively determined in the concentrations from picograms to nanograms per milliliter. Results of the replicated determinations of urine samples are summarized in Table 2. The analytical precision was satisfactory for the quantitative determination of all OH-MTBCs.

When MTBC-d_4 (20 μg/kg, orally) was administered, MTBC-d_4, 6-OH-MTBC-d_4, and 7-OH-MTBC-d_4 were excreted in urine every 1.5 h, as shown in Fig. 4. The excretion rate per hour of both OH-MTBCs-d_4 was greater than that of MTBC-d_4 throughout all urine collections. The ratio of free to total 6-OH-MTBC-d_4 was in the range 0.54–0.70. 7-OH-MTBC-d_4, in contrast to 6-OH-MTBC-d_4, showed no significant amount of free form, and the ratio of free to total 7-OH-MTBC-d_4 was only ≤ 0.02.

When we applied the GC-NICIMS method to urine analysis, the mean concentrations and SD ($n = 47$) of MTBC, total 6-OH-MTBC, and total 7-OH-MTBC were 3.51 ± 5.34, 93.3 ± 145.7, and 110.7 ± 131.2 ng/ml, respectively. The mean values expressed as nanograms per milligram creatinine and SD were 2.64 ± 3.17 for MTBC, 76.4 ± 95.8 for total 6-OH-

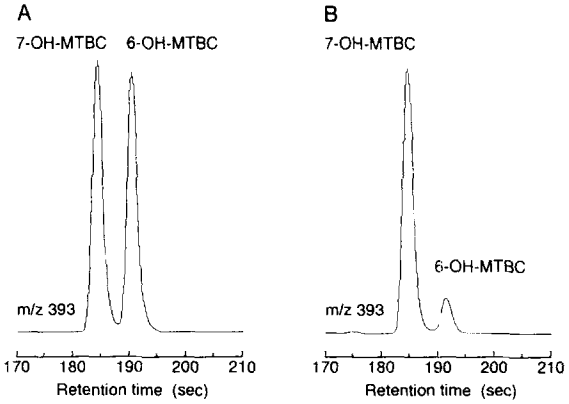

FIG. 3. Mass fragmentograms obtained from authentic 6-OH-MTBC and 7-OH-MTBC (A), and a urine sample (B).

TABLE 2

ANALYTICAL PRECISION FOR THE DETERMINATION OF TOTAL AND FREE 6-OH-MTBC AND 7-OH-MTBC IN HUMAN URINE

Compound	n	Mean concentration (ng/ml)	CV (%)
Total			
6-OH-MTBC	8	92.2	3.6
7-OH-MTBC	8	297.9	1.9
Free			
6-OH-MTBC	12	24.3	3.6
7-OH-MTBC	12	4.1	6.1

MTBC, and 92.9 ± 96.8 for total 7-OH-MTBC, respectively. Both urinary excretion of 6-OH-MTBC and 7-OH-MTBC were greater than that of MTBC ($p < 0.001$). The excretion ratio of 6-OH-MTBC plus 7-OH-MTBC to MTBC ranged from 10.5–353.7. Its mean value indicated that the total hydroxylated metabolites were excreted in about 95-fold excess over MTBC.

DISCUSSION

We recently found that (R)-(+)-MTBC-d_4 predominated in urine over (S)-(−)-MTBC-d_4 when the MTBC-d_4 racemate was orally administered to a human subject (36). Because participation of the biosynthesis was excluded from that experimental system, such an unequal urinary excretion of the enantiomers indicates that the in vivo metabolism of MTBC is enzymatically assisted in humans. To specify the MTBC metabolites, MTBC-d_4 was orally administered, and thereafter its tetradeuterated metabolites excreted in urine were characterized. The results of GC-NICIMS analysis only reflect the metabolism of MTBC, because the deuterated metabolites (OH-MTBC-d_4) are distinguishable from nondeuterated products (OH-MTBC-d_0), which originate in the in vivo condensation of serotonin. The appearance of the deuterated metabolites was confined to MTBC-d_4 administration, and they were identified as 6-OH-MTBC and 7-OH-MTBC. Although we analyzed different urine samples from a single subject, the consistent results indicate that MTBC is metabolically hydroxylated at the 6- and 7-positions in humans.

It is possible that some OH-MTBCs are artifactually formed by analytical procedures, during which urinary serotonin condenses with acetaldehyde in biologic samples and/or

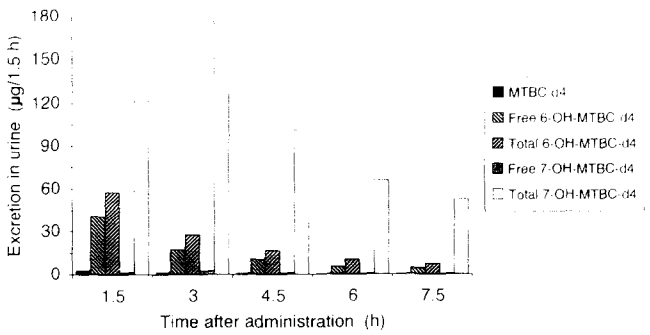

FIG. 4. MTBC-d_4, free and total 6-OH-MTBC-d_4, and free and total 7-OH-MTBC-d_4 excreted in urine every 1.5 h after MTBC-d_4 was administered (20 μg/kg, orally).

