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Gas chromatographic–mass spectrometric screening procedure for the identification of formaldehyde-derived tetrahydro-β-carbolines in human urine☆

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ABSTRACT

A gas chromatographic–mass spectrometric method for the identification of 1,2,3,4-tetrahydro-β-carboline and four metabolites extracted from urine is described. In a first step the substances, formed by reaction of formaldehyde with biogenic amines, were derivatized in aqueous solution with methyl chloroformate to eliminate an artificial formation of these compounds via condensation of endogenous indole ethylamines with aldehydes or α-keto acids during the work-up procedure. This initial derivatization formed stable hydrophobic compounds and improved the extractability for a liquid–liquid extraction. Further clean-up was performed by solid-phase extraction on C_{18} sample preparation columns. The method can identify these compounds in the picogram range.

INTRODUCTION

Pictet–Spengler reaction of indole ethylamines with aldehydes produce tricyclic indole derivatives known as tetrahydro-β-carbolines (THBC; Fig. 1). The formation occurs readily under physiological conditions [1] and produces substances that can function as neurotransmitters and/or neuromodulators. They evoke neuropharmacological and psychopharmacological actions in humans and animals, including changes in brain 5-hydroxytryptamine concentrations [2–5], inhibition of monoamine oxidase (MAO) [6,7], especially A-type [8,9], and inhibition of membrane translocating mechanisms [10,11]. THBC compounds also possess potent psychometric effects [12], as well as tremorogenic and convulsive actions [13].

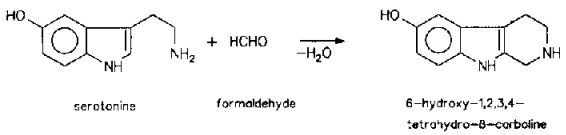

Fig. 1. Pictet–Spengler reaction.

During the past two decades interest has focused on the hypothesis that endogenously formed THBC compounds could contribute to psychotic effects observed in alcoholism, especially in discrimitative stimulus properties, or in schizophrenia [14–25]. Acute and chronic administration of selected THBC compounds to rats has been reported to alter alcohol consumption significantly [26–28]. Alcoholism usually is understood as ethanolism, and normally the ethanol oxidation product acetaldehyde has been re-

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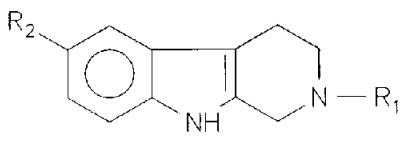
garded as the reaction partner to form THBC compounds. Recent investigations showed that alcoholic beverages contain not only ethanol but also congener alcohols, especially methanol. This alcohol is used as an alcoholism marker and as a decisive factor in the research on the aetiology of chronic alcoholism [29–34]. *In vitro* studies showed that its oxidation product, formaldehyde, is a more potent reaction partner for THBC formation than acetaldehyde [35,36]. There are some known metabolic pathways of formaldehyde synthesis, which may account for THBC formation in tissues [37–41], but up to now nothing is known about how THBC formation in humans depends on the oxidation product of ingested methanol. In our opinion, chronic consumption of alcoholic beverages coupled with methanol accumulation may cause an induction of THBC-forming mechanisms. So formaldehyde-derived THBC compounds could be involved in the aetiology of alcoholism and therefore could be considered as biochemical alcoholism markers. The aim of the present paper is to present a sensitive screening procedure for measurement of formaldehyde-derived THBC compounds in the urine of healthy volunteers and chronic alcoholics.

A variety of analytical methods has been developed to identify THBC compounds as *in vivo* products [42–55]. Examination of these reports revealed a range of analytical methods with different sensitivities or specifities, and exposed analytical pitfalls produced by the work-up procedure [56,57]. So we developed an analytical method, which allows the determination of the following formaldehyde-derived THBC compounds: N-methyl-1,2,3,4-tetrahydro-β-carboline (N-Me-THBC), 1,2,3,4-tetrahydro-β-carboline (THBC), 6-methoxy-1,2,3,4-tetrahydro-β-carboline (6-MeO-THBC), 6-methyl-1,2,3,4-tetrahydro-β-carboline (6-Me-THBC) and 6-hydroxy-1,2,3,4-tetrahydro-β-carboline (6-OH-THBC) (Fig. 2). Glucuronidase is used to hydrolyse the samples, because THBC compounds in urine may be excreted as glucuronides [58–61]. The procedure includes an initial chemical derivatization in aqueous solution to prevent artificial formation of



	R_1	R_2
N–methyl–1,2,3,4–tetrahydro–β–carboline	$-CH_3$	–H
1,2,3,4–tetrahydro–β–carboline	–H	–H
6–methyl–1,2,3,4–tetrahydro–β–carboline	–H	$-CH_3$
6–methoxy–1,2,3,4 tetrahydro–β–carboline	–H	$-OCH_3$
6–hydroxy 1,2,3,4–tetrahydro–β–carboline	–H	–OH

Fig. 2. Structures of the tetrahydro-β-carbolines identified with the procedure described.

