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ORIGINAL ARTICLE

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Quantitative determination of taxine B in body fluids by LC–MS–MS

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Abstract A new specific and sensitive LC–MS–MS method for the detection of taxine B and isotaxine B, the main toxic pseudo-alkaloids from yew (*Taxus* sp.), in biological samples (blood, urine, gastric content) was developed. Biological samples were prepared for LC–MS–MS by means of solid-phase extraction (SPE) procedure and yielded a recovery of 86%. Chromatographic separation was achieved using an RP_{18} column. Detection of taxine B and isotaxine B was performed using multiple reaction monitoring with *m/z* 584.2 as precursor ion, i.e. $[M+H]^+$, of both isomers and *m/z* 194.3 and *m/z* 107.1 as product ions after collision-induced dissociation. Docetaxel was applied as internal standard. The method was fully validated for the analysis of blood samples. Linearity was proven in the range from 0.1–500 ng/g. The limit of detection and the limit of quantitation are 0.4 and 2 ng/g, respectively. The method was applied to the determination of taxine B and isotaxine B in four fatal cases (two humans, two horses) with suspected yew intoxication. Blood levels were 105, 168, 174 and 212 ng/g.

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Introduction

Plants of the genus *Taxus* L. (yew) are widespread over the Northern hemisphere. The most common species in central Europe (*Taxus baccata* L., also known as English Yew) is often found as an ornamental shrub or tree. This evergreen conifer is known to contain toxic substances such as cyanogen glycosides and a mixture of diterpene pseudo-alkaloids known as taxine [1, 2]. The latter compounds can be found in all parts of the plant except for the scarlet red aril. The pseudo-alkaloid concentration in the yew can differ in a wide range, depending on the season. Highest concentrations (up to 2%) have been found in needles harvested in January [2–4].

The lethal dose is not exactly known, but it is usually assumed that 50–100 g of leaves (a handful) are fatal [4, 5]. Ingestions of needles or yew decoctions for suicidal purposes have been reported. Accidental ingestions repeatedly occur with children eating the red aril including the seeds [1, 4–10].

Due to its widespread distribution, yew is also known to be a danger to domestic livestock [11–14].

The main pseudo-alkaloid of the taxine fraction is taxine B, and it is assumed to be responsible for the toxicity of the plant [5, 15, 16]. The toxic action of taxine B is based on cardiac effects. The pseudo-alkaloid inhibits the calcium as well as the sodium transport across the cell membrane of cardiac myocytes. This causes nausea, dizziness, pain, tachycardia as well as bradycardia and will eventually lead to fatal heart failure [16–20].

In the past, intoxications were usually confirmed by examination of the gastrointestinal tract for intact plant material since macro-morphological findings are non-specific [4–6, 12, 13]. However, there are known cases where plant material was already degraded in the stomach or not present at all because a decoction of yew has been

ingested [9, 14]. In these cases, determination of taxine in biological samples is essential.

For detecting *Taxus* poisoning in stomach content, methods using gas chromatography were published. Since the taxine alkaloids are non-volatile, detection of 3,5-dimethoxyphenol, the aglycon of the *Taxus* ingredient taxicatin, was used here to achieve an indirect evidence of *Taxus* poisoning [8].

Latest analytical methods aim for liquid chromatography–mass spectrometry (LC–MS) as a highly specific and sensitive method for the detection of non-volatile or unstable substances [21]. This technique permits the determination of intact alkaloids [22] and was successfully applied to determine *Taxus* ingredients as well as *Taxus* pseudo-alkaloids itself [14, 23–25]. One method provided semi-quantitative data in reference to the whole taxine fraction [9].

Nevertheless, with these methods, quantification of taxine B could not be achieved. Since purified taxine pseudo-alkaloids are not available, in this work taxine B and isotaxine B were extracted and isolated from yew leaves. After extensive cleaning-up, this substance was used as reference material. Blood and tissue samples of four fatal cases (two humans and two horses) were extracted and analysed. With this new method, we present the first quantitative data on the sum of isomers taxine B and isotaxine B (taxine B/isotaxine B) concentrations in cases of lethal intoxications.

