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Endogenous alkaloids in man XXXVIII. ''Chiral'' and ''achiral'' determination of the neurotoxin TaClo (1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline) from blood and urine samples by high-performance liquid chromatography–electrospray ionization tandem mass spectrometry☆

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Abstract

An improved sensitive assay for the determination of the dopaminergic and serotonergic neurotoxin 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo) is presented, based upon on-line coupling of high-performance liquid chromatography with electrospray ionization tandem mass spectrometry (HPLC–ESI-MS–MS). Applying synthetic [D_4]TaClo as a fourfold deuterated internal standard, TaClo was detected and reliably quantified as a trace constituent of blood samples (0.5 up to 70 ng g^{-1} of clot) obtained from six patients orally treated with the hypnotic chloral hydrate. Unambiguous identification of this tricyclic ''endogenous alkaloid'' was achieved by selected reaction monitoring (SRM) experiments. The molecular ion peaks of TaClo, m/z 289 (for [$^{35}Cl_3$]TaClo) and m/z 291 (for its [$^{37}Cl^{35}Cl_2$]isotopomer), were both monitored to undergo a retro-Diels–Alder fragmentation by loss of a CH_2=NH portion (−29 u) as typical of a tetrahydropyrido ring system of tetrahydro-β-carbolines. Detection of the resulting fragments, m/z 260 and m/z 262, with the expected statistical chlorine isotopic intensities of 100:96 confirmed the identity of the TaClo molecule. In addition, an enantiomer-specific device was developed for TaClo, by employing a chiral reversed-phase HPLC column in combination with circular dichroism (CD) spectroscopy and MS–MS analysis (LC–CD and LC–MS–MS coupling). In a human clot sample, both TaClo enantiomers were found in equimolar concentration (i.e., as a racemate) corroborating a spontaneous, non-enzymatic formation of TaClo

☆For part XXXVII, see Ref. [12].

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from biogenic tryptamine and therapeutically administered chloral. In urine samples of TaClo-treated rats, by contrast, the (*S*)-antipode was found to predominate, hinting at an enantiomer-differentiating metabolism of the compound.

Keywords: Mammalian; Alkaloids; 1-Trichloromethyl-1,2,3,4-tetrahydro-β-carboline

1. Introduction

The highly chlorinated harman derivative 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo) (Fig. 1) is discussed to be a potent neurotoxic agent [1–4] because of its ability to easily penetrate the blood–brain barrier [3,5], to severely affect the striatal dopamine [6,7] and extracellular serotonin [8] metabolism, and to trigger a slowly-developing neurodegeneration in rats resulting in a Parkinsonian-type diminished locomotion of the animals [9]. The mechanism of TaClo-induced cell death is ascribed to the strong inhibition of complex I of the mitochondrial respiratory chain [7,10,11], the formation of reactive oxygen species such as hydroxyl radicals [8,12], or to DNA damaging processes [12]. TaClo, structurally closely related to the well-established merely synthetic dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [13,14], has to be taken into account as one of the genuine factors inducing neuronal degeneration in man. This became obvious from the discovery of TaClo in blood samples of elderly patients suffering from Parkinson's disease who had been treated orally with chloral hydrate [15], a clinically widely used hypnotic for the induction of sedation and sleep in children and adults [16,17]. Chloral (Clo), which is rapidly distributed throughout the body within 30 min after intake, is a chemically highly reactive aldehyde that is speculated to spontaneously condense with the biogenic amine tryptamine (Ta) by a Pictet–Spengler cyclization, to afford the alkaloid-type neurotoxic heterocycle TaClo (see Fig. 1) [1,3,18].

In previous papers [2,5,15], we reported on a gas chromatographic screening procedure for the reliable determination of TaClo from mammalian body fluids (e.g., serum, clot, urine) or tissues (e.g., brain, liver, kidney) based upon electron-capture and mass-selective detection after conversion of TaClo to volatile perfluoroacyl compounds. This combined assay, however, turned out to be time-consuming and quite difficult-to-handle for a growing number of body fluid samples (e.g., urine, serum, clot). For a more rapid and less complex identification of even smallest traces of TaClo in biological matrices, high-performance liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–ESI-MS–MS) appeared to be a well-suited technique,

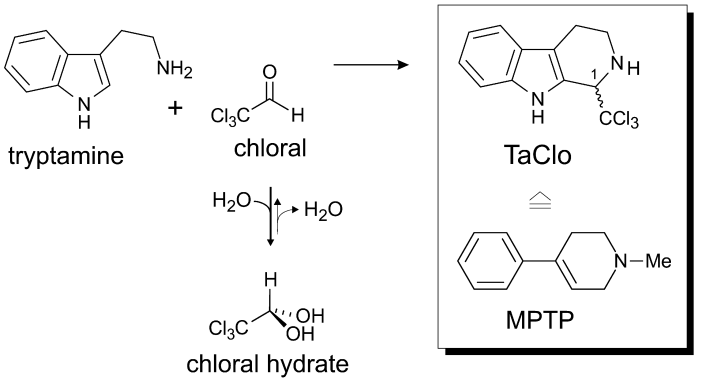

Fig. 1. Formation of the neurotoxin TaClo from the biogenic amine tryptamine (Ta) and the unnatural aldehyde chloral (Clo), and its structural similarity to the neurotoxin MPTP, which produces selective nigral neuronal death in human and experimental models.

since it allows a simple and specific detection of TaClo without the necessity of a derivatization step prior to analysis. Combining selectivity and sensitivity of ESI-MS–MS, selected reaction monitoring (SRM) experiments were applied for the profiling of TaClo down to a picogram scale in human blood samples drawn from (mostly elderly) patients after a consecutive intake of chloral hydrate.

