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Determination of tetrahydro-β-carbolines in urine by high-performance liquid chromatography with suppression of artefact formation

HIRONORI TSUCHIYA,* SHIGERU OHTANI, KUNIAKI YAMADA, NOBUHIKO TAKAGI and TOKISHI HAYASHI†

Department of Dental Pharmacology, Asahi University School of Dentistry, Gifu 501-02, Japan
†GRELAN Pharmaceutical Company Ltd, Tokyo 205, Japan

Abstract: A high-performance liquid chromatographic method has been developed for the determination of urinary tetrahydro-β-carbolines. When standing tryptamine with formaldehyde and acetaldehyde under extraction conditions, the significant amounts of artefact 1,2,3,4-tetrahydro-β-carboline (TBC) and 1-methyl-1,2,3,4-tetrahydro-β-carboline (MTBC) were formed in a short time. Urine samples added with 2-ethyl-1,2,3,4-tetrahydro-β-carboline (an internal standard) were treated with fluorescamine, and then with glycine, followed by serial solvent extractions. Such a pretreatment using two-step reactions removed a precursor (trypamine) by extracting its fluorescamine derivative, and enhanced the detection response by consuming excess fluorescamine. It solved the analytical problem that artefact TBC and MTBC are formed during analysis. Reversed-phase ion-pair chromatography using a C_8-column and trifluoroacetic acid as a counter ion completed a base-line separation of three analytes within 10 min. The calibration graphs showed a good linearity in the range 0.1–50.0 ng ml^{-1} of urine samples spiked with standard TBC and MTBC. In the spike experiment, the recovery and relative standard deviation were almost 100% and less than 3.0%, respectively, for both TBC and MTBC. The proposed method enables the determination of the genuine urinary concentrations of TBC and MTBC without involving their artefacts.

Keywords: *Tetrahydro-β-carbolines; HPLC; fluorescence detection; suppression of artefact formation; urine.*

Introduction

The formation of artefacts by analytical procedures themselves is liable to be overlooked, although it is important, especially for trace bioactive compounds not fully elucidated on their presence and contents [1, 2]. A class of β-carbolines belong to such artefact compounds formed during analysis. Among them, 1,2,3,4-tetrahydro-β-carboline (TBC) and 1-methyl-1,2,3,4-tetrahydro-β-carboline (MTBC) known not only as plant alkaloids [3], but as constituents in mammalian urine and tissues [4, 5] have been attracting much concern because of their various physiological and pharmacological effects [6–8]. The determination of urinary concentrations of TBC and MTBC is required to study their metabolism which has been controversial in mammalian systems.

TBC and MTBC are mainly formed by the condensation between tryptamine and either formaldehyde or acetaldehyde (Fig. 1), and this reaction readily takes place under laboratory as well as under physiological conditions. TBC, MTBC and the related compounds were reported to possibly be formed from tryptamine in biological samples, and aldehydes contained in and/or contaminating the samples and extraction reagents [9, 10], while other reports described that the artefact formation seemed to be less in the analysis of acetaldehyde-derived tetrahydro-β-carbolines [11, 12]. However, such a possibility has not been quantitatively investigated. If substantial amounts of TBC and MTBC are actually formed by analytical procedures, suppression of the artefact formation is essential to their accurate determination.

The present study was done with the following objectives: (1) determination of TBC and MTBC formed under analytical conditions; (2) suppression of the artefact formation; and (3) analysis of the genuine concentrations of TBC and MTBC in human urine by high-performance liquid chromatography (HPLC).

* Author to whom correspondence should be addressed.