Materials and methods

Isolation of reference material

Reagents and chemicals

Dichloromethane and methanol were obtained from LabScan (Dublin, Ireland). Diethyl ether, ethyl acetate, acetonitrile, ammonium acetate, neutral aluminium oxide for column separation and sulfuric acid were from Merck (Darmstadt, Germany) and were of analytical grade.

Plant material

Branches of *Taxus baccata* were dried for 1 week at room temperature in a dark drying room.

Isolation procedure

The crude taxine fraction was obtained by soaking the dried needles of *Taxus baccata* in 0.5% sulfuric acid. This step was followed by a liquid–liquid extraction and column separation over neutral alumina according to Jenniskens et al. [15].

Further purification was done by preparative HPLC in two successive runs. Gradients consisting of 50 mM ammonium acetate buffer-acetonitrile and 50 mM ammonium acetate buffer-methanol were used as eluents.

The purity of the gained substance was determined by analytical HPLC (254 nm) and LC–MS–MS. The identity of the substance was confirmed by NMR data [15, 26], exact mass measurement and LC–MS–MS.

Instrument conditions and settings

The apparatus for preparative HPLC consisted of a Varian ProStar 210 Delivery system equipped with Varian ProStar 325 UV-VIS detector. Preparative separation was achieved using a C-18 Guard Cartridge (Phenomenex 10×10 mm) and a Kromasil 100 C-18 semi-preparative column (mz-Analysentechnik Mainz, 250×10 mm, 5 μm) at a flow rate of 3 ml/min. All data were processed using Star Chromatography Workstation (Version 6.2).

Electrospray-exact mass measurement was achieved with a MicroTof (Bruker Daltonics, Bremen) using loop injection. Mass calibration was done directly prior to mass measurement with sodium formate clusters.

^{1}H-NMR data ($CDCl_3$) was attained using a Varian Mercury 400 plus.

Analytical determination

Reagents and chemicals

A stock solution of pure taxine B/isotaxine B (1 mg/ml) was prepared in acetonitrile. Work solutions were obtained just before use by appropriate dilutions with water.

In order to determine the linearity of response, the stock solution was diluted with HPLC solvent (70% 0.02 M ammonium acetate, 30% MeOH).

Docetaxel as internal standard (IS) was purchased from Sigma (Taufkirchen, Germany). A stock solution of 1 mg/ml as well as an IS working solution of 50 μg/ml was prepared in acetonitrile. All stock solutions and the IS working solution were stored at −18°C.

Acetonitrile, methanol, ammonia and ammonium carbonate were obtained from Merck (Darmstadt, Germany). Purified water was from Baker (Griesheim, Germany). For solid-phase extraction (SPE), octadecyl-modified silica columns (Chromabond 200 mg) were purchased from Macherey-Nagel (Düren, Germany).

Carbonate buffer for sample preparation consisted of 0.01 M ammonium carbonate, adjusted to pH 9.3 with 25% ammonia.

Spiked blood calibrators

Blank blood samples were collected from seven healthy volunteers. These samples were spiked with aqueous taxine solutions for the validation.

Table 1 MS ionization and detector settings

Compound	Transition *m/z*	Cone voltage (V)	Collision energy (eV)
Taxine B/isotaxine B			
Main transition	584.2>194.3	30	25
Secondary trace	584.2>107.1	30	60
Docetaxel			
Main transition	830.2>549.2	45	35
Secondary trace	808.3>527.2	20	13

Sample preparation and extraction

Blood and tissue samples (each 0.5 g) were mixed with 2 ml 0.01 M ammonium carbonate buffer pH 9.3, and 50 µl of IS working solution was added . This mixture was homogenized and centrifuged at 5,000×*g* for 10 min. The supernatant was decanted and loaded on an activated SPE C-18 column. The column was pre-conditioned with 1 ml methanol, 1 ml water and 1 ml of 0.01 M carbonate buffer pH 9.3. The sample was allowed to pass through the column under gravity before a wash step with 3 ml carbonate buffer was performed. Columns were dried under vacuum for 30 min. The analyte was eluted with two aliquots of 0.7 ml methanol. Eluents were pooled and evaporated to dryness under a stream of nitrogen at 40°C before reconstitution with 1 ml HPLC solvent (70% 0.02 M ammonium acetate, 30% MeOH).