Furthermore, we describe the elaboration of a stereospecific device for the two TaClo enantiomers in order to clarify the mechanism of the endogenous formation of TaClo in man with particular emphasis on the question whether the observed condensation of tryptamine with chloral occurs spontaneously as postulated or with enzymatic assistance. TaClo generation from an enzyme-assisted and thus stereoselective process would be reflected in an unequal abundance of the two enantiomers in human blood samples, while a merely chemical cyclization reaction should lead to fully racemic material. In addition, we also analyzed urine samples obtained from TaClo-treated rats to study a presumable participation of enzymatically mediated pathways in the metabolic degradation of TaClo, as evident from a predominant urinary excretion of one of the TaClo enantiomers. Thus, in order to take advantage of the sensitive and specific ESI-MS–MS method developed for an ''achiral'' determination of TaClo, we have adopted the chromatographic conditions to the use of a reversed-phase chiral HPLC column for the separation of the two TaClo enantiomers. We also report on the stereochemical attribution of (1*R*)- and (1*S*)-TaClo in human blood and rat urine samples ultimately achieved by online circular dichroism (CD) spectroscopy.

2. Experimental

2.1. Chemical and reagents

For chromatography and sample preparation, analytical-grade solvents and reagents were used. Acetonitrile (MeCN) was available from Riedel-de Haën (Pestanal grade; Seelze, Germany) and trifluoroacetic acid (TFA, spectroscopic grade) was purchased from Merck (Darmstadt, Germany). Water purified by the Milli-Q system (Millipore, Bedford, MA, USA) was used throughout the study. 1-Trichloromethyl-1,2,3,4-tetrahydro-9*H*-pyrido[3,4-*b*]indole (TaClo) was synthesized via its formamide by refluxing tryptamine and chloral in formic acid and subsequent deformylation reaction with methanolic hydrochloric acid to afford TaClo hydrochloride (TaClo·HCl) [3,18]. The deuterium-labeled analog [3,3,4,4-$^{2}H_{4}$]1-trichloromethyl-1,2,3,4-tetrahydro-9*H*-pyrido[3,4-*b*]-indole ([D_4]TaClo] was prepared by a procedure similar to that described in a previous paper [5].

2.2. Blood samples of patients therapeutically treated with chloral hydrate

Whole blood samples were obtained from eight patients (age: 67 to 82 years) suffering from Parkinson's disease after a daily oral treatment with a single dose of chloral hydrate (Chloraldurat [19]). Detailed data of drug application and duration of the treatment are described in Table 3. Patient 9 (Table 3, entry 9) was an 11-year old boy suffering from intractable epilepsy since the age of 7 months. A regular evening application of 1 g of chloral hydrate had been started from the age of 5.5 years. For control experiments, whole blood samples (ca. 30 ml) were taken from six healthy volunteers (age: 25 to 60 years), who had not been treated with chloral hydrate. All heparinized blood samples (volume: ca. 12–15 ml) were separated into serum (ca. 4 ± 0.5 g) and clot (ca. 7 ± 0.5 g) by centrifugation. The samples were immediately frozen and stored at -40 °C prior to analysis.

2.3. Animal experiments on rats

Han–Wistar rats (aged 3–4 months), weighing from 280 to 350 g, were acclimated for at least 2 days in stainless steel cages with water and food ad libitum before treatment with TaClo hydrochloride. During dosing, the rats were housed with free access to food and water in individual stainless steel metabolism cages at room temperature (21 °C), in a 12/12 h light–dark cycle. Four rats were injected intraperitoneally with daily doses of 0.8 mg kg^{-1} of TaClo hydrochloride. Urine samples (ca. 3 ml) were collected from two rats, each, in 48-h fractions on the second and on the third day (24 to 72 h), and on the seventh (120 to 168 h) and the eighth day (144 to

192 h) of treatment. All samples were immediately frozen using dry ice, and stored at approximately −40 °C prior to LC–MS–MS analysis.

2.4. Clean up procedure for blood and urine samples

Prior to workup, 50 μl of a methanolic solution of the internal standard [D_4]TaClo (175 pg μl^{-1}; $5.31 \cdot 10^{-13}$ mol μl^{-1}) were added to human serum (ca. 3 g) and clot (ca. 6 g) samples. Sample purification by solid-phase extraction (SPE) was accomplished on C_{18} sorbent Chrompack Bond-Elut SPE cartridges (250 mg of sorbent) using an SPE 12-G-system vacuum chamber (J.T. Baker, Philipsburg, PA, USA). Cartridge pretreatment was carried out by flushing with 2 ml of methanol and 2 ml of Milli-Q water. The conditioned C_{18} cartridge was loaded with the blood sample solution (after homogenization and centrifugation as described in Ref. [5]) or with rat urine (ca. 2 ml), and washed with 1 ml of water. After gentle vacuum aspiration, the analyte TaClo and the internal standard [D_4]TaClo were eluted by passing 2 ml of methanol through the column. Finally, the solvent was evaporated to dryness in a stream of nitrogen. The purified samples were redissolved in 30 μl of methanol, and a 5-μl volume was directly applied to HPLC–ESI-MS–MS analysis. Each HPLC run of a human blood or a rat urine sample was followed by a water/methanol injection to undoubtedly guarantee the exclusion of any memory effects possibly present on the HPLC column used.

