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Endogenous alkaloids in man XXVI.[1] Determination of the dopaminergic neurotoxin 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo) in biological samples using gas chromatography with selected ion monitoring

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Abstract

Highly chlorinated β-carbolines have a potential in vivo relevance to Parkinson's disease. In this paper, a gas chromatographic method for the determination of the neurotoxic 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo), the condensation product of tryptamine and chloral hydrate, is described. The specific and sensitive assay involves purification of the biological samples by solid-phase extraction with C_{18} cartridges, derivatization with heptafluorobutyric anhydride, and chromatography on a non-polar fused-silica capillary column. Detection of TaClo was achieved by the registration of characteristic mass fragments of the TaClo heptafluorobutyric amide derivative using selected ion monitoring. The method was utilized to detect and quantify TaClo in blood, urine, bile, faeces, and brain tissue of rats treated with this alkaloid-type heterocycle. Four-fold deuterium-labelled TaClo was used as an internal standard.

Keywords: Alkaloids; 1-Trichloromethyl-1,2,3,4-tetrahydro-β-carboline; β-Carbolines; Chloral hydrate

1. Introduction

Despite tremendous research efforts in the past, the etiology of Parkinson's disease (PD) is still not fully understood [1,2]. The hypothesis that environmental factors might play a crucial role in the pathogenesis of PD, was stimulated by the observation of a severe irreversible Parkinson syndrome in a number of addicted individuals after (illegal) uptake

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of a drug contaminated by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [3–5]. This initiated an intensive search for further neurotoxins with which humans can come into contact, either by exogenous uptake (nutritional or environmental) or by endogenous formation [6–8].

β-Carbolines have been considered, among other compounds, as potential natural inducers of neurodegenerative processes, due to their occurrence in mammalian organisms [9–16], their neuropharmacological potential [10–12], and their striking structural similarity to MPTP [17–20].

We have recently described a new class of tetrahydro-β-carbolines derived from trichloroacetaldeyde (chloral) [20–27]. In contrast to earlier reports in the literature [28], we could demonstrate that tryptamine (Ta) and chloral hydrate (Clo) readily undergo a Pictet–Spengler-type condensation reaction under quasi-physiological conditions, to give 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo) (see Fig. 1) [21]. Hence, whenever chloral occurs in the organism, i.e. after uptake of the hypnotic drug chloral hydrate [29] or after exposure to the frequently used industrial solvent trichloroethylene (TCE) [30], a spontaneous in vivo formation of highly chlorinated β-carbolines is possible. These alkaloid-type heterocyles might originate from endogenously present biogenic amines (e.g. tryptamine or serotonin) or from natural or therapeutically administered amino acids (e.g. L-tryptophan or 5-hydroxy-L-tryptophan) (Fig. 1).

This hypothesis was supported by GC–MS investigations confirming an endogenous formation of TaClo in rats (found in blood and brain samples) after continuous administration (21 days) of its precursors tryptamine hydrochloride (5 mg/kg i.p.) and chloral hydrate (10 mg/kg i.p.) [24,27]. Thus, TaClo has to be considered as a potential mammalian alkaloid, i.e. a heterocyclic compound that may be formed endogenously under particular conditions. Recently, TaClo has also been detected in whole blood samples obtained from patients treated with chloral hydrate [24,31].

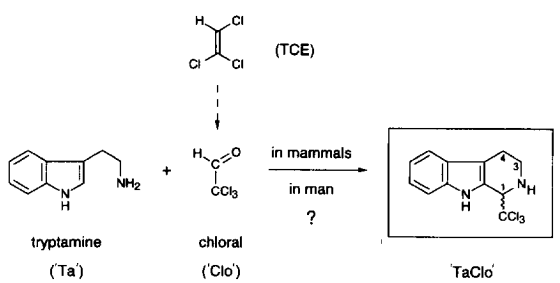

Fig. 1. Possible in vivo formation of 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo) from endogenously occurring tryptamine (Ta) and the non-natural aldehyde chloral (Clo) after application as the hypnotic drug chloral hydrate or after its formation by metabolic degradation of the solvent trichloroethylene (TCE).

This is of special interest due to the fact that TaClo was found to be a potent neurotoxic agent that induces a slow degeneration of dopamine neurons [24,27]. A daily subchronic treatment of rats with small doses of TaClo (0.2 mg/kg i.p.) for seven weeks resulted in significant behavioural changes observable up to nine months after the end of the injection period [32]. Biochemical investigations on the extracellular striatal dopamine metabolism in rats showed that the dopaminergic system was severely affected by TaClo [33]. Pathological changes induced by TaClo (e.g. disintegration of dendrites, swelling of axons) were observed in both neuronal and glial cell cultures [34]. Most recently, TaClo was found to inhibit highly selectively complex I of the mitochondrial respiratory chain in rat brain homogenates, the required concentrations being 10 times lower than those needed for 1-methyl-4-phenylpyridinium ion (MPP^+), the toxic metabolite of MPTP [25,35].

Consequently, the investigation of the formation and metabolism of TaClo in the living organism is of great importance for the question whether (and by what mechanism) this highly lipophilic tetrahydro-β-carboline might be involved in the pathogenesis of PD. For this reason, we have developed a reliable analytical assay for the determination of TaClo in body fluids, organs, and tissues.