Ion suppression test

For the evaluation of matrix effects, a systematic procedure according to Matuszewski et al. [27] was used. Different blank blood extracts were spiked with analyte and injected. Neat standard solutions in the same concentration were measured, resulting in the reference peak area. A comparison of the areas obtained from the spiked blood extracts with the reference peak area can indicate matrix effects.

HPLC conditions and detector settings

A Quattro micro tandem mass spectrometer fitted with Z-spray ion interface was used for all analyses. Ionization was achieved using electrospray in the positive ionization mode (ESI+). The following ESI settings were used for the analysis of taxine B/isotaxine B and docetaxel: capillary voltage 0.8 kV, source temperature 120°C; desolvation gas (nitrogen) was heated to 350°C and delivered at a flow rate of 500 l/h. Detection was performed using multiple reaction monitoring (MRM). Two transitions were measured for each compound. The main transition was used for quantification purposes, and a further secondary transition was monitored for confirmatory purposes (Table 1). Collision gas (argon) pressure was maintained at 6×10^{-3} mbar.

For HPLC analyses, a Waters Alliance 2695 separations module (equipped with auto sampler) was used. Chromatographic separation was achieved with a Varian Pursuit C-18 MetaGuard pre-column (2 mm I.D., 3 µm) and a Pursuit C-18 analytical column (2.0×150 mm, 3 µm). The column was maintained at 40°C. Gradient elution was carried out at a constant flow rate of 200 µl/min.

The HPLC solvent A consisted of 0.02 M ammonium acetate, solvent B of methanol. Initial conditions were 20% solvent B for 2 min, increasing to 80% B at 25 min and holding for 10 min (total run time 50 min).

All aspects of system operation and data acquisition were controlled using Mass Lynx NT 4.0 software with automated data processing using the QuanLynx software (Waters).

Results and discussion

After extraction and isolation out of yew leaves, a purity of 91% for taxine B/isotaxine B was attained (HPLC-DAD 254 nm).

The identification of taxine B/isotaxine B was confirmed by exact mass measurement and NMR data. Exact mass measurement of the extracted taxine resulted in a mass of 584.32 *m/z* for $[M+H]^+$. This mass agrees with the published structure $C_{33}H_{45}NO_8$ of taxine B/isotaxine B.

The ^{1}H-NMR spectrum showed that although a sophisticated clean-up procedure was performed, a preparative separation of the isomers could not be achieved. Our following studies with this mixture confirmed the observations of Jenniskens et al. that pure taxine B easily isomerizes to isotaxine B and vice versa due to acetyl migration (Fig. 1) [14, 15, 28].

In the HPLC solvent, an equilibrium between those isomers is reached. Peak areas of extracted standard material and from extracted case material showed identical distribution patterns between the two isomers. For this reason, the prepared isomeric mixture could be used as reference substance for quantification. As it is not known whether taxine B or isotaxine B is more toxic, the results of our studies show the sum of taxine B and isotaxine B. The