2.5. High-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry

The HPLC system used was an Applied Biosystems dual-syringe pump Model 140B (Bai, Bensheim, Germany), equipped with a Rheodyne valve fitted with a 5-μl loop, and a SunChrom Triathlon autosampler (Bai, Bensheim, Germany). All achiral chromatographic separations were performed on a Symmetry C_{18} column (150×2.1 mm I.D., 5 μm) from Waters (Eschborn, Germany). The flow-rate was set at 200 $\mu l\ min^{-1}$. Solvent A was 0.01% (v/v) TFA in water, and solvent B 0.01% (v/v) TFA in acetonitrile. A nonlinear solvent gradient was programmed as follows: pressurizing with 50% B, equilibration time 10 min at 10% B; gradient starting at $t=0$ min with 10% B (1 min) programmed to 100% B within 10 min, 100% B kept constant for 4 min.

HPLC–ESI-MS–MS analyses were performed on a triple stage quadrupole (Q1q2Q3) TSQ 7000 mass spectrometer equipped with a pneumatically assisted electrospray interface (Finnigan MAT, Bremen, Germany). Data acquisition and processing were done on a Personal DECstation 5000/33 (Digital Equipment, Unterföhring, Germany) with ICIS 8.1 software (Finnigan MAT). Nitrogen served both as sheath (70 p.s.i.; 1 p.s.i.=6894.76 Pa) and auxiliary gas (10 units), argon as collision gas. The electrospray capillary voltage was 4 kV, and the temperature of the heated inlet capillary, serving simultaneously as a repeller electrode (20 V) was maintained at 250 °C. Positive ions were detected by scanning from 200 to 500 u with a total scan duration of 1.0 s for a single spectrum. Product ions were monitored by selecting the protonated precursors $[M+H]^+$ in Q1, performing collision-induced dissociation (CID) in the collision cell q2, and detecting the product ions in Q3. MS–MS experiments were performed at a collision gas pressure of 2.0 mTorr (1 Torr=133.322 Pa), and collision energies ranging from 15 to 35 eV (laboratory frame of reference) scanning a mass range from 20 to 400 u with a total scan duration of 3.0 s. The mass spectra were measured in the centroid mode and referenced to 100% intensity of the base peak. From the resulting characteristic MS–MS fragmentation pattern of TaClo and its fourfold deuterated analog, the following most abundant ion pairs were chosen for selected reaction monitoring (SRM) experiments: m/z 289→260 and m/z 291→262 (for TaClo), m/z 293→262 and m/z 295→264 for [D_4]TaClo. These ion pairs represent the protonated molecules $[M+H]^+$ and the product ions resulting from the cleavage of a CH_2NH fragment (−29 u) (cp. Figs. 2 and 3).

2.6. Method validation

The stability of TaClo and [D_4]TaClo in whole blood and in processed extracts of spiked serum and clot samples was assessed at four different con-

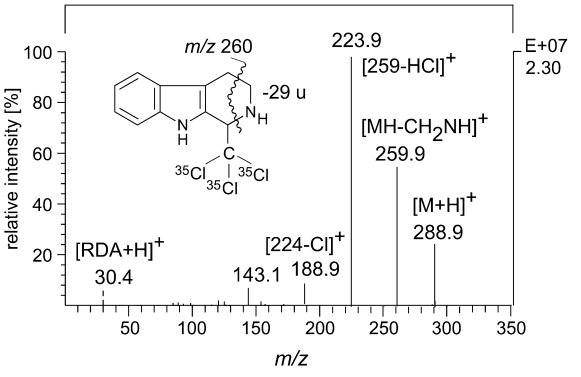

Fig. 2. Product ion spectrum (15 eV, 2.0 mTorr Ar) of TaClo. The inset structure shows the origin of product ion m/z 260 originating from the protonated molecule (m/z 289) by neutral loss of the CH_2=NH moiety (−29 u) by retro-Diels–Alder reaction.

centration levels (85, 17, 6.8, and 1.02 ng g^{-1}) prior to validation. Both tetrahydro-β-carbolines were found to be stable in human whole blood stored at −40 °C and in purified extracts of spiked human serum and clot samples at nominally −40 °C for at least 3 months. Visual examination and comparison of the LC–MS–MS chromatograms of blank and validation control samples obtained during validation experiments revealed the method to provide an excellent specificity for TaClo and for its deuterium-labeled analog [D_4]TaClo. The analyte TaClo and [D_4]TaClo used as the internal standard (I.S.) showed good chromatographic peak shapes, and no significant interferences with the retention times of TaClo and [D_4]TaClo by endogenous components were observed.