A key step of this analytical procedure is the sample preparation technique. Solid-phase extraction (SPE) on C_{18} adsorbent material was applied to guarantee an effective elimination of most of the interfering matrix components. After conversion of TaClo to a volatile perfluoroacyl compound, using heptafluorobutyric anhydride (HFBA) as a derivatizing reagent, the trichloromethyl heterocycle can be identified using gas chromatography in combination

with mass selective detection (GC–MSD). Furthermore, we report on the synthesis and use of the four-fold deuterium-labelled [D_4]TaClo as an internal standard for a sensitive and reproducible quantification of this trichloromethyl heterocycle in urine, blood, and brain tissue. This GC–MS assay was successfully applied to gain preliminary results from animal studies of the in vivo fate of TaClo.

2. Experimental

2.1. Chemicals

For chromatography and sample preparation, analytical grade solvents and reagents were used. Water was purified by the Milli-Q system (Millipore, Bedford, MA, USA).

[α,α,β,β-$^2H_4$]Tryptamine was purchased from MSD Isotopes (Montreal, Canada). Heptafluorobutyric anhydride (HFBA) was obtained from Macherey and Nagel (Düren, Germany). *Helix pomatia* juice with enzymatic activities of β-glucuronidase (10^5 Fishman U/ml) and arylsulphatase ($8 \cdot 10^5$ Roy U/ml) was purchased from Boehringer Mannheim (Mannheim, Germany).

2.2. Chemical synthesis

1-Trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo) was synthesized according to a procedure described in a previous paper [21]. Its deuterium-labelled analog [3,3,4,4-$^2H_4$]-1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline ([D_4]TaClo) was prepared following a similar procedure. [$^2H_4$]Tryptamine and chloral were suspended in toluene and refluxed for 20 min; after removal of the solvent, the residue was purified by column chromatography on silica gel (eluent: chloroform–hexane = 3:1). The pure compound [D_4]TaClo was crystallized as its hydrochloride salt from methanol–ether. The ^1H NMR spectrum (250 MHz; [$^2H_4$]methanol) indicated the following signals: δ [ppm] = 6.24 (1H, s, 1-H), 7.36 (1H, m_c, 6-H), 7.43 (1 H, m_c, 7-H), 7.67-7.72 (2H, m, 5- and 8-H), identical to those of the non-labelled compound, except for the missing signals corresponding to the protons at C-3 and C-4. The mass spectrum (EI, 70 eV) showed the following mass charge (m/z) ratios and relative intensities of the typical ion fragments: m/z (%) = 298/296/294/292 (0.11/0.87/1.98/2.05) $[M]^{+\cdot}$, 261/259/257 (0.14/0.39/0.90) [M−Cl], 260/258/256 (0.38/0.46/0.58) [M−HCl], 224/222 (1.32/3.69) [M−2Cl], 187 (2.92) [M−3Cl], 175 (100) [M−CCl_3]. Direct insertion probe electron-impact high-resolution mass spectrometry was done on a MAT 90 (Finnigan MAT, San José, CA, USA) mass spectrometer. Exact mass measurement of an ion fragment $C_{12}H_7D_4^{35}Cl_3N_2$ recorded at 292.0233 amu confirmed the elemental composition of $C_{12}H_7D_4Cl_3N_2$ (calculated. 292.0239 amu). Derivatization with heptafluorobutyric anhydride (HFBA) led to the HFB derivative (see Fig. 2), from which the isotopic composition of [D_4]TaClo was calculated from the electron impact (EI) mass spectrum according to a method introduced by Biemann [36]. The degree of labelling was determined as follows: $^2H_0 = 0.3\%$, $^2H_1 = 0.2\%$, $^2H_2 = 0.2\%$, $^2H_3 = 4.1\%$,

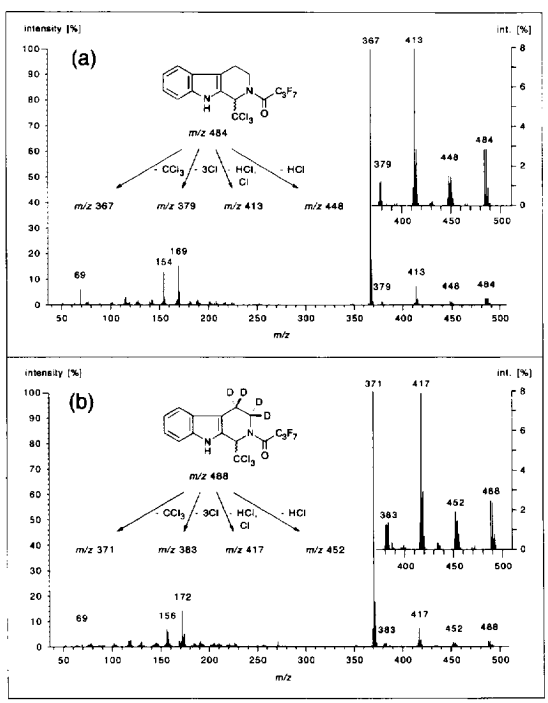

Fig. 2. EI ionization (70 eV) mass spectra of the HFB derivatives of (a) 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo) and (b) its deuterium-labelled analog [D_4]TaClo used in this study as the internal standard. The inset structures show the origin of the characteristic fragment ions.