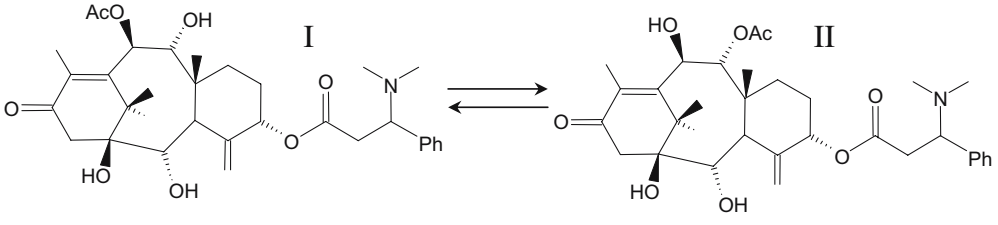

Fig. 1 Taxine B (*I*) isomerizes to isotaxine B (*II*) due to acetyl group migration. The ratio of the equilibrium depends on the used solvent

assay was validated for the determination of taxine B/isotaxine B in whole blood samples.

Linearity

A series of neat standard solutions was prepared in HPLC solvent (0, 0.1, 0.5, 1.0, 2.5, 5.0, 7.5, 10, 25, 50, 75, 100, 250 and 500 ng/ml) and analysed in duplicate. The response curve using linear regression was found to be linear (R^2=0.998) over the range investigated.

Specificity

Blank human blood samples (seven individuals, samples were not pooled) and spiked blood samples were extracted and measured in duplicate. The spiked samples contained taxine B/isotaxine B (1 ng/g). The chromatograms were compared with each other. No interfering signals were caused by extracted matrix.

Calibration

A series of calibrators (0.4–400 ng/g) were prepared in duplicate by adding taxine standard to blank blood samples in appropriate concentrations. Fifty microlitres IS work solution was added to each sample. The response curve was generated using the peak area ratios of the analyte and the IS. The linear regression of the calibration graph is described by the function y=0.096x−0.023 and showed a correlation coefficient R^2=0.998. The graph was linear and of acceptable quality.

Table 2 Inter-assay and intra-assay precision and accuracy for the determination of taxine B/isotaxine B in blood samples

	Concentration taxine B/isotaxine B added (ng/g blood)		
	2.0	100.0	200.0
Determined taxine concentration (ng/g blood)			
Series 1 (n=5)	2.32	95.7	195.0
Series 2	2.28	99.2	183.0
Series 3	2.12	99.8	198.3
Mean value series 1–3 (n=15)	2.2	98.2	192.1
Intra-assay precision (CV %), arithmetic mean value			
Series 1 (n=5)	5.7	5.3	3.5
Series 2	5.1	5.1	4.7
Series 3	3.5	7.5	7.1
Inter-assay precision (CV %), arithmetic mean value			
Series 1–3	3.8	1.8	3.4
Intra-assay accuracy (%), arithmetic mean value			
Series 1 (n=5)	116	95	97
Series 2	114	99	91
Series 3	106	99	99
Inter-assay accuracy (%), arithmetic mean value			
Series 1–3	112	99	96

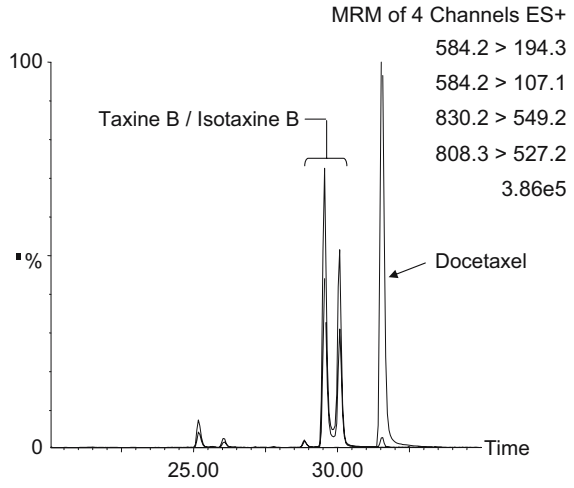

Fig. 2 MRM chromatogram of the blood sample from human case no. 1 with 174 ng taxine B/isotaxine B per gram

Recovery

Recovery was calculated from the ratio of the slope of the regression line obtained with standard aqueous solutions without extraction procedure and spiked blood samples after SPE. The recovery was 86% across the concentration range of the calibration.