For validation, standard solutions of TaClo were prepared in methanol at a concentration of 960 ng ml^{-1} (solution A), 40.6 ng ml^{-1} (solution B), 510 ng ml^{-1} (solution C), and 51 ng g^{-1} (solution D). As the internal standard, a methanolic solution of [D_4]TaClo was used at a concentration of 175 ng ml^{-1} (I.S. solution). Various aliquots of solution A

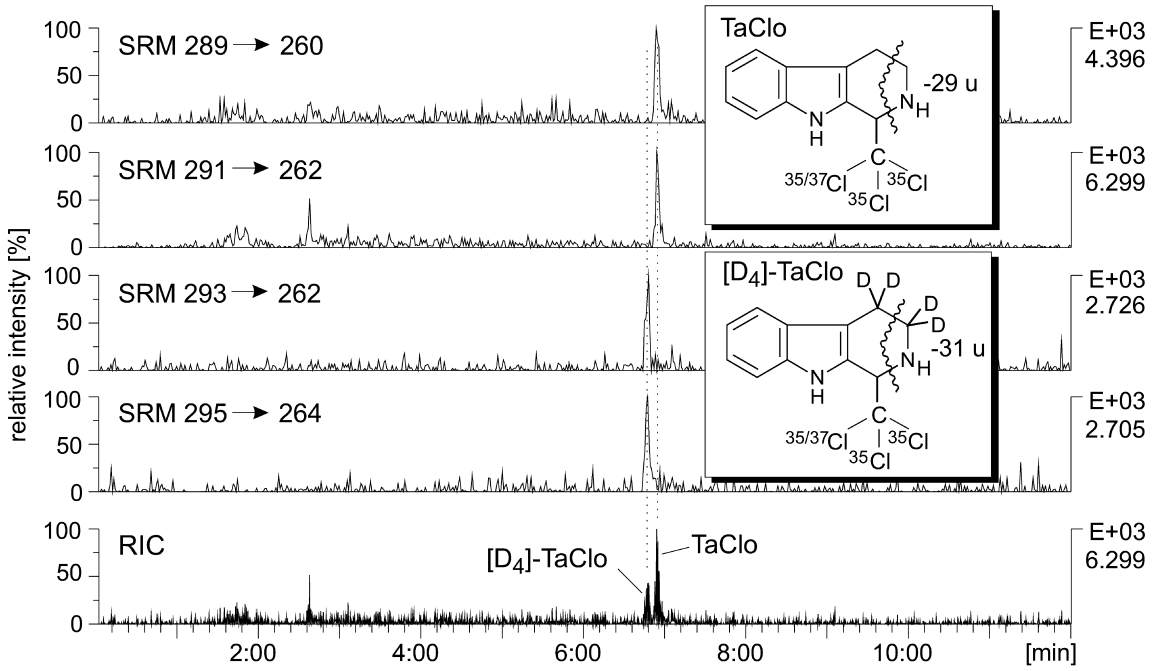

Fig. 3. Identification and quantification of TaClo in a human clot sample (cp. Table 3, entry 2). The recorded HPLC–MS–MS chromatogram is illustrated by the reconstructed ion current (RIC), and the ion traces obtained from SRM experiments (15 eV, 2.0 mTorr Ar) using the ion pairs m/z 289→m/z 260 (for [$^{35}Cl_3$]TaClo) and m/z 291→m/z 262 (for [$^{37}Cl^{35}Cl_2$]TaClo). Detection of m/z 260 and m/z 262 with the statistically expected intensity distribution of 100:96 unequivocally confirmed the determination of TaClo. The internal standard, [D_4]TaClo, added to verify the presence of endogenously occurring TaClo by retention time analysis (t_R=6.8 min) and to quantify the TaClo content detected in blood, was monitored in SRM mode by scanning the loss of a CD_2=NH moiety (−31 u).

Table 1
Linear regression analysis of TaClo in matrix-free solution, in spiked serum, and in spiked clot samples using [D_4]TaClo as the internal standard (I.S.)

Matrix	Concentration range of TaClo	[D_4]TaClo added as the I.S.	Slope,[a] *b*	*y*-Intercept,[a] *a*	Correlation coefficient, *r*
Saline	6.4–80 ng ml^{-1}	2.9 ng ml^{-1}	0.9172±0.0432	−0.0043±0.0018	0.9988
Saline	270–5420 pg ml^{-1}	2920 pg ml^{-1}	0.4822±0.0142	−0.0027±0.0011	0.9996
Serum	6.3–82.5 ng g^{-1}	2.9 ng g^{-1}	0.7417±0.0228	−0.0038±0.0022	0.9975
Serum	280–5300 pg g^{-1}	2920 pg g^{-1}	0.8993±0.0581	−0.0068±0.0038	0.9994
Clot	6.5–79.5 ng g^{-1}	2.2 ng g^{-1}	1.1832±0.0663	−0.0088±0.0042	0.9982
Clot	210–4090 ng g^{-1}	2190 pg g^{-1}	1.0133±0.0611	−0.0041±0.0019	0.9990

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

(20, 50, 100, 150, 200, and 250 μl) or solution B (20, 50, 100, 150, 200, and 400 μl) spiked with a volume of 50 μl of [D_4]TaClo as the I.S. were added to 3 ml of saline, to ca. 3 g of bovine serum, or to ca. 4 g of bovine clot. The spiked samples were submitted to the sample clean up procedure described above (see Section 2.4), and analyzed by HPLC–MS–MS experiments as described above (see Section 2.5). Standard curves (see Table 1) were established by regression analysis of the relationship between the peak-area ratios versus the concentration ratios of the analyte TaClo and the internal standard [D_4]TaClo. The limit of detection (LOD=4*x*) was calculated by taking the mean value *x* of 10 matrix-reagent blanks: the LOD was found to be 20 pg g^{-1} (experimental value: 19.6 pg g^{-1}). The limit of quantification (LOQ=10*x*) determined in the same manner was calculated to be 50 pg g^{-1} (experimental value: 49 pg g^{-1}).