THBC compounds during the work-up procedure. The use of this kind of derivatization facilitates the isolation by liquid liquid extraction. In a second step, solid-phase extraction is used to eliminate most of the interfering matrix compounds. For separation and identification a gas chromatographic–mass spectrometric (GC–MS) method was developed.

EXPERIMENTAL

Materials

Methanol, ethyl acetate (Uvasol, Merck, Darmstadt, Germany), water (HPLC grade, Baker, Gross-Gerau, Germany), THBC , 5-hydroxytryptamine creatinine sulphate, β-glucuronidase type H-1 (all from Sigma, Deisenhofen, Germany), methyl chloroformate, 5-methoxytryptamine hydrochloride, 5-methyltryptamine hydrochloride and N-methyltryptamine (Aldrich, Steinheim, Germany) were used. Inorganic chemicals used were boric acid, sodium tetraborate, potassium dihydrogenphosphate, potassium hydroxide, orthophosphoric acid, sodium hydroxide, hydrochloric acid, acetic acid, glyox-

ylic acid and semicarbazide (p.a., Merck). Chem-Elut extraction columns (20 ml volume, diatomaceous earth as sorbent) were purchased from Analytichem International (ICT-Handelsgesellschaft, Frankfurt a.M., Germany). Worldwide Monitoring Clean Up C_{18} end-capped extraction columns (100 mg, 1 ml) were purchased from Amchro (Sulzbach/Taunus, Germany).

Samples of 6-OH-THBC, 6-MeO-THBC and 6-Me-THBC were prepared by the reaction of 5-hydroxytryptamine, 5-methoxytryptamine and 5-methyltryptamine, respectively, with glyoxylic acid, according to the method of Ho and Walker [62]. A slight modification of this procedure yielded N-Me-THBC from N-methyltryptamine according to the procedure of Elliott [63].

Borate buffer (pH 9.0) consisted of 835 ml of solution A [12.37 g of boric acid + 100 ml of 1 *M* sodium hydroxide with sodium tetraborate (0.05 *M*) made up to 1 l] and 165 ml solution B (0.1 *M* hydrochloric acid).

Instrumentation

A Model 5890A gas chromatograph (Hewlett-Packard) with a 5970A mass-selective detector (MSD) was used for analysis. Data acquisition and manipulation were performed using standard software supplied by the manufacturer. Perfluorotributylamine was used for a daily automatic tune. For sample analysis the electron multiplier voltage of the detector was set in the range 200–400 V above the autotune voltage. A fused-silica capillary column OV1 (12 m × 0.2 mm I.D., film thickness 0.33 μm) was used. The temperature was programmed from an initial value of 100°C, held for 2 min, followed by a linear increase to 300°C at 40°C/min. The final temperature was held for 5 min. The split–splitless injector was maintained at 260°C.

Sample preparation

The sample preparation procedure is outlined in Fig. 3. The sample consisted of 10 ml of urine adjusted to pH 4.5 with acetic acid, and to which were added 0.1 ml each of 1 *M* semicarbazide and β-glucuronidase. For enzymic hydrolysis the sample was incubated at 55°C for 2 h. Then 7.2 ml of dipotassium hydrogenphosphate were added to adjust the pH to 7.2, followed by 0.1 ml of methyl chloroformate. The sample was vortex-mixed and allowed to stand for 10 min. The pH was increased to 9.5 by addition of 2.5 ml of saturated sodium carbonate. Then 0.1 ml of methyl chloroformate was again added, and the sample was allowed to stand for 15 min.