$^2H_4$=94.3%, $^2H_5$=0.9%. Calculation was done from the relative intensities of the mass peaks *m/z* 367–372 [M−CCl_3] measured for the HFB derivatives of TaClo and [D_4]TaClo, assuming that the relative peak heights of these ions are analogous for each species.

2.3. Instrumentation

Gas chromatographic–mass spectrometric (GC–MS) analyses were performed using EI ionization on a Hewlett-Packard (HP) 5890 Series II gas chromatograph coupled with a HP 5971A single quadrupole (Hewlett-Packard, Palo Alto, CA, USA). Operations of the GC–MSD system as well as data acquisition and processing were controlled by a HP Vectra personal computer in combination with the HP Chem Station software (HP G 1030 A). Chromatography was carried out on a HP-1 fused-silica capillary column (12 m×0.2 mm I.D.; 0.33 μm film thickness). A 'retention gap' consisting of uncoated fused-silica (1 m×0.53 mm I.D.) was connected between the on-column injection port and the analytical column. Helium was used as a carrier gas with a column head pressure of 90 kPa, giving a linear velocity of 50 cm/s. The GC oven temperature was maintained at 130°C for 2 min, then programmed to increase by 20°C/min up to 200°C, held at this temperature for 10 min, then warmed up to 250°C at a rate of 20°C/min.

EI ionization with 70 eV energy was used. The temperature of the transfer line was 280°C and the ion source was maintained at 180°C. The data were obtained in full scan mode from 50 to 500 atomic mass units (amu), or in selected ion monitoring (SIM) mode. SIM was done with a dwell time of 100 ms per ion and 3.6 cycles/s. In SIM analyses ions at *m/z* 484, 448, 413, 367 and *m/z* 488, 452, 417, 371 were monitored for TaClo and its deuterium-labelled analog [D_4]TaClo, respectively.

2.4. Animal studies

2.4.1. Collection of blood, urine, and faeces

A female Wistar rat (body weight, 223 g) was injected intraperitoneally with a solution of TaClo that had been prepared by dissolving 9.0 mg of TaClo hydrochloride in a mixture of 200 μl of DMSO and 100 μl of saline (0.9% NaCl in water). A solution of 8.9 mg TaClo in a mixture of 100 μl of corn oil, 300 μl of ethanol, and 200 μl of DMSO was administered by gavage to a male Wistar rat weighing 216 g. The doses of TaClo applied to the animals in both of the experiments were calculated as 40 mg/kg and 41 mg/kg, respectively. Blood samples were collected by heart puncture under ether anaesthesia, 120 min and 240 min after TaClo administration. Urine samples were taken in fractions for the periods of 0–12 h, 12–24 h, and 24–48 h after the administration. Faeces was collected over the whole duration of the experiment (48 h). All samples were immediately frozen in dry ice.

2.4.2. Collection of bile

A female Wistar rat (body weight: 235 g) was injected intraperitoneally with diazepam (1 mg/kg body weight). A solution of 2 mg (i.e. 8.5 mg/kg) of TaClo, dissolved in a mixture of 100 μl of methanol and 500 μl of water, was administered intravenously (via the tail vein) to the rat. Cannulation of the bile duct was done 15 min after anaesthesia (application of 1 mg/kg of Hypnorm®), by introduction of a polyethylene tube. Bile samples were then collected in 2-h fractions from time 0 to 8 h after TaClo administration, and cooled immediately.

2.4.3. Recovery of TaClo in organs, tissues, and brain

Male Wistar rats (n=4; 11–12 months old) weighing 280–300 g were injected intraperitoneally with TaClo hydrochloride (dissolved in 0.9% saline). Daily doses of 4 mg/kg body weight were administered to the rats over a period of six days. The animals were sacrificed by decapitation 24 h after the last injection. Brains, livers, kidneys as well as the hearts and muscle tissues were removed and immediately frozen in dry ice. The brains were separated according to the regions: cortex frontalis, cortex parietalis, cortex occipitalis, telencephalon, diencephalon, mesencephalon, metencephalon, medulla, and cerebellum.

All the samples were stored at −20°C until analyzed.

2.5. Clean-up procedure

The pretreatment of biological samples was carried out in the following manner:

2.5.1. Blood, urine

Using screw-cap vials 500 μl of whole blood or 1 ml of urine were each diluted with 1 ml of Milli-Q water. For precipitation of proteins, 500 μl of acetonitrile was added and the sample solution was briefly vortex-mixed (Bender and Hobein AG, Zürich, Switzerland) and centrifuged (10 min, 15 000 g_{max}) in a Sorvall RC-5B refrigerated superspeed centrifuge (Du Pont Instruments, Wilmington, DE, USA). The residue was again washed with 500 μl of acetonitrile, vortex-mixed and centrifuged. Both supernatant fractions were transferred to a second vial and diluted with 5 ml of a sodium phosphate buffer (0.5 *M*, pH=6). The sample solution was then adjusted to pH 7.5–8.5 with 1 *M* NaOH. For urine samples, a single precipitation step with acetonitrile for removing the proteins was sufficient.

