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ORIGINAL ARTICLE

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LC-MS determination of Taxus alkaloids in biological specimens

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Abstract A semi-quantitative LC-MS method was developed for the detection of the pseudo alkaloids of *Taxus baccata* (yew) from human body fluids and tissue samples. This method was used to examine the cause of death of a 43-year-old man who died several hours after he drank a decoction of taxus leaves. Autopsy and histology demonstrated early signs of myocardial hypoxia. Since investigation of the stomach content did not yield evidence of taxus ingestion, the taxus alkaloids were determined in blood, stomach content and tissue samples of the deceased by LC-MS. The samples were prepared by solid phase extraction on RP-18 columns. Chromatographic separation was achieved by HPLC on a RP-8 column, coupled to an ion trap mass spectrometer (Finnigan LCQ). An atmospheric pressure electrospray ionisation was performed. Spectra of the alkaloids were recorded in the single MS mode and in the MS-MS mode and compared with reference spectra obtained from an extract of yew leaves. In the stomach content, the kidneys, the liver and a heart blood sample of the deceased, alkaloids of *Taxus baccata*, predominantly taxine B and iso-taxine B, were identified. The semi-quantitative evaluation of the heart blood revealed a taxine concentration of 11 µg taxine/g. As far as we know this is the first case in which a semi-quantitative analysis of taxine alkaloids has been performed.

Keywords *Taxus baccata* · Yew leaves · Fatal intoxication · HPLC-MS

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Introduction

The yew tree (*Taxus baccata L.*, also known as English yew) is an evergreen conifer widely spread all over central and southern Europe. The toxicity of yew has been well known since ancient times and extracts of yew leaves have been used for homicides as well as suicides [1, 2]. The seeds, the bark and the leaves are known to contain toxic substances. Whereas the seeds with a scarlet red and sweet tasting arillus (the arillus contains no toxic substances) are mainly responsible for accidental intoxication [3], the bark and the leaves are used in homicidal or suicidal poisoning, especially also as decoctions [4, 5].

Several alkaloids are responsible for the toxicity and have been isolated and identified, especially the diterpene alkaloids of the taxine fraction [6, 7]. The cardiotoxic effects of these alkaloids have been studied on isolated perfused heart of guinea-pigs [8]. Current investigations have revealed that the toxic action of these taxine alkaloids also in man is especially on cardiac myocytes resulting in fatal heart failure [9]. In cases of ingestion of leaves or bark, typical fragments can often be identified morphologically in the stomach content and the duodenum [2, 10, 11]. Also, in casework the comparison of the peak pattern obtained by GC analysis of the stomach content with that of an extract of Taxus leaves revealed Taxus ingredients as the cause of death [11]. Furthermore, GC-MS has been applied to detect poisoning by Taxus [12]. Indirect evidence of yew ingestion has been achieved by the detection of 3,5-dimethoxyphenol, the aglycon of the Taxus ingredient taxicatin [13].

Since high performance liquid chromatography-mass spectrometry (LC-MS) equipment is available to several laboratories, this technique was also successfully applied to determine Taxus ingredients from extracts of *Taxus ssp*. [14, 15, 16, 17] as well as to clarify the cause of death, even if rare or recently developed substances are involved [18]. A direct determination of Taxus alkaloids by LC-MS has been reported to analyse the stomach content of horses which were suspected of having been poisoned [19]. LC-MS

was also successfully applied to determine the anticancer agent paclitaxel (Taxol) in the plasma of cancer patients [20].

In the following we describe a rapid method for the qualitative and semi-quantitative analysis of taxine alkaloids in human blood and tissue samples by solid phase extraction and LC-MS.

Case history

A 43-year-old man suffered from schizophrenia. He had been in hospital for treatment several times in the last years and he had attempted suicide before by incision of the wrist. His prescribed anti-psychotic long-term medication was perazin (Taxilan). Due to common adverse effects, the man had been seeking an alternative medication for a long time. For this purpose, he had bought a small yew tree and had prepared and consumed a decoction from the leaves a few days previously. He later told his sister that he had tolerated taxus a lot better than perazin and that he intended to replace the anti-psychotic drug by regular intake of tea from taxus.

It is not exactly known when he drank the second tea from taxus. Afterwards, he sat together with his sister when he complained about nausea and circulatory insufficiency. He told her that he had used leaves of *Taxus baccata* but the quantity was unknown. He started vomiting and his sister brought him to bed. When she looked for him 3 h after the onset of the symptoms she found him lying dead in his bed. There was an empty tea strainer in the kitchen which was cleaned by the sister later.