For extraction, the whole sample volume was applied to a Chem-Elut column and eluted twice with 20 ml of 1-chlorobutane. The eluate was

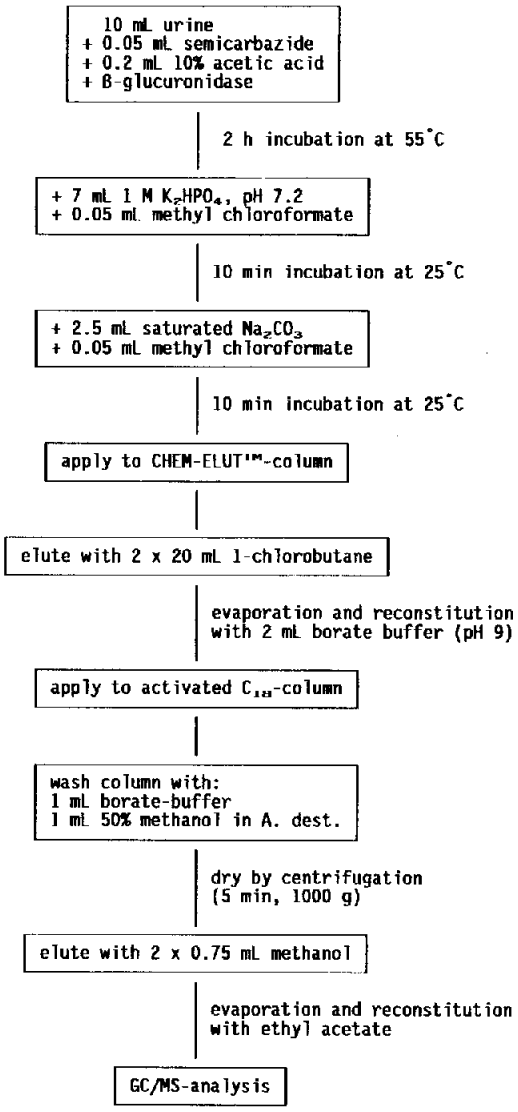


Fig. 3. Sample preparation procedure.

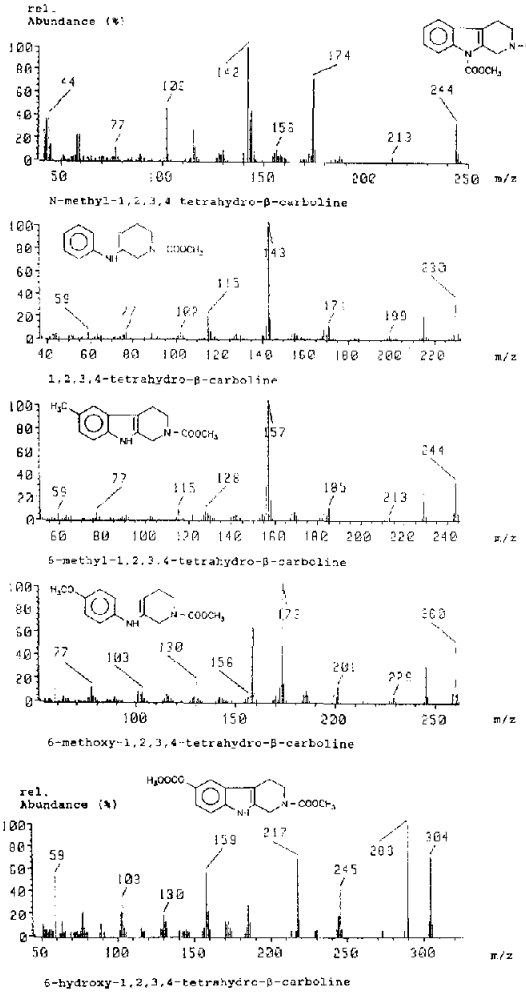


Fig. 4. Mass spectra of derivatized tetrahydro-β-carbolines identified with this procedure.

evaporated to dryness under vacuum, and the residue was prepared for solid-phase extraction by reconstitution in 2 ml of borate buffer (pH 9).

Before application, the C_{18} extraction columns were conditioned by washing with 2 ml of methanol, followed by 2 ml of water and 1 ml of borate buffer. Prepared samples were applied to the columns under vacuum at a flow-rate of *ca.* 1 ml/min. The columns were washed with 1 ml of water, followed by 1 ml of 50% methanol in water, and dried by centrifugation (5 min, 1000 *g*). The THBC compounds were eluted with two 0.75-ml volumes of methanol and collected in a vial. The eluate was evaporated to dryness at 50°C under a stream of nitrogen. The residue was dissolved in 20 μl of methanol, and a 2-μl aliquot was subjected to GC–MS analysis.

RESULTS AND DISCUSSION

Electron impact (EI) mass spectra of the compounds were recorded by total ion monitoring. The mass spectra are shown in Fig. 4. Only monocarbomethoxy derivatives of THBC, 6-Me-THBC and 6-MeO-THBC, formed by reaction with the aliphatic (ring C) nitrogen, could be detected. Under the reaction conditions described no bis derivatives (rings B and C) seem to be produced. However, N-Me-THBC was derivatized at the aromatic indole nitrogen, owing to its increased basicity and hence its increased reactivity.