2.5.2. Bile

Samples of bile (ca. 1 ml collected in 2-h fractions, see Section 2.4.2 above) obtained from TaClo-treated rats were deproteinized with 50 μl of perchloric acid. After centrifugation at 12 000 g_{max} for 10 min, an aliquot of 350 μl of the supernatant was directly dissolved in 5 ml of sodium phosphate buffer (0.5 *M*, pH 6), and adjusted to pH 7.5–8.5 with 80 μl of 1 *M* NaOH. A second aliquot of 350 μl was used for enzymatic hydrolysis of possibly present conjugates (see below). Further workup of these samples was done by addition of 400 μl of acetonitrile for deproteinization. After centrifugation the residue was washed twice with 2 ml of a sodium carbonate buffer (10 m*M*, pH 9). All supernatant fractions were collected and the sample pH was adjusted to 7.5–8.5 with 1 *M* NaOH.

2.5.3. Faeces

After addition of 15 ml of methanol to faeces (ca. 6 g) obtained from TaClo-treated rats, the samples were homogenized using an ultraturrax (Janke and Kunkel, Staufen, Germany), and subsequently centrifuged (20 min, 15 000 g_{max}). The yellow supernatant fractions were pipetted into new vials and evaporated to dryness. The residues were then dissolved in 7 ml of 0.1 *M* sodium acetate buffer (pH 6). Aliquots of 3 ml of these solutions were incubated with *Helix pomatia* for enzymatic hydrolysis (see below). The remaining volumes (4 ml per sample) were directly purified according to the procedure described above for SPE pretreatment of blood and urine samples.

2.5.4. Brain

Brain tissue (ca. 300 mg) was weighed and immediately added to a 5-ml volume of methanol. The sample tubes were placed in an ice bath and the cooled brain tissue was homogenized using an ultraturrax. After sonication assisted extraction for 15 min, the samples were centrifuged at 5°C (15 min, 15 000 g_{max}). The supernatant fraction was poured into a new tube and then evaporated to dryness in a Speed Vac vacuum concentrator (Savant, Vanves, France). After addition of 400 μl of a mixture of methanol–water (1:1) to the sample residue, 4 ml of water was added and the sample pH was adjusted to 7.5–8.5 with 50 μl of 1 *M* NaOH.

2.5.5. Liver

Frozen liver tissue (ca. 2–3 g) was homogenized at 4°C for 10 min with 15 ml of methanol using an ultraturrax. The homogenate was then centrifuged at 12 000 g_{max} for 20 min at 4°C. The clear supernatant was carefully pipetted into a round bottom flask and concentrated to a volume of about 1 ml. After addition of 50 μl of 1 *M* NaOH, the alkalinized sample solution was extracted three times with 400 μl of methyl-*tert.*-butyl ether (MTB) assisted by sonication. The combined extracts were evaporated to dryness by a stream of nitrogen. The residue was dissolved in 500 μl of methanol–water (1:1) and centrifuged at 4°C (15 min, 15 000 g_{max}). Subsequently, the supernatant was diluted with 4 ml of Milli-Q water, and the sample pH was adjusted to 8–9 with 50 μl of 1 *M* NaOH.

2.5.6. Enzymatic hydrolysis of conjugates

Urine, blood, faeces, and bile samples from TaClo-treated rats were adjusted to pH 5.2 with glacial acetic acid, mixed with 3 ml of 0.1 *M* sodium acetate buffer (pH 6), and then added with *Helix*

pomatia juice (10 μl per 1 ml of sample). These mixtures were incubated at 37°C for 24 h, followed by precipitation of proteins, centrifugation, and SPE.

2.5.7. Solid-phase extraction (SPE)

Further workup of blood, urine, bile, faeces, brain, and liver samples by SPE was accomplished with C_{18} sorbent Bakerbond SPE-cartridges (100 mg of sorbent) using a Baker SPE 12-G-system vacuum chamber (J.T. Baker, Phillipsburg, PA, USA). Cartridge pretreatment was carried out by flushing with 2 ml of methanol, 2 ml of Milli-Q water and 1 ml of 10 m*M* sodium carbonate buffer (pH 9). The conditioned C_{18} cartridge was loaded with the diluted sample solutions and, after gentle vacuum aspiration, washed with 1 ml of sodium carbonate buffer (10 m*M*, pH 9). Then 50 μl of methanol–water (4:1) were passed through the column and the sorbent material was dried under vacuum. Subsequently, the analyte TaClo (and $[D_4]$TaClo if added as the internal standard, see Section 2.7) was eluted by passing 2 ml of chloroform–MTB (1:1) through the column. Traces of water in the eluate were removed with small amounts of solid magnesium sulphate. The organic phase was then pipetted into a vial and evaporated to dryness under a stream of nitrogen.

2.6. Derivatization

The dry residues of matrix-free solutions or biological samples (after SPE) were dissolved in 150 μl of 0.1 *M* triethylamine in toluene. For derivatization 5 μl of heptafluorobutyric anhydride (HFBA) was added. The samples were incubated at room temperature for 10 min. After extraction of the solutions with 200 μl of sodium phosphate buffer (0.5 *M*, pH 6), the toluene layer was transferred to a screw-cap vial and dried with 10 mg of anhydrous sodium sulphate. A 1-μl volume of the organic layer was injected into the GC–MSD system.