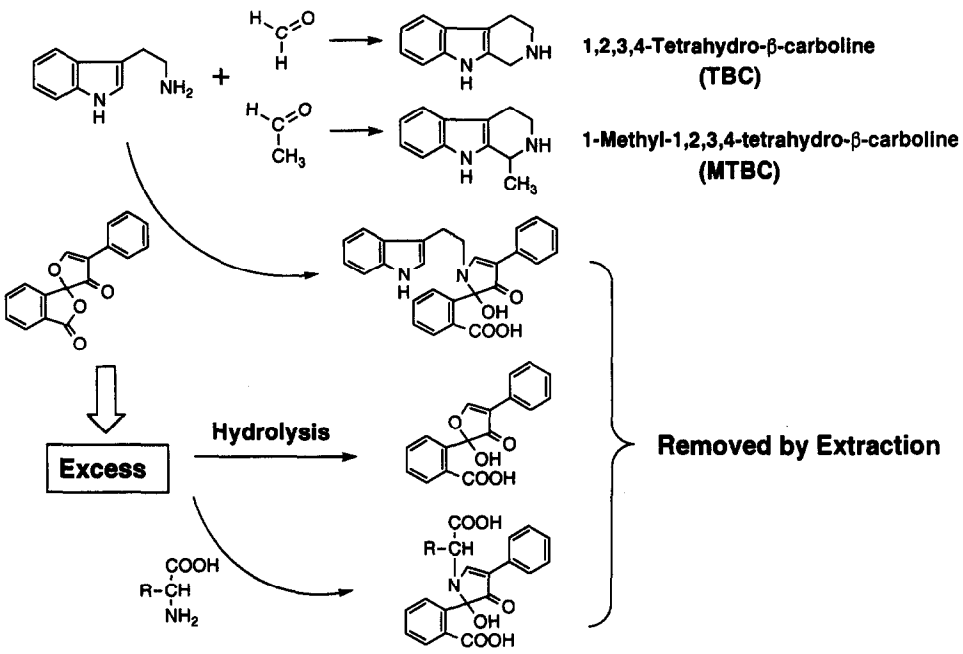

Figure 1
Formation of TBC and MTBC, and analytical strategy for their determination with suppression of artefact formation.

Experimental

Chemicals and reagents

TBC and MTBC were prepared by the methods of Ho and Walker [13], and of Hayashi *et al.* [14], respectively. 2-Ethyl-1,2,3,4-tetrahydro-β-carboline (ETBC) used as an internal standard was synthesized as previously reported [15]. Their ethanolic solutions (1.0 mg ml^{-1} of each) were stored at 4°C and were diluted with water as required. Fluorescamine, L-amino acids and tryptamine hydrochloride were purchased from Fluka (Buchs, Switzerland), Ajinomoto (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Acetonitrile was of HPLC grade and the other reagents were of the highest available quality. Water used was redistilled by an all-glass apparatus.

Sample preparation

One-day urine was collected from 10 healthy subjects aged from 21 to 25 years. Urine specimens were also collected from three male subjects aged from 28 to 40 years immediately after a drinking party. All urine samples were filtered through a 0.22-μm pore-size and the filtrates were treated as follows.

A 50-μl aliquot of the ETBC solution (50.0 ng ml^{-1}) and 0.5 ml of 3.0 M potassium phosphate buffer (pH 8.5) were added to 0.25 ml of the urine filtrate. To the mixture, 0.5 ml of a fluorescamine solution in acetonitrile (5.0 mg ml^{-1}) was added, under vortex-mixing for 20 s. Immediately after that, 0.5 ml of 3.0 M potassium phosphate buffer (pH 8.5) containing glycine (100.0 mg ml^{-1}) was added under vortex-mixing for 20 s. The mixture was extracted with 7.0 ml of ethyl acetate after adding 2.0 ml of 0.5 M NaOH. The organic phase was extracted with 1.0 ml of 0.2 M HCl, and then the aqueous phase was re-extracted with 7.0 ml of diethyl ether after adding 2.0 ml of 0.5 M NaOH. The organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was dissolved with 0.2 ml of 0.2% (v/v) trifluoroacetic acid and a 50–100-μl aliquot of the resulting solution was injected to an HPLC column.

Urinary creatinine was determined by a creatinine assay kit (Sanko Junyaku, Tokyo, Japan).

Artefact formation of TBC and MTBC

Tryptamine (0.25–25.0 μg ml^{-1}) and aldehydes (formaldehyde and acetaldehyde, 1.0–100.0 μg ml^{-1} of each) were dissolved in 1.0 ml of 0.1 M phosphate buffer (pH 7.0), 0.1 M HCl or 0.1 M NaOH, and the mixtures were allowed to stand at room temperature. At specified time intervals, 0.25-ml aliquots of the solutions were analysed as described.

Optimization of analytical conditions

According to the described procedure, the solutions containing TBC, MTBC and ETBC (10.0 ng ml^{-1} of each), and tryptamine (1.0–25.0 μg ml^{-1}) were treated by varying the concentration of fluorescamine (0–10.0 mg ml^{-1}) or the kind and concentration (0–100.0 mg ml^{-1}) of amino acids. The analytical conditions were optimized by comparing the relative peak heights of TBC, MTBC, ETBC and tryptamine. The results were expressed as the means of duplicated experiments.