Each compound was characterized with respect to its base peak (normalized to 100%) and other prominent secondary mass fragments. The retention times were recorded and the chosen diagnostic mass fragments were monitored for each compound in the selected-ion monitoring (SIM)

TABLE I

CHARACTERISTIC MASS FRAGMENTS, RETENTION TIMES AND RECOVERIES DETERMINED WITH EXTERNAL STANDARD

Substance	*m/z*	Retention time (min)	Recovery[a] (mean ± S.D., n = 5) (%)
N-Me-THBC	102, 142, 174, 244	9.60	79.9 ± 8.3
THBC	115, 143, 230	9.75	85.4 ± 7.5
6-Me-THBC	157, 229, 244	10.56	83.2 ± 7.3
6-MeO-THBC	158, 173, 260	11.74	80.3 ± 5.9
6-OH-THBC	158, 217, 289, 304	13.77	84.7 ± 6.4

mode in several acquisition groups (Table I). The mass fragments *m/z* 102, 142, 174 and 244 were chosen to monitor for the presence of N-Me-THBC, *m/z* 115, 143 and 230 were used for THBC, *m/z* 157, 229 and 244 were used for monitoring of 6-Me-THBC, *m/z* 158,173 and 260 for 6-MeO-THBC, and the mass fragments selected for 6-OH-THBC were *m/z* 158, 217, 289 and 304. Reference standards were examined in the SIM mode, with retention times and ion ratios being recorded, prior to and following injection of the samples obtained from urine extracts.

Fig. 5 shows a gas chromatogram of a reference standard with 100 pg per substance. Experiments with spiked urine samples (100 pg/ml) showed that the peaks observed had the same retention times, mass fragments and ion mass ratios as those observed for the external standards. The biological matrix effected no shift in the three identification marks. Recoveries determined with an external standard ranged from 79.9 ± 8.3 to 85.4 ± 7.5% (n = 5). Using the routine method described above, the minimum detectable concentrations of the THBC compounds were 50–100 pg/ml of urine.

In agreement with Bosin and Jarvis [50] this method is based on the known chemical reactivity of alkyl chloroformates, especially methyl chloroformate, with amines and phenols in aqueous solution to produce carbamate and carbonate derivatives, respectively. By derivatizing the aqueous sample initially, the potential for the artificial formation of THBC compounds is markedly decreased or eliminated, since endogenous indole ethylamines are no longer free and

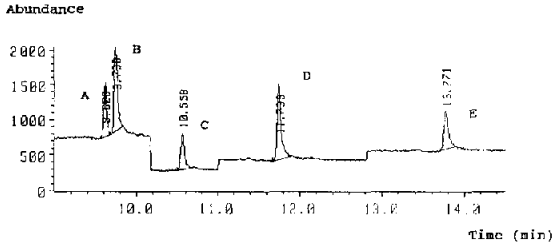


Fig. 5. Gas chromatogram obtained with 100 pg of each substance. Peaks: A = N-Me-THBC; B = THBC; C = 6-Me-THBC; D = 6-MeO-THBC; E = 6-OH-THBC.

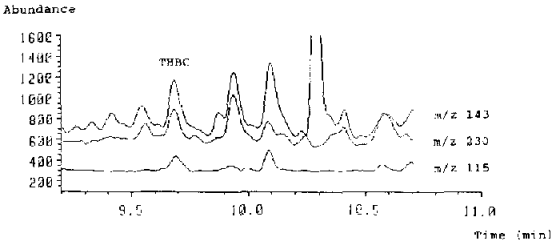


Fig. 6. Chromatogram obtained from a urine sample from a chronic alcoholic.

available to react with an aldehyde or α-keto acid. Additionally, semicarbazide was added to the sample to remove any formaldehyde present or arising during the work-up procedure. The derivatization products were stable at least for one week and delivered hydrophobic compounds, which were well recovered by liquid–liquid extraction. The following solid-phase extraction, including a washing step with 50% methanol in water, produced highly purified extracts. These derivatives possess good GC properties. Therefore the retention times, together with the diagnostic mass fragments (at least three) and the specific ion ratios, can be used to identify THBC compounds in human urine (Fig.6). The procedure described can be considered as an effective screening method and should facilitate the study of *in vivo* formation of THBC compounds.

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