2.7. Calibration curves and quantification using $[D_4]$TaClo

Standard solutions of TaClo were prepared in methanol at a concentration of 34 μg/ml (solution A) and 335 ng/ml (solution B). As the internal standard (I.S.), methanolic solutions of the deuterium-labelled analog $[D_4]$TaClo were used at concentrations of 33 μg/ml (I.S. solution I) or 330 ng/ml (I.S. solution II). For quality control of the method, calibration curves (see Table 1) were established using bovine whole blood and human urine spiked with TaClo in the range of 8.4–201 ng/ml. Each curve was drawn using six levels of concentration (8.4, 16.8, 33.5, 67, 134, and 201 ng/ml). A volume of 200 μl of the I.S. solution II (66 ng/ml blood or urine) was added to each tube prior to SPE.

Calibration curves (see Table 1) used for the quantification of TaClo in body fluids and tissues after administration of this tetrahydro-β-carboline to rats were constructed in the following manner:

2.7.1. Blood and faeces

The amount of TaClo recovered in rat blood (see Section 3.4) and faeces samples (see Table 2) was determined using a calibration curve for TaClo in the range of 3.4–40.8 μg/ml of blood (3.4, 6.8, 13.6,

Table 1
Linear regression analysis of TaClo in matrix-free solution as well as in spiked blood, urine, and brain samples using $[D_4]$TaClo as internal standard (I.S.)

Matrix	Concentration range of TaClo	$[D_4]$TaClo added as I.S.	Slope[a] *b*	*y*-Intercept[a] *a*	Correlation coefficient *r*
Methanol	1.7–20.4 μg/ml	9.9 μg/ml	0.917±0.043	−0.003±0.001	0.998
Methanol	33.5–201 ng/ml	110 ng/ml	0.48±0.014	−0.019±0.008	0.999
Blood	3.4–40.8 μg/ml	19.8 μg/ml	0.797±0.015	−0.046±0.017	0.997
Blood	8.4–201 ng/ml	66 ng/ml	0.65±0.022	−0.028±0.012	0.996
Urine	1.7–20.4 μg/ml	9.9 μg/ml	6.05±0.006	−0.026±0.011	0.999
Urine	8.4–201 ng/ml	66 ng/ml	1.18±0.058	−0.008±0.0016	0.997
Brain	29–217 ng/g	110 ng/mg	1.01±0.069	−0.002±0.0005	0.998

Linear regression parameters: $m/m_{I.S.} = b \cdot R/R_{I.S.} + a$. R=detector response; m=mass of the analyte.
[a] Mean±S.D. is given.

Table 2
Elimination of authentic TaClo after its peripheral administration to rats

Administration of TaClo		Intraperitoneal	Per os
Dose applied		8.97 mg/rat	8.90 mg/rat
Dose (mg/kg)		40 mg/kg	41 mg/kg
Urine collected	0–48 h	14.7 ml	9.8 ml
Amount of TaClo	0–12 h	10.9 μg (2.0 μg/ml)	1.7 μg (840 ng/ml)
in urine fractions[a]	12–24 h	2.2 μg (630 ng/ml)	2.3 μg (820 ng/ml)
	24–48 h	1.9 μg (330 ng/ml)	5.5 μg (1.1 μg/ml)
Total	0–48 h	15 μg (1.0 μg/ml)	9.5 μg (970 ng/ml)
% of TaClo excreted in urine		0.2%	0.1%
Faeces collected	0–48 h	5.92 g	6.40 g
Amount of TaClo in faeces[a]	0–48 h	42 μg	90 μg
% of TaClo detected in faeces		0.5%	1.0%

[a] Values represent average amount calculated from three independent GC–MSD runs.

27.2, and 40.8 μg/ml). The graph was obtained by spiking a 500-μl volume of bovine blood with various aliquots (50, 100, 200, 400, and 600 μl) of the standard solution A. [D_4]TaClo was added as the I.S. at a concentration of 19.8 μg/ml (300 μl of I.S. solution I).

2.7.2. Urine

For the quantification of the renal elimination of TaClo after its administration (intraperitoneally or by gavage) to rats (see Table 2), a calibration curve was constructed in the range of 1.7–20.4 μg/ml of urine (1.7, 3.4, 6.8, 13.6, and 20.4 μg/ml) by spiking a 1-ml volume of human urine using solution A. [D_4]TaClo was added as the I.S. at a concentration of 9.9 μg/ml (150 μl of I.S. solution I).

2.7.3. Brain

A calibration curve for the determination of the amount of TaClo in different rat brain regions (see Fig. 4) was established in the range of 29–217 ng/g of wet tissue. The graph was obtained by spiking frozen pig brain tissue (292, 299, 318, 297, and 309 mg) with various aliquots (25, 50, 100, 150, and 200 μl) of the standard solution B. A volume of 100 μl (33 ng per sample) of the I.S. solution II was added to each brain sample.

The sample solutions of each calibration point ($n=2$) were derivatized with HFBA after purification by SPE and analyzed by GC–MSD in the SIM mode. In all cases calibration graphs were constructed by plotting the peak-area ratio of TaClo at m/z 367 to [D_4]TaClo at m/z 371 against the corresponding concentration ratio of TaClo/[D_4]TaClo. Standard linear regression analysis was used to determine the slope, intercept, and the strength of correlation for the calibration curves (see Table 1).