Autopsy showed unspecific findings compatible with intoxication: acute congestion of parenchymatous organs, massive oedema of the brain, haemorrhagic oedema of the lungs and dilation of the stomach. In addition, a chronic duodenal ulcer and mild arteriosclerosis were present. Histology of the myocardium showed a pronounced interstitial oedema and areas with early signs of hypoxia such as swollen nuclei, perinuclear vacuolization, homogenization and eosinophilia of the sarcoplasm. Immunohistochemical staining for troponin C was positive with strong and intermediate depletion in many areas of the left ventricle (Fig. 1) but staining for C5b–9 was negative. The lungs showed alveolar haemorrhagic oedema and focal haemorrhages. In addition, a mild fatty degeneration of the liver and massive dilation of the submucous gastric vessels were found. Conventional haematoxylin-eosin staining and numerous smear preparations of the aqueous stomach content were not successful in identifying particulate matter typical for *Taxus baccata*. The blood alcohol concentration at the time of death was 0.0‰. Extensive toxicological analysis of urine, heart blood, stomach content and liver after acidic and alkaline extraction and GC-MS analysis of the derivated extracts did not reveal any cause of death.

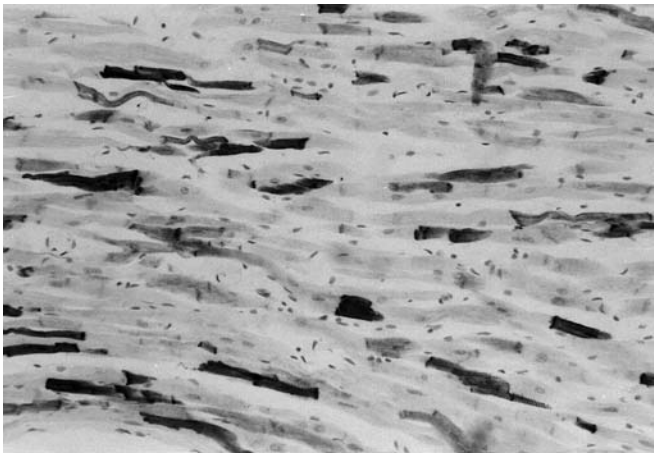


Fig. 1 Left ventricle, troponin C. Clear depletion of the structural protein troponin C indicating early ischaemic damage of the myocardium

Material and methods

Reagents and chemicals

Methanol, ethanol, dichloromethane and acetone, all of Picograde quality, were purchased from Promochem (Wesel, Germany). Ammonium acetate, ammonium carbonate, hydrochloric acid, acetic acid and ammonia solution (25%) were of analytical grade and obtained from Merck (Darmstadt, Germany). For solid phase extraction octadecyl-modified silica columns (chromabond 200 mg) were purchased from Macherey-Nagel (Düren, Germany). Water was deionised and bi-distilled.

Extraction of Taxus leaves

Fresh *Taxus baccata* leaves (2.5 g), harvested in March, were homogenized in 100 ml ethanol. After filtration the solid residue was discarded, the filtrate was evaporated, the residue was mixed with 50 ml 1 M hydrochloric acid and extracted with 50 ml dichloromethane. The organic phase was discarded, the aqueous phase was alkalised with ammonia solution (25 %) and extracted 3 times with 50 ml dichloromethane. Evaporation of the solvent under reduced pressure resulted in 20.6 mg of a pale yellow, oily substance, the crude alkaloid extract. A solution of this fraction (1 mg/ml in acetonitrile) was used for the identification of the alkaloids by LC-MS.

Extraction of blood and intestines

Blood samples and samples of stomach content, kidney and liver (2 g each) were homogenized in 4 ml of 0.01 M ammonium carbonate buffer, pH 9.3. The mixtures were centrifuged and the supernatants transferred to C_{18} columns which were preconditioned by rinsing with methanol, water and ammonium carbonate buffer [21].

HPLC conditions and detector settings

A Waters Alliance 2695XE series HPLC pump with autosampler was interfaced to a Waters TM 486 UV detector and a Finnigan MAT LCQ ion trap mass spectrometer in a serial connection.

Chromatographic separation was achieved with a LiChrospher 60 RP-select B (250×4 mm, 5 µm) analytical column (Merck, Darmstadt Germany). Gradient elution was carried out at a constant flow rate of 1 ml/min.

HPLC solvent A consisted of 0.01 M ammonium acetate, solvent B of methanol.

Initial conditions were 20% HPLC solvent B for 2 min, increasing to 60% B at 30 min and hold for 10 min (total run time 40 min).

The LCQ instrument was equipped with an electrospray ionisation (ESI) source. The ESI settings were as follows: polarity positive, sheath gas flow (N_2) 80 psi, source voltage 4.25 kV, capillary voltage 42 V, capillary temperature 200°C. After chromatographic separation of the alkaloids from the crude taxine fraction the mass spectra were recorded in the full scan mode in the range m/z 150–1000. For the semi-quantitative determination of taxine B and iso-taxine B the MS-MS mode without further collision energy was chosen. The ion m/z 584, which is the protonated molecule mass ($[M+H]^+$) of taxine B and isotaxine B was isolated with an ion isolation width of 2 Dalton. For the confirmation of the semi-quantitative results collision-induced fragment ions were generated with a collision energy of 12%. The collision gas was helium.