HPLC analysis

A chromatographic system was substantially the same as used in a previous study [15]. The separation was performed by delivering the mobile phase consisted of acetonitrile–trifluoroacetic acid–water (25.0:0.2:74.8, v/v/v) to a 250 mm × 4.6 mm i.d. Shim-pack CLC-C8 (M) column (5-μm particle size; Shimadzu, Kyoto, Japan) at a flow rate of 1.0 ml min^{-1} and at a column temperature of 35°C. Fluorescence intensity of the eluates was detected at 275 nm for excitation and at 350 nm for emission.

Urinary TBC and MTBC were determined by reference to the peak height ratios to ETBC which were prepared from the standard solutions treated in a similar manner to urine samples. The results were corrected by recoveries obtained from the spike experiment (5.0 and 10.0 ng ml^{-1} of TBC and MTBC spiked to urine, $n = 8$).

Results and Discussion

Artefact formation under analytical conditions

There are analytical pitfalls in the determination of tetrahydro-β-carbolines and the related compounds, due to the possible formation of artefacts during analytical procedures [9, 10, 16]. Figure 1 shows the formation mechanism of TBC and MTBC in which tryptamine condenses with either formaldehyde or acetaldehyde. To determine whether this condensation reaction really proceeds under analytical conditions, the mixtures of tryptamine, formaldehyde and acetaldehyde were allowed to stand at room temperature under the postulated extraction and back-extraction conditions: in acidic, neutral and alkaline media. Thereafter, TBC and MTBC formed were determined at specified time intervals. Figure 2 shows the concentrations of artefact TBC formed from tryptamine (2.0 μg ml^{-1}) and formaldehyde (5.0 μg ml^{-1}). Under any conditions, ng ml^{-1} levels of TBC were formed in a very short time at room temperature. Its formation depended on the tryptamine and formaldehyde concentrations, and the reaction time. MTBC was also formed under all conditions, although its concentrations tended to be about 10 times less than those of TBC. Such difference may be due to the higher reactivity of formaldehyde than acetaldehyde. Even when tryptamine was serially extracted without adding standard aldehydes, TBC and MTBC were detected, which are derived from the aldehyde impurities in extraction reagents [17, 18]. From these results, the artefact formation would occur during extraction procedures which are performed for the pretreatment of urine samples. The difference in concentrations of the formed artefacts indicates that the artefact formation is a more serious problem in the analysis of TBC than of MTBC, as reported previously [11, 12, 16].

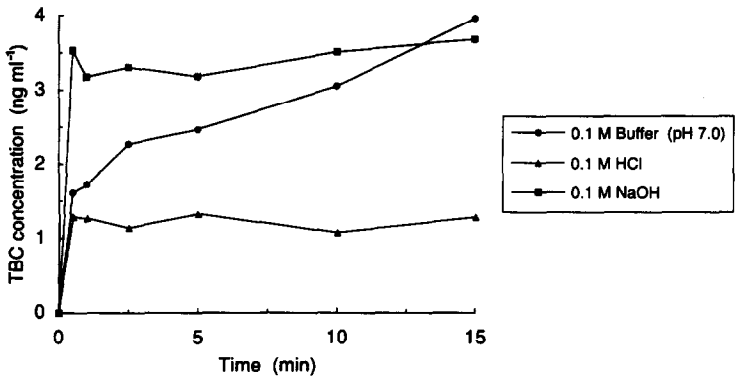

Figure 2
Artefact formation of TBC. Tryptamine (2.0 μg ml^{-1}) and formaldehyde (5.0 μg ml^{-1}) were allowed to stand in 0.1 M phosphate buffer (pH 7.0), 0.1 M HCl and 0.1 M NaOH, and then TBC was determined at specified time intervals.

Treatment with fluorescamine

To suppress the artefact formation during analysis, previous studies treated one precursor, aldehydes with an aldehyde-trapping reagent such as semicarbazide [19–21]. However, this technique was reported to be unable to completely exclude the artefacts, especially TBC [16]. Even if aldehydes are removed prior to extraction, the aldehydes exist as impurities in extraction solvents and thus recontamination may occur, causing artefact formation since basic tryptamine is co-extracted with TBC and MTBC throughout all extractions.