Accuracy (expressed as recovery) and precision (expressed as relative standard deviation, RSD) of the assay (see Table 2) were tested by replicate measurements of eight human serum and clot samples spiked with TaClo concentrations ranging between 0.34 and 68 ng g^1: These test samples were prepared by spiking 3 g of human serum and 6 g of human clot with [D_4]TaClo (50 μl of the I.S. solution≡8.75 ng per sample) and various aliquots of the TaClo standard solution C (40, 60, 100, 200, and 400 μl for serum and 80, 120, 200, 400, and 800 μl

Table 2
HPLC–MS–MS analysis of TaClo in spiked human serum and clot samples: determination of accuracy and precision of the assay at different concentration levels

Concentration (ng g^{-1}) added	Spiked human serum samples					Spiked human clot samples				
	Found (mean±SD)[a] (ng g^{-1})	Recovery (%)	SE[b]	RSD[c] (%)	*n*	Found (mean±SD)[a] (ng g^{-1})	Recovery (%)	SE[b]	RSD[c] (%)	*n*
0.34	0.308±0.027	90.7	0.011	8.76	6	0.306±0.011	90.2	0.005	3.58	6
1.02	0.913±0.049	89.5	0.020	5.36	6	0.950±0.027	93.1	0.011	2.84	6
1.70	1.62±0.072	95.7	0.029	4.43	6	1.61±0.038	94.7	0.015	2.36	6
6.80	6.60±0.102	97.0	0.046	1.55	5	6.63±0.040	97.5	0.089	0.60	5
10.20	9.79±0.050	96.0	0.022	0.51	5	9.83±0.085	96.4	0.038	0.86	5
17.00	15.38±0.49	90.4	0.246	3.19	4	16.63±0.096	97.8	0.048	0.58	4
34.00	32.08±0.72	94.4	0.292	2.24	6	32.25±0.31	95.1	0.126	0.96	6
68.00	65.40±1.12	96.2	0.501	1.71	5	65.26±1.25	96.0	0.511	1.91	6

[a] SD, Standard deviation.
[b] SE, Standard error of mean.
[c] RSD, Relative standard deviation.

for clot) or TaClo standard solution D (20, 60, and 100 μl for serum and 40, 120, and 200 μl for clot).

2.7. Chiroptical stereoanalysis of TaClo

Resolution of the two TaClo enantiomers, (1*R*)-TaClo and (1*S*)-TaClo, on an analytical scale was carried out on a reversed-phase Chiralcel OD-R column (250×4.6 mm I.D., 5 μm) from Daicel (Tokyo, Japan). The flow-rate was set at 0.5 ml min^{-1}. Solvent A was 0.1% (v/v) TFA in water and solvent B 0.1% (v/v) TFA in acetonitrile. After pressurizing with 50% B, followed by an equilibration time of 15 min at 40% B, an isocratic solvent system with A–B (60:40) was used for enantiomeric separations. Simultaneous UV and CD detection (see Fig. 4) was performed on a J-715 spectropolarimeter (Jasco Deutschland) with a standard flow cell at 238 nm. CD spectra were measured in stop-flow mode, and analyzed by comparison with CD curves of the pure TaClo enantiomers, which had stereochemically been assigned previously [3,20]. HPLC–ESI-MS–MS analysis in SRM mode was applied as described above (cf. Section 2.5) to determine the enantiomeric composition of TaClo (racemic or enantiomer-enriched) in human blood and rat urine samples.

3. Results and discussion

3.1. Electrospray ionization tandem mass spectrometry

First of all, in order to successfully apply HPLC–ESI-MS–MS for the identification and quantification of the neurotoxic mammalian alkaloid TaClo from serum, clot, or urine matrices, even down to a picogram scale, parameters dealing with the mass fragmentation processes relevant for a reliable and unequivocal characterization of the tetrahydropyrido