extraction solvents to form 6-OH-MTBC (11,17,19). Such an analytical pitfall was reported to be a less serious problem for the acetaldehyde-derived tetrahydro-β-carbolines (5,11) compared with the formaldehyde-derived ones (13,14). However, 6-OH-MTBC-d_4 was artifactually formed by extracting the urine samples spiked with serotonin-d_4 corresponding to the urinary level (33,34). The artifactual formation should be also noticed in the analysis of OH-MTBCs. The proposed method could effectively suppress the artifactual formation of OH-MTBCs by treating with fluorescamine. In such a pretreatment, the precursors, serotonin and tryptamine, are converted to the carboxylic fluorescamine derivatives, which are removed from the analytical system by the following extractions (21,22,35).

Urinary OH-MTBCs are presumed to be present in a conjugated form (17). For the determination of total OH-MTBCs, urine samples were treated with sulfatase to convert them to the free forms (8,11). To suppress condensation during the enzymatic reaction, the incubation was performed in the presence of the hydrazide gel (23), which trapped the carbonyl compounds in biologic samples.

By these devices, it has become possible quantitatively to determine only the OH-MTBCs originally contained in urine. The proposed GC-NICIMS method has been proven to be sensitive, selective, and reproducible enough to determine the 6-OH-MTBC and 7-OH-MTBC of the urinary levels.

A larger fraction of MTBC was excreted in urine as the two hydroxylated metabolites, 6-OH-MTBC and 7-OH-MTBC. In the administration experiment, urinary 6-OH-MTBC occurred in both free and conjugated forms, whereas most of the 7-OH-MTBC was excreted in urine as a conjugated form. These excretion profiles agree with previous reports (5,8,11,12).

Although the detailed metabolic process is unclear at this time, it would be possible to speculate on the enzymatic reaction responsible for the hydroxylation of MTBC. Both 6-OH-MTBC and 7-OH-MTBC show the urinary excretion to be different between their enantiomers, indicating that MTBC is hydroxylated by a substrate-stereoselective reaction (8,12). When MTBC was incubated with different tissue homogenates of rats, the formation of 6-OH-MTBC and 7-OH-MTBC occurred exclusively by incubating with hepatocytes, and the highest hydroxylation activity was associated with liver microsomes (10). The rats used in that study were pretreated with 3-methylcholanthrene to induce microsomal enzymes, suggesting that certain forms of cytochrome P-450s participate in the metabolic hydroxylation of MTBC.

The urinary concentrations of 6-OH-MTBC and 7-OH-MTBC, and the 6-OH-MTBC/7-OH-MTBC ratios varied with human subjects. Whereas the predominant excretion of both OH-MTBCs over MTBC was consistent with the other reports, the urinary concentrations of total 6-OH-MTBC and total 7-OH-MTBC were different from them (5,6,12). For 6-OH-MTBC, the in vivo condensation between serotonin and acetaldehyde may be unlikely, because the acetaldehyde levels in the body are low under normal conditions (6). Although a biosynthetic route via serotonin and pyruvate is presumed (6,17), its in vivo occurrence may be ruled out by the fact that deuterated MTBC is not detected in human urine after the administration of deuterated tryptophan, whereas deuterated tryptamine is excreted (21,22). For 7-OH-MTBC, metabolic hydroxylation is the only route for its formation, because 6-hydroxytryptamine as a precursor is not available in vivo (17). Another interpretation is the possibility that 6-OH-MTBC and 7-OH-MTBC are exogenously supplied in an individually different manner. OH-MTBCs and their potent precursor tetrahydro-β-carbolines are present in various foods and beverages

(1,4,9,15,25). The dietary sources would influence the urinary excretion of 6-OH-MTBC and 7-OH-MTBC.

When substantially the same tracer experiment was carried out by administrating TBC-d_4, the two hydroxylated isomers of TBC were excreted in urine as well as MTBC (data not shown). The in vivo hydroxylation at the 6 and 7 positions is considered to be a metabolic route common to a class of tetrahydro-β-carbolines (8,17,20). Lipophilic MTBC and the related tetrahydro-β-carbolines easily cross the blood-brain barrier and potentially influence brain functions, although hydroxylated metabolites such as 6-OH-MTBC and 7-OH-MTBC are not advantageous for crossing the blood-brain barrier, because of their higher hydrophilicity (2,28). The metabolic hydroxylation of MTBC in liver and the subsequent excretion in urine may be relevant to elimination of the neuropharmacologically active MTBC in humans. Changes in the levels of MTBC and related compounds have been considered in association with psychiatric disorders and alcoholism (16, 17,24,27). If the enzymatic system responsible for the in vivo hydroxylation of MTBC was suppressed, the elimination of MTBC would be reduced, possibly resulting in the enhancement of its influences on psychological or mental functions. Deficiency in the hepatic metabolism of MTBC may be related to the pathogenesis of certain psychiatric disorders.

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