3. Results and discussion

3.1. Gas chromatography

Derivatization of TaClo and [D_4]TaClo with HFBA in the presence of triethylamine led to the corresponding heptafluorobutyric amides (see inset structure in Fig. 2). Gas chromatography of these perfluorinated compounds was done on fused-silica capillary columns coated with non-polar stationary phases (dimethyl- or dimethylphenylpolysiloxane; DB-1, DB-5) [37]. Using the split-injection mode, a peak discrimination was observed, probably by dehydrohalogenation or hydrodehalogenation of TaClo

(G. Bringmann, R. God and H. Friedrich, unpublished results), similar to organochloro pesticides of the DDT-type [38,39]. This unfavorable side effect can be reduced using the milder technique of the on-column injection mode.

3.2. Mass spectrometry

EI mass spectra of the heptafluorobutyric amides derived from TaClo and [D_4]TaClo were recorded in full scan mode (see Fig. 2). As expected, the EI mass spectrum of the HFB derivative of [D_4]TaClo in principle shows the same signals as the corresponding non-deuterium-labelled TaClo-HFB derivative, but shifted by four mass units. The EI mass spectra of both of the HFB derivatives demonstrate the presence of one HFB group: molecular ion at 490/488/486/484 (intensity 0.09/0.78/2.46/2.50) and 494/492/490/488 (intensity 0.06/0.75/2.17/2.25). According to the expected pattern for three chlorine atoms in the molecule, based on the natural distribution of the chlorine isotopes ^{35}Cl and ^{37}Cl, the $[M]^{+\cdot}$ signals *m/z* 484 respectively 488 are accompanied by three isotope peaks. The exclusive HFBA acylation of TaClo only in the tetrahydropyridine ring system and not on the indol nitrogen atom, is in agreement with former work in the field of "normal", i.e. chlorine-free tetrahydro-β-carbolines [40]. The base peaks *m/z* 367 respectively 371 (100%) of the mass spectra of TaClo and [D_4]TaClo correspond to the entire loss of the trichloromethyl group as indicated in Fig. 2. These chlorine-free fragments were used as target ions in mass fragmentography for the quantification of TaClo in biological samples obtained from TaClo-treated rats (see below). Other prominent ion fragments (used as qualifier ions) are observed at *m/z* 448 and 452 originating from an elimination of HCl, the signals *m/z* 413 and 417 resulting from a loss of HCl and Cl, as well as the mass fragments *m/z* 379 and 384 resulting from the cleavage of three chlorine atoms (see Fig. 2).

3.3. Determination and quantification of TaClo in spiked biological samples

3.3.1. Sample preparation

The SPE workup procedure used for the purification of TaClo from various biological material is described in detail in the experimental section, and is based on sample loading onto a conditioned C_{18} cartridge (at pH 7.5–8.5), washing of the cartridge with sodium carbonate buffer and selective elution of TaClo and [D_4]TaClo with a solvent mixture of chloroform and MTB (1:1). While brain samples were immediately worked up by SPE after homogenization and extraction with methanol, liver tissue had to be purified by a second extraction step using MTB to eliminate most of the interfering unpolar matrix components.

3.3.2. Quantification of TaClo in biological samples

Using [D_4]TaClo as the I.S., the calculated values for the content of TaClo in spiked biological samples differed from the actual quantity of TaClo added, by only 2.5% (blood), 7.5% (urine), and 4.7% (brain), respectively. As indicated in Table 1, regression analysis of the relationship between the concentration ratio of TaClo/[D_4]TaClo and the chromatographic peak area ratio of the target ions 367/371 corresponding to the HFB derivatives of TaClo/[D_4]TaClo yielded linear regression lines over all concentration ranges analyzed, with correlation coefficients $r>0.997$ (see Table 1).

The estimated limits of detection in body fluids and tissues (based upon a signal-to-noise-ratio of 2:1) using EI were 4 ng/ml (whole blood), 2 ng/ml (urine), and 5 ng/g (brain). The limit of quantification was defined by a mean accuracy between 95 and 105% and a coefficient of variation (standard deviation/mean) lower than 10%. The estimated limits of quantification in biological matrices using EI were 12 ng/ml (whole blood), 10 ng/ml (urine), and 15 ng/g (brain). The coefficient of variation for five replicate measurements of TaClo (whole blood: 134 ng/ml; urine: 67 ng/ml; brain: 105 ng/g) was calculated to be 5.2%, 4.1% and 9.8%, respectively.

3.4. Initial animal study concerning the pharmacokinetics of TaClo: analysis of urine, faeces and blood

First of all, we have investigated whether TaClo is resorbed by the organism and how far it is subsequently eliminated from the body in its authentic, non-metabolized form. Wistar rats were treated with relatively high doses of TaClo (40 mg/kg). After

workup of the biological samples (urine, faeces, blood), the following results were obtained by GC–MSD analysis (see Table 2):

3.4.1. Analysis of urine samples

TaClo was identified in the urine of the animals, both after i.p. and p.o. administration, indicating resorption of the compound by the organism. The two modes of application resulted in different courses of the elimination profiles: After i.p. application, the highest TaClo value (11 μg/sample) was measured in the 0–12 h urine fraction. In the two next 12 h fractions, the amount of TaClo recovered decreased down to 1.9 μg/sample. By contrast, the urinary TaClo concentration after oral application gradually increased from 1.7 μg/urine sample, reaching the highest experimentally determined value of 5.3 μg/sample in the fraction collected from 24–48 h. For the cleavage of conjugates possibly formed, aliquots of each of the urine fractions were treated with a mixture of arylsulphatase and β-glucuronidase prior to workup. Still, no significant increase of renal excretion of TaClo was observed after enzymatic hydrolysis.