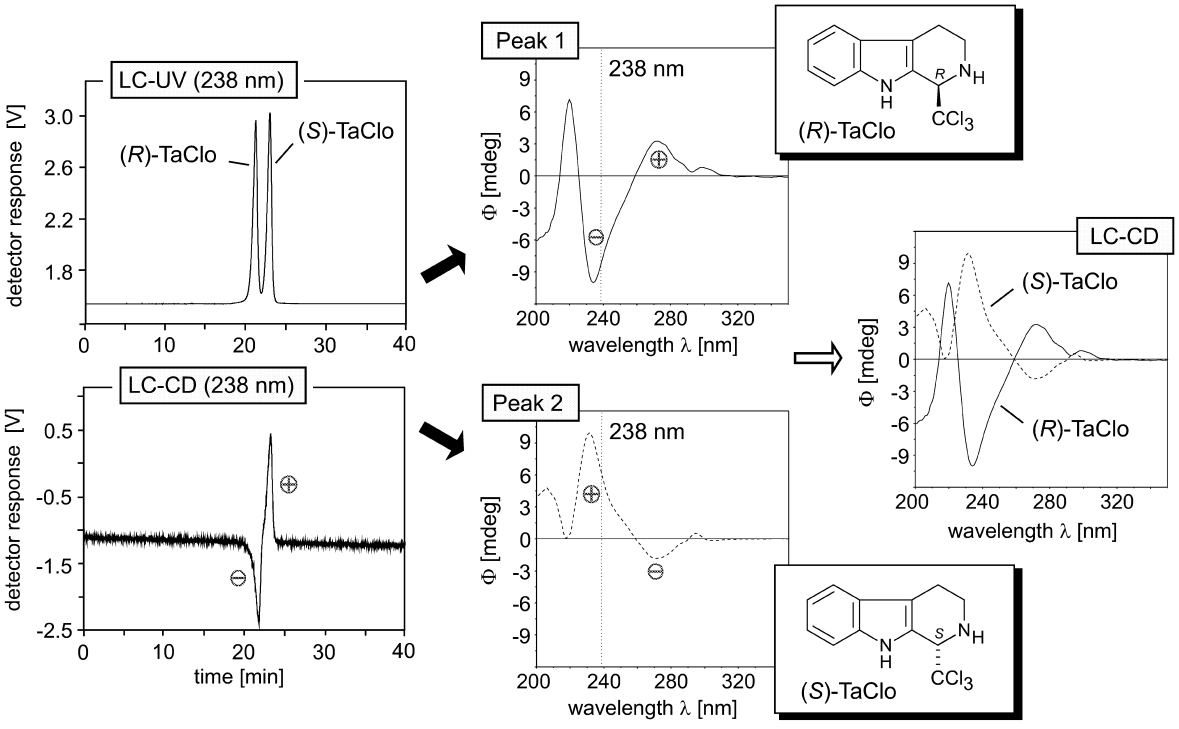

Fig. 4. LC–UV and LC–CD chromatograms of the two TaClo enantiomers at 238 nm after separation on a chiral reversed-phase system (left); LC–CD spectra of (*R*)-TaClo and (*S*)-TaClo were measured in stop-flow mode (center), exhibiting opposite curves (right).

ring system of TaClo were investigated. Low-energy CID of the protonated TaClo molecule $[M+H]^+$ resulted in the formation of the product ion *m/z* 260 by elimination of a CH_2=NH fragment (−29 u) by a retro-Diels–Alder reaction as typical of 1,2,3,4-tetrahydro-β-carbolines [21]. As outlined in Fig. 2, the most abundant product ion *m/z* 224 originated from the combined losses of a CH_2=NH and an HCl moiety (−65 u).

Based on the characteristical neutral loss of an imine (CH_2=NH) fragment, we developed SRM experiments with a high sensitivity and selectivity, and an effective reduction of chemical noise. In comparison to other tetrahydro-β-carbolines, TaClo exhibits the huge trichloromethyl group at C-1 as an additional structural pecularity that dominates the molecule. Due to the natural occurrence of 1:0.32 of the chlorine isotopes ^{35}Cl and ^{37}Cl [22], the isotopic pattern of a Cl_3 unit is expected to give a statistical intensity distribution of $^{35}Cl_3$:$^{35}Cl_2^{37}Cl$:$^{35}Cl^{37}Cl_2$:$^{37}Cl_3$=100:96:31:3. Hence, besides its HPLC retention time, TaClo was identified by monitoring the two SRM experiments *m/z* 289→*m/z* 260 (for [$^{35}Cl_3$]TaClo) and *m/z* 291→*m/z* 262 (for [$^{35}Cl_2^{37}Cl$]TaClo) expected to arise with nearly similar intensities of about 100:96 for a $^{35}Cl_3$ versus a $^{35}Cl_2^{37}Cl$ portion. The internal standard [D_4]TaClo, which had been added to the respective samples (e.g., human blood) prior to the clean up procedure on C_{18} cartridges, was also determined by applying the two SRM experiments described above. Thus, analogously to the non-deuterium-labeled TaClo molecule, but shifted by four mass units, the retro-Diels–Alder reactions *m/z* 293→*m/z* 262 and *m/z* 295→*m/z* 264 were scanned (cp. Fig. 3).

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 their target ions yielded linear lines over all concentration ranges analyzed, with correlation coefficients $r>0.9975$. To investigate the applicability and reliability of the LC–MS–MS method described above, the determination of TaClo was carried out on eight human serum and clot test samples spiked with the internal standard [D_4]TaClo and various quantities of TaClo (see Table 2). The extraction procedure presented in detail in Experimental (see also Ref. [5]) was found to permit high recoveries of TaClo ranging from 89.5 to 97% (for serum) and 90.2 to 97.8% (for clot) with RSDs varying between 0.51 and 8.76% (for serum) and 0.58 to 3.58% (for clot) (see Table 2).

3.2. Identification of TaClo in human blood samples

Blood was drawn from nine patients (cp. Table 3) after a longer consecutive oral treatment with Chloraldurat (active agent: chloral hydrate [19]). Generally, a daily dose of 250 mg up to 750 mg administered in gelatin capsules containing 250 mg of the drug had been applied to elderly patients (cp.