In this study, the other precursor, tryptamine was treated with fluorescamine to produce a carboxylic derivative which was removed from the analytical system by extractions (Fig. 1). Once tryptamine is removed, the artefact formation is not expected to occur, even if aldehydes contamination occurs during the following procedures.

Tryptamine (5.0 μg ml^{-1}), corresponding to 20–100 times over the urinary level [22, 23], was removed by treating with fluorescamine (>2.0 mg ml^{-1}) as shown in Fig. 3. When tryptamine (1.3 μg ml^{-1}) was extracted in the presence of aldehydes (0.8–16.6 μg ml^{-1}), pg–ng ml^{-1} levels of artefact TBC and MTBC were formed. The peaks of such artefacts completely disappeared when extraction took place after the fluorescamine treatment.

The effectiveness of the fluorescamine treatment is evaluated by comparing the quantitative results between treated and non-treated urine. However, urine samples not treated with fluorescamine contained interfering peaks and, therefore, a reliable determination of TBC and MTBC could not be achieved. The fluorescamine treatment was essential not only for the removal of a precursor (tryptamine) but also for the purification of both tetrahydro-β-carbolines [15]. While the evaluation by urine analysis is practically difficult, it is possible with the use of a procedural blank. TBC (0.1–0.7 ng ml^{-1}) and MTBC (0.3 ng ml^{-1} or less than the quantitative limit) were detected when tryptamine solutions of the urinary level [22, 23] were extracted and chromatographed without the fluorescamine treatment. The varition in concentrations of the formed artefacts depended upon the reagent grade of the extraction solvents used, which correlates to the aldehyde contamination [18]. When standard solutions of TBC and MTBC (10.0 ng ml^{-1} of each) were analysed, both peak height ratios to ETBC were consistently higher in samples without, rather than with, the fluorescamine treatment. These results apparently indicate that the treatment to suppress the artefact formation is necessary for the determination of the TBC and MTBC originally contained in urine.

Fluorescamine was chosen as a pretreatment reagent because of its property to react exclusively with primary amines in a very short time [24, 25]. Fluorescamine reacts with tryptamine to produce a carboxylic derivative and excess fluorescamine is hydrolysed to a carboxylic product (Fig. 1). Both acidic compounds should be removed by the following extractions. However, the peak heights of TBC and MTBC were reduced with an increase in the fluorescamine concentration, suggesting that secondary amines such as TBC and MTBC also react with fluorescamine, and are converted to carboxylic compounds [26]. The fluorescamine

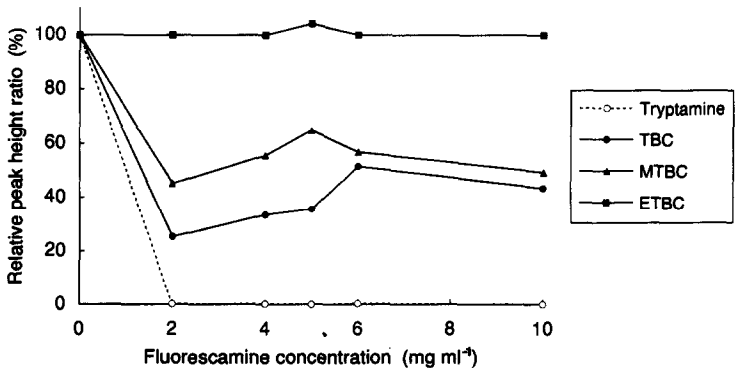

Figure 3
Effect of fluorescamine concentration on the peak heights of tryptamine, TBC, MTBC and ETBC. Tryptamine (5.0 μg ml^{-1}) and tetrahydro-β-carbolines (10.0 ng ml^{-1} of each) were treated with fluorescamine, extracted and chromatographed.

treatment completely eliminated the tryptamine peak, whereas it provided reduced, but significant, peaks of TBC and MTBC, and failed to influence ETBC. These results are ascribed to the reaction rate which differs between primary and secondary amines, and to the reactivity of ETBC which is lost due to substitution with an ethyl group at the 2-position [15].

Additional treatment with amino acids

Different amino acids (12.5 mg ml^{-1} of each) were added immediately after the reaction of fluorescamine with tryptamine to consume the remaining fluorescamine (Fig. 1). The peak heights of TBC and MTBC incraesed uniformly as shown in Fig. 4. Short-chain, acidic and hydroxylic amino acids were chosen because of the ease with which the fluorescamine derivatives of such amino acids were removed by extraction. Glycine and threonine showed relatively good results for increasing the decreased peak heights.