3.4.2. Analysis of faeces

Since a relatively high TaClo content had been found in the bile (see below), we had expected to detect significantly higher amounts of TaClo in the faeces than those measured in the urine samples. By contrast, in faeces collected from 0–48 h, we could detect only traces of the compound both in free and in conjugated form. The total amount of TaClo determined by GC–MSD was calculated to be 42 μg/sample after i.p. administration, and 90 μg/sample after p.o. application. From these findings, it can be assumed that TaClo or its corresponding phase II metabolites, such as glucuronides or sulphates, after biliar excretion, are subjected to a renewed resorption (presumably in their non-conjugated form) by the small intestine because of their highly lipophilic character, thus forming an enterohepatic cycle (Table 2).

Although a statistical interpretation of the results obtained from this preliminary animal study is not possible because of the small number of rats used in the experiments described above, it can be deduced that a renal or biliar elimination of TaClo in its authentic or in a conjugated form does not play an important role.

3.4.3. Detection of TaClo in erythrocytes

Whole blood was centrifuged to separate plasma from the erythrocyte containing fraction. GC–MS investigations of these samples indicated that the highly nonpolar TaClo molecule is capable of passing membranes. TaClo was mainly determined in the fraction of the lysed erythrocytes and not in plasma, the overall recovery in blood being relatively low. After intraperitoneal or oral administration, 6 μg and 4 μg per ml of blood analyzed were measured after a 2-h duration of the experiment. In each case, the identification of TaClo was based on retention time analysis and registration of characteristic mass fragments in SIM mode.

3.5. Biliar excretion of TaClo

After intravenous administration of 8.5 mg/kg of TaClo to a Wistar rat, a distinct part of this compound was recovered in the bile mainly in the sample collected between 0–2 h (see Fig. 3). For the hydrolysis of possibly formed phase II metabolites, aliquots of all of the bile fractions were treated with a cocktail consisting of the enzymes β-glucuronidase and arylsulphatase. In these samples, the TaClo content was increased by a factor of 2.5, as compared with the values that were measured without previous enzymatic cleavage. In both series of experiments, TaClo was unequivocally identified according to a full scan mass spectrum. Apparently, a metabolic transformation of the compound by conjugation had taken place.

In Fig. 3, a bar graph is presented showing the content of TaClo found in bile of treated rats with and without preceding enzymatic hydrolysis as a function of the duration of the experiment. It can clearly be seen that particularly in the fractions 0–2 h and 2–4 h, distinctly higher concentrations of the tetrahydro-β-carboline were detected after incubation with a mixture of β-glucuronidase and arylsulphatase. Including the enzymatically hydrolyzed material, a total amount of up to 35% of the administered TaClo was excreted in the bile. These results are consistent with experiments on 6-methoxy-1,2,3,4-tetrahydro-β-carboline: after administration of this rather nonpolar harmane representative to rats