Precision and accuracy

To determine the intra-assay accuracy and precision (Table 2), five replicate analyses were performed with spiked blood samples at 2.0, 100 and 200 ng/g taxine B/isotaxine B. IS was added and the samples were extracted and analysed within 1 day. Inter-assay accuracy and precision were determined by repeating this procedure at the same concentrations on 3 different days. Data thus obtained were evaluated in reference to the IS. The results show excellent coefficients of variation (CV %), ranging

Table 3 Concentrations of taxine B/isotaxine B in samples from four cases of fatal yew intoxication

	Taxine B/isotaxine B concentration		
	Blood (ng/g)	Stomach content (μg/g)	Urine (μg/ml)
Human case no. 1	174	≈50	
Human case no. 2	105	≈2	3
Horse case no. 1	212		40
Horse case no. 2	168		

from 1.8 to 7.5. The accuracies were between 91.5 and 116%.

LOD and LOQ

Blood samples spiked with taxine B/isotaxine B at low concentrations were analysed in order to determine the limit of detection (LOD). The signal intensity of the secondary trace *m*/*z* 584.2>*m*/*z* 107.1 was 55% of the main transition *m*/*z* 584.2>*m*/*z* 194.3. According to the requirements for qualitative substance identification [29], the maximum permitted tolerance for relative ion intensities for this ratio is 20%. This criterion is met by the LOD of 0.4 ng/g (S:N=100:1).

The limit of quantitation (LOQ) was defined as the lowest concentration point of the calibration curve. It also has to be of sufficient accuracy and precision (CV>20). As seen in the intra- and inter-assay precision experiments, the LOQ with 2 ng/g taxine B/isotaxine B is within the limits of acceptability for the validation of bioanalytical assays [30].

Ion suppression

The test with extracted human blood samples showed no ion suppression effects caused by the matrix in the selected retention time window. It is however possible that other complex matrices, for instance decomposed tissue samples, can cause ion suppression. In these cases, standard addition procedure is recommended.

Authentic samples

A published LC–MS method describes the semi quantitative determination of taxine pseudo-alkaloids [9]. Crude taxine extract was used as reference substance. Composition and amount of alkaloids in yew leaves and therefore also in the crude alkaloid extract depend not only on the species but also on the season, location and temperature. Results with the semi-quantitative method will differ depending on the crude extract used as reference. For this reason, it is not possible with this semi-quantitative method to draw conclusions concerning the concentration of a single component, as for instance taxine B.

This study presents for the first time quantitative data on taxine B/isotaxine B in cases of yew intoxications. Taxine B/isotaxine B was detected in blood samples (Fig. 2), urine and gastric content of two humans and two horses (Table 3). All intoxications were fatal and were not related to each other.

Gastric content of human case no. 1 (24-year-old man) contained many yew leaves. It is known that he ingested yew needles in order to commit suicide. A post-mortem extraction of the leaves caused by the gastric acid can explain the high taxine B/isotaxine B concentration. Since taxine concentrations in the gastric content can change extremely after death, these results can only give a qualitative indication of the presence of taxine pseudo-alkaloids. However, they cannot give an evidence of the amount of alkaloids absorbed prior to death. Therefore, determination of the pseudo-alkaloids in stomach content only is not reasonable.

In the human case no. 2 (33-year-old woman), a determination out of biological samples was also essential. There was no plant material found in the body. The finding of a glass of red yew berries near the dead body gave an indication on the cause of death. The analysis of blood, urine and stomach content was able to confirm this assumption. Yew plant material was ingested; however, it remains unknown if she ate any of the berries or drank a decoction of them.

Both horses were found dead on their feedlot. In horse case no. 2, yew needles were found in the gastric content, whereas in case no. 1, no characteristic plant material was found at all. Although this method demonstrated its utility, it is necessary to collect more data in cases with fatal outcome in order to estimate the toxicity of taxine B.

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