TBC and MTBC restored the reduced detection response with an increase of the glycine concentration. Their peak heights became stable over 50 mg ml^{-1}, as shown in Fig. 5.

Due to these results, the fluorescamine treatment was performed as a two-step process. The first step involved the reaction of fluorescamine with tryptamine to remove the precursor of TBC and MTBC, whilst the second involved consumption of the remaining fluorescamine by glycine reaction. Thereafter, the procedure involved serial extractions under alkaline conditions, back-extraction to the acidic aqueous phase, and re-extraction.

HPLC separation

TBC, MTBC and ETBC were separated by reversed-phase ion-pair chromatography using a C_8-column and trifluoroacetic acid as a

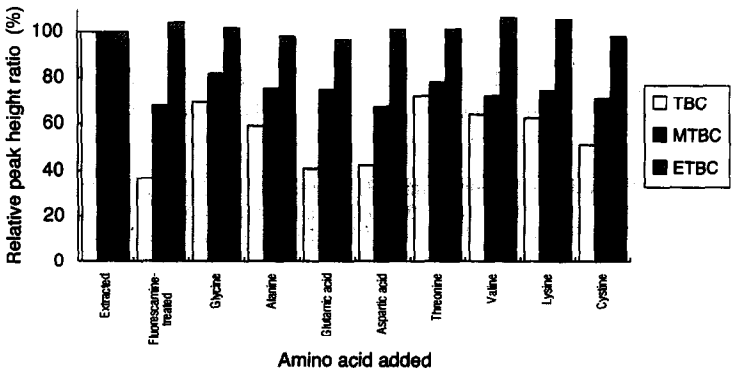

Figure 4
Effect of amino acids on the peak heights of TBC, MTBC and ETBC. After the fluorescamine treatment of tetrahydro-β-carbolines (10.0 ng ml^{-1} of each), their solutions were reacted with different amino acids (12.5 mg ml^{-1} of each), extracted and chromatographed.

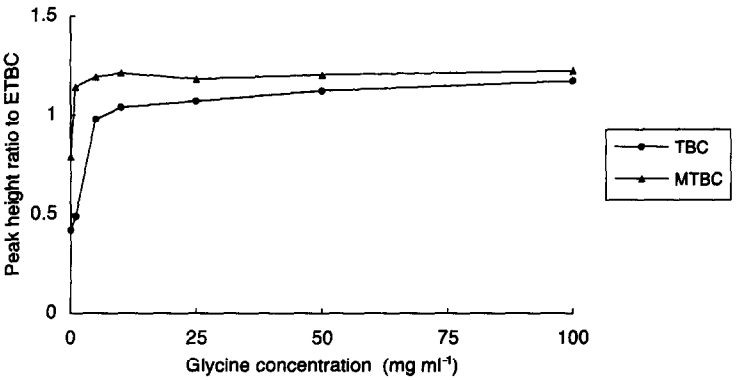

Figure 5
Effect of glycine concentration on the peak heights of TBC and MTBC. After the fluorescamine treatment of tetrahydro-β-carbolines (10.0 ng ml^{-1} of each), their solutions were reacted with glycine, extracted and chromatographed.

counter ion. The trifluoroacetic acid concentration, the acetonitrile concentration and the column temperature were important factors for the efficient separation of the three analytes. Compromise conditions between separation time and resolution were optimized as described.

Representative chromatographic results are shown in Fig. 6. No major interference peak was observed on any chromatograms obtained from urine samples. A base-line separation of TBC, MTBC and ETBC was completed within 10 min.

Analysis of urine

Urine samples spiked with standard TBC and MTBC of different concentrations were analysed, and their peak height ratios to ETBC were plotted against concentration. The relationship was linear within the concentration range of 0.1–50.0 ng ml^{-1} for both TBC and MTBC. The regression equation and correlation coefficient were found to be $Y = 0.499X + 1.150$ and $r = 0.9992$ for TBC, and $Y = 0.538X + 2.383$ and $r = 0.9993$ for MTBC.