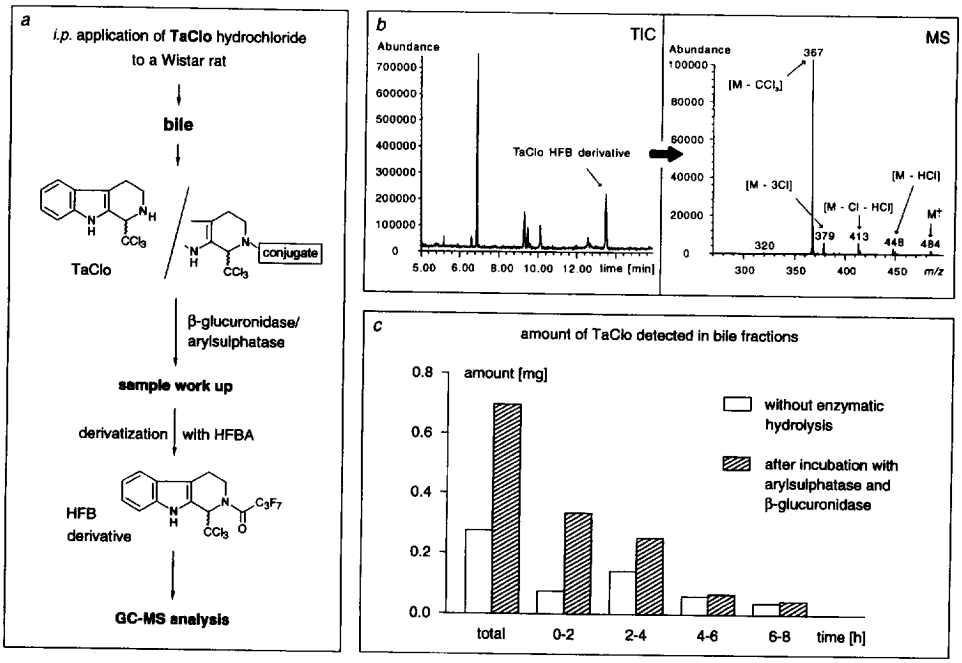

Fig. 3. GC–MS analysis of bile after administration (i.p.) of TaClo to a Wistar rat. The bile was collected from 0–8 h in 2-h fractions. (a) TaClo was determined directly and after enzymatic hydrolysis of possibly present conjugates. GC–MS analyses were done after purification of bile aliquots on C_{18} cartridges and derivatization with HFBA. (b) Identification of TaClo was achieved by a full scan mass spectrum showing the typical chlorine-containing fragments of the HFB–TaClo derivative. (c) The content of TaClo detected in each of the bile fractions is illustrated in a bar graph showing the amount of TaClo found without enzymatic hydrolysis and after incubation with an enzyme mixture consisting of β-glucuronidase and arylsulphatase. For further details, see Section 2 and Section 3.5.

in a radioactively labelled form, Ho et al. [41] recovered 46% of the applied total radioactivity within 24 h in the bile of the animals.

3.6. Detection of TaClo in various brain regions, organs, and tissues after its peripheral administration

The capability of TaClo of penetrating membranes was confirmed by its identification in brain material obtained from rats ($n=7$) systemically treated with this highly lipophilic compound (dose: 4 mg/kg, applied daily for six days). The finding that TaClo can pass the blood–brain barrier is of particular importance with respect to the observed neurotoxicological potential of this compound [24,27,32–35]. By spiking the brain samples with the I.S. prior to workup, quantification of the amounts of TaClo detected in different brain regions was accomplished (see inset table in Fig. 4). The data thus obtained indicate a nearly equal distribution of the peripherally administered trichloromethyl heterocycle in all the brain areas investigated. Apart from the cerebellum, with ca. 30 ng TaClo/g wet tissue, the detected average TaClo content in the brain material was calculated to be ca. 80 ng/g wet tissue (Fig. 4).

Due to the highly lipophilic character of TaClo, it may be assumed that an accumulation of this compound in organs, especially in fatty tissues, could play a role. For this reason, also muscle tissues, fatty tissues, lungs, hearts, kidneys, and livers from this series of TaClo-treated rats were analyzed. Using selective ion registration, TaClo was detected only in very low amounts (less than 50 ng/g wet tissue) in most of the tissues and organs investigated. Because of the central role of the liver in metabolic transformations and biliar excretion of xenobiotics, the liver tissues were examined even more closely, giving an average TaClo content of less than 1 μg/g wet tissue.

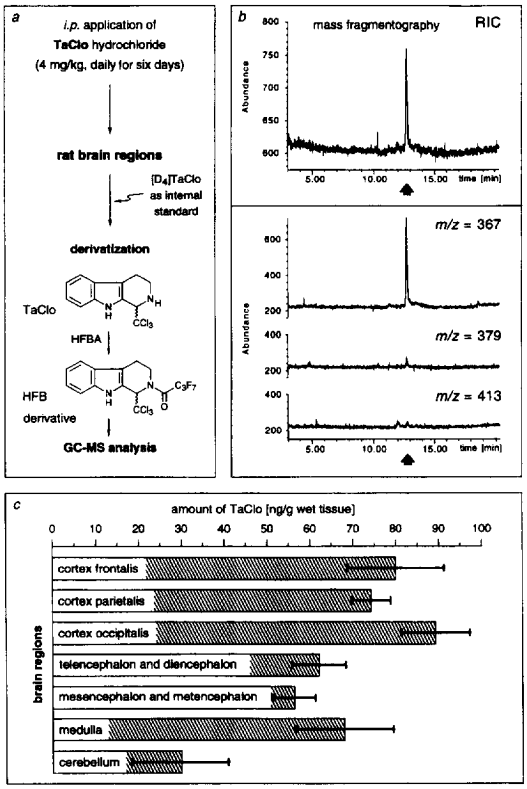

Fig. 4. Identification and quantification of TaClo in various brain regions obtained from TaClo treated rats ($n=4$). (a) After selective work up of brain matrices by SPE, followed by derivatization of the purified sample solutions with HFBA, characteristic ion fragments of the resulting TaClo–HFB derivative were detected by GC–MS. (b) The recorded gas chromatogram of a brain sample is illustrated by the reconstructed ion current (RIC) and the ion traces at the mass numbers m/z 367, 379, and 413, respectively. In nearly all brain samples investigated, TaClo was clearly identified as its HFB derivative at the retention time $t=12.5$ min. (c) Quantification of the recovered amounts of TaClo in each of the analyzed brain regions was done by adding [D_4]TaClo as the I.S. to the sample solutions prior work up. The values given in the bar graph represent means±S.E.M. of four independent GC–MS runs.

4. Outlook

In conclusion, this preliminary animal study on rats concerning the pharmacokinetics of TaClo has revealed a rapid metabolic turnover of the compound and its ability to pass the blood–brain barrier. Current research is now focussing on the identification of metabolites after administration of [^{14}C]-radiolabelled TaClo to rats.

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