Table 3
Identification of the neurotoxin TaClo in blood samples of patients after intake of the hypnotic chloral hydrate as determined by HPLC–ESI-MS–MS

Patient (sex)	Age (years)	Duration of daily treatment	Dose of chloral hydrate (mg) per day	Approx. concentration of TaClo (ng g^{-1})	
				Serum	Clot
1 (♀)	67	3 days	500	n.d.	n.d.
2 (♀)	76	>6 month	250	0.5	4.0
3 (♂)	64	1 month	250	n.d.	n.d.
4 (♀)	82	10 days	500	n.e.	0.5
5 (♀)	81	14 days	250	0.14	n.e.
6 (♀)	77	8 days	250	n.e.	0.96
7 (♀)	79	26 days	750	n.d.	n.d.
8 (♀)	74	5 days	500	0.14	n.d.
9 (♂)	10	4.5 years	1000	1.6	70

n.d.=Not detected; n.e.=not examined.

Table 3, entries 1–8) suffering from Parkinson's disease. Furthermore, the blood sample of a 11-year-old epileptic boy (cp. Table 3, entry 9) was investigated. From the age of 5.5 years, this pediatric patient had received a regular evening dose of 1 g of chloral hydrate to control recurrent nightly episodes of severe seizures. For control purposes, blood was also taken from healthy volunteers ($n=6$) who had not been treated with chloral hydrate.

All blood samples were separated into serum and clot, purified by solid-phase extraction, and finally analyzed by HPLC–ESI-MS–MS. As outlined in Table 3, TaClo was unambiguously determined in serum and/or clot samples of six patients, in five of the elderly persons in quantities of approximately 0.2 up to 4.0 ng g^{-1}. Fig. 3 shows the characteristic ion traces of TaClo and [D_4]TaClo as monitored by SRM experiments in the clot fraction of patient 2. Remarkably, the by far highest TaClo content of about 70 ng g^{-1} was measured in clot obtained from the epileptic boy. TaClo was not found in control blood samples of the untreated volunteers.

Three features are most striking about our findings: first of all, the identification of TaClo in the human organism is in accordance with the results from a previous study [15] on five elderly patients suffering from Parkinson's disease who had been treated orally with 500 mg of chloral hydrate per night on up to seven consecutive days. The TaClo quantities detected varying from less than 1 ng up to 35 ng per milliliter of blood correlated very well with the doses of chloral hydrate administered and with the duration of treatment. Secondly, in nearly all of the human blood samples investigated TaClo was mainly found to occur in clot (i.e., in lyzed erythrocytes) due to the highly lipophilic character of the TaClo molecule. Thirdly, and most importantly, it is conspicuous how drastically the concentration of TaClo determined in the young epileptic was increased. For example, in comparison to the 76-year-old patient (cp. Table 3, entry 2) subchronically treated with chloral hydrate for more than 6 months, the TaClo content in the child was found to be more than one order of magnitude enhanced. Thus, the high TaClo quantities in the boy hint at an accumulation of the neurotoxin TaClo in the human body obviously as a consequence of the long-term application of chloral hydrate for several years.

3.3. Enantiomer-differentiating analysis of TaClo in blood and urine samples

Since soft ESI techniques require an aqueous or polar mobile phase [23], a reversed-phase Chiralcel OD-R HPLC column was employed for the separation of the two TaClo enantiomers. As presented in Fig. 4, (*R*)-TaClo and (*S*)-TaClo can easily be resolved, and by applying the LC–CD coupling technique in stop-flow mode for the online measurement of the full CD spectra, the two antipodes can unequivocally be attributed stereochemically by comparison with CD curves of enantiomerically pure material assigned previously [3,20]. The CD spectrum of the *R*-configured TaClo enantiomer shows a significant positive Cotton effect at 273 nm and a strong negative one at 234 nm, while (*S*)-TaClo exhibits—as expected—an opposite CD curve (cp. Fig. 4, right).

Control experiments using standard solutions of both TaClo enantiomers as well as LC–CD investigations on human blood and rat urine samples spiked with the pure antipodes, (*R*)- or (*S*)-TaClo, clearly revealed the reference materials to be stereochemical homogeneous. No racemization at C-1 was observed in matrix-free solutions or in biological matrices, i.e., the stereochemical integrity of the chiral center was preserved, no inversion of the configuration has taken place in human blood or rat urine during sample handling and processing.

The clot sample obtained from that epileptic boy (cp. Table 3, entry 9) was chosen to analyze, for the first time, the enantiomeric composition of TaClo endogenously present in a living organism due to the extremely high TaClo level of 70 ng g^{-1}. Applying the SRM experiments described above, TaClo was found to occur as a racemate, i.e., as a 1:1 mixture of (*R*)- and (*S*)-TaClo, in this blood sample (cp. Fig. 5a). It therefore seems likely that TaClo is formed in vivo by a spontaneous, merely chemical Pictet–Spengler condensation and not via an enzyme-catalyzed reaction. This 1:1 ratio is not even changed by any subsequent enantiomer-differentiating metabolism, which is possibly slow—or likewise non-stereospecific—compared to the formation of the racemic neurotoxin under these ''saturation conditions''. From other 1,2,3,4-tetrahydro-β-carbolines (e.g., for 1-methyl-1,2,3,4-tetrahydro-β-carboline) it