The recovery throughout all procedures, and the analytical reproducibility, were evaluated

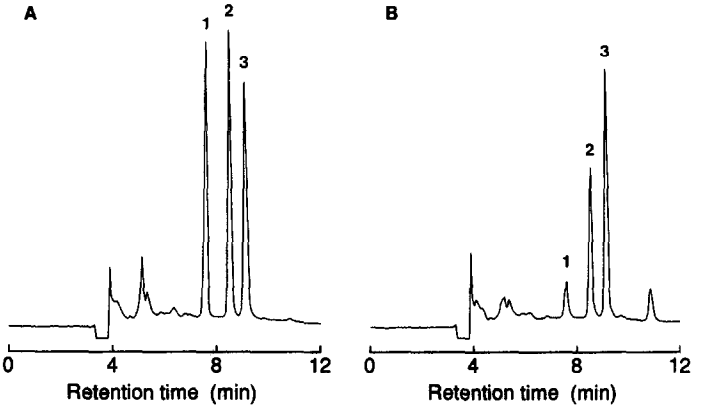

Figure 6
Chromatograms of a standard solution (A) and urine (B). Peaks: 1 = TBC, 2 = MTBC and 3 = ETBC (internal standard). Standard (10.0 ng ml^{-1} of TBC and MTBC) and urine samples were pretreated, extracted and chromatographed. Column: C_8 (250 mm × 4.6 mm i.d.). Mobile phase: acetonitrile–trifluoroacetic acid–water (25.0:0.2:74.8, v/v/v). Flow rate: 1.0 ml min^{-1}. Column temperature: 35°C. Fluorescence detection: 275/350 nm.

Table 1
Concentrations of urinary TBC and MTBC determined by the proposed method

Subject		Concentration			Ratio to creatinine (ng mg^{-1})		Excretion (μg day^{-1})	
Sex	Age	TBC (ng ml^{-1})	MTBC (ng ml^{-1})	Creatinine (mg ml^{-1})	TBC	MTBC	TBC	MTBC
M	21	0.36	2.45	1.70	0.21	1.44	0.40	2.72
M	21	0.28	2.40	1.70	0.16	1.41	0.28	2.38
M	22	0.22	1.11	0.96	0.23	1.16	0.80	6.28
M	22	0.17	1.67	1.21	0.14	1.38	0.27	2.62
M	25	0.26	2.42	1.05	0.25	2.30	0.30	2.81
M	25	0.20	2.84	1.47	0.14	1.93	0.20	2.86
F	22	0.33	0.80	0.63	0.52	1.27	1.21	4.76
F	22	0.58	1.10	0.82	0.71	1.34	0.20	2.75
F	22	0.57	7.29	1.93	0.30	3.78	0.23	2.88
F	22	0.89	5.61	1.49	0.60	3.77	0.79	4.99
Mean (SD)		0.39 (0.22)	2.77 (1.99)	1.30 (0.41)	0.33 (0.20)	1.98 (0.91)	0.47 (0.11)	3.51 (1.27)
After drinking alcoholic beverages:								
M	28	1.73	7.69	1.22	1.42	6.30		
M	32	2.07	22.29	1.53	1.35	14.57		
M	40	2.16	9.31	0.79	2.73	11.78		
Difference from drinkers:					$P < 0.005$	$P < 0.005$		

by pretreating, extracting and chromatographing replicate spiked urine samples at concentrations of 5.0 ng ml^{-1} for TBC and 10.0 ng ml^{-1} for MTBC. The mean recovery and relative standard deviation (n = 8) were 99.6 and 2.7% for TBC, and 102.3 and 1.7% for MTBC.

Table 1 shows the results obtained from application of the proposed method to urine analysis. The quantitative values agreed with those of previous reports which paid sufficient attention to the artefact formation [10, 14, 15]. The contents of TBC, MTBC and the related compounds in the body are influenced by drinking alcoholic beverages, possibly resulting in an elevation of their urinary excretion [7, 28, 29]. Urine samples were collected from three volunteers after a drinking party, in which they drank mainly beer. The ratios with respect to creatinine were greater for both TBC and MTBC (with a statistically significant difference) than for the subjects who had not been drinking. Alcoholic drinking increases the aldehyde contents in the body as the result of detoxication. Such an increase may enhance the potential of TBC and MTBC formation *in vivo* [28, 29]. On the other hand, certain non-distilled alcoholic beverages such as beer and wine contain relatively large amounts of TBC and MTBC as constituents [27, 30]. It has not been conclusively shown whether the increased urinary excretion of TBC and MTBC after drinking alcoholic beverages reflects enhancement of the biosynthesis or of the exogenous supply.

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