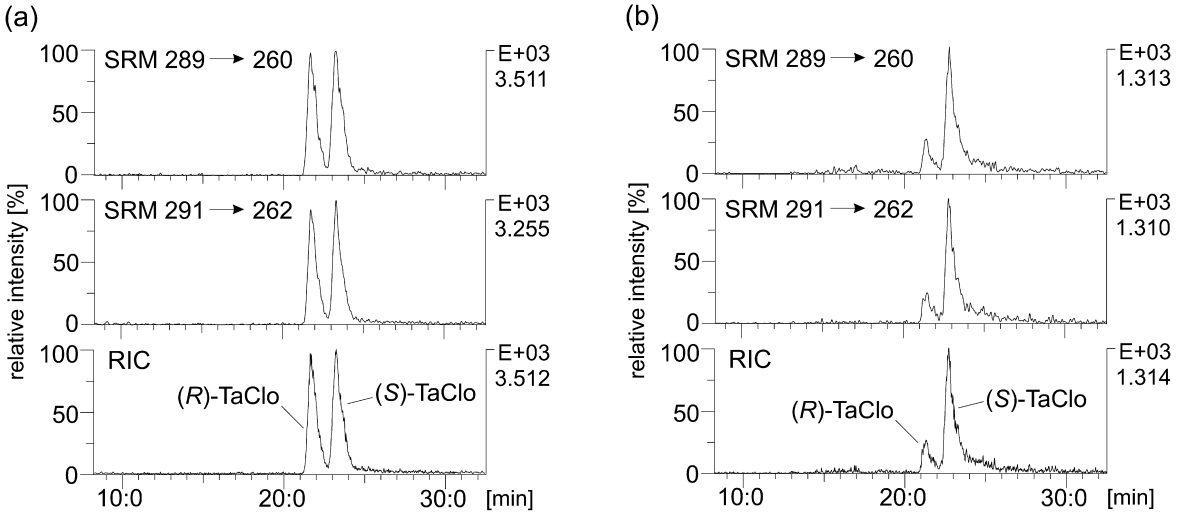

Fig. 5. Determination of both of the TaClo enantiomers by ''chiral'' HPLC–ESI-MS–MS analysis from human blood and rat urine by employing the SRM experiments m/z 289→m/z 260 and m/z 291→m/z 262. (a) TaClo occurring as a 1:1 mixture of (*R*)- and (*S*)-TaClo (i.e., as a racemate) in a human clot sample (cp. Table 3, entry 9). (b) Urinary excretion of the TaClo enantiomers in unequal abundance evidences an enzymatic metabolism in rats (cp. Table 4, entry 1).

is also known that their biosynthesis in mammals is not associated to an enzymatic reaction [24]. Different proportions of their enantiomers excreted in urine, however, gave strong hints at the participation of enzymatically mediated reactions in the metabolism [24,25].

To study more closely the metabolic pathway of the two TaClo enantiomers, the stereoselective HPLC–ESI-MS–MS assay was used to evaluate urine samples of Wistar rats that had been injected intraperitoneally with a solution of racemic TaClo (dose applied: 0.8 mg kg^{-1}) for 3 or 8 days. In this case, a non-racemic mixture of the two TaClo enantiomers was found, with the (*S*)-enantiomer being enriched (cp. Fig. 5b) in nearly all of the samples analyzed (cp. Table 4).

Thus, (*R*)-TaClo and (*S*)-TaClo seem to behave pharmacokinetically different. Our findings hint at an enzyme-catalyzed degradation of the initially racemic TaClo with a significant preference for the (*R*)-configured enantiomer to be converted into (presumably more polar) metabolites. The predominant excretion of (*S*)-TaClo may also account for a better absorption or distribution of the (*R*)-enantiomer in the organism, and therefore, a more rapid renal elimination of the (*S*)-antipode.

4. Conclusion and discussion

A rapid, simple and highly sensitive HPLC–ESI-MS–MS assay was developed for the ''achiral'' and ''chiral'' detection of the tetrahydro-β-carboline TaClo in complex biological matrices. In a series of chloral-treated patients, TaClo has been clearly iden-

Table 4
Enantiomeric ratios of TaClo in urine samples of rats treated with *rac*-TaClo

Rat	48 h urine collected on days *x*, *y* of treatment	er[a] (*R*:*S*) of TaClo
1	2, 3	16:84
2	2, 3	50:50
3	7, 8	43:57
4	7, 8	27:73

[a] er=Enantiomeric ratio.

tified, the most remarkable finding being the strongly increased TaClo level (presumable due to accumulation) in a young epileptic after a long-term administration of chloral hydrate. Furthermore, ''chiral'' analysis of TaClo in human blood revealed this heterocycle to originate merely chemically as a racemate, while racemic TaClo administered to rats is obviously metabolized with enzyme assistance leading to a predominant urinary excretion of the (*S*)-enantiomer.

This study confirms and considerably extends our earlier report [15] on the identification of the neurotoxin TaClo in chloral-treated patients. Due to the fact that TaClo can initiate a slowly progressing neurodegeneration in rats with symptoms comparable to those of Parkinsonism [9], the occurrence and presumable storage of TaClo in man may also contribute to the gradual onset of Parkinson's disease as one of the causative factors of this severe illness. Although, up to now, there is no concrete evidence that TaClo has triggered neuronal denervation in a patient becoming manifest with age, a chronic administration of chloral hydrate should, from our point of view, be avoided whenever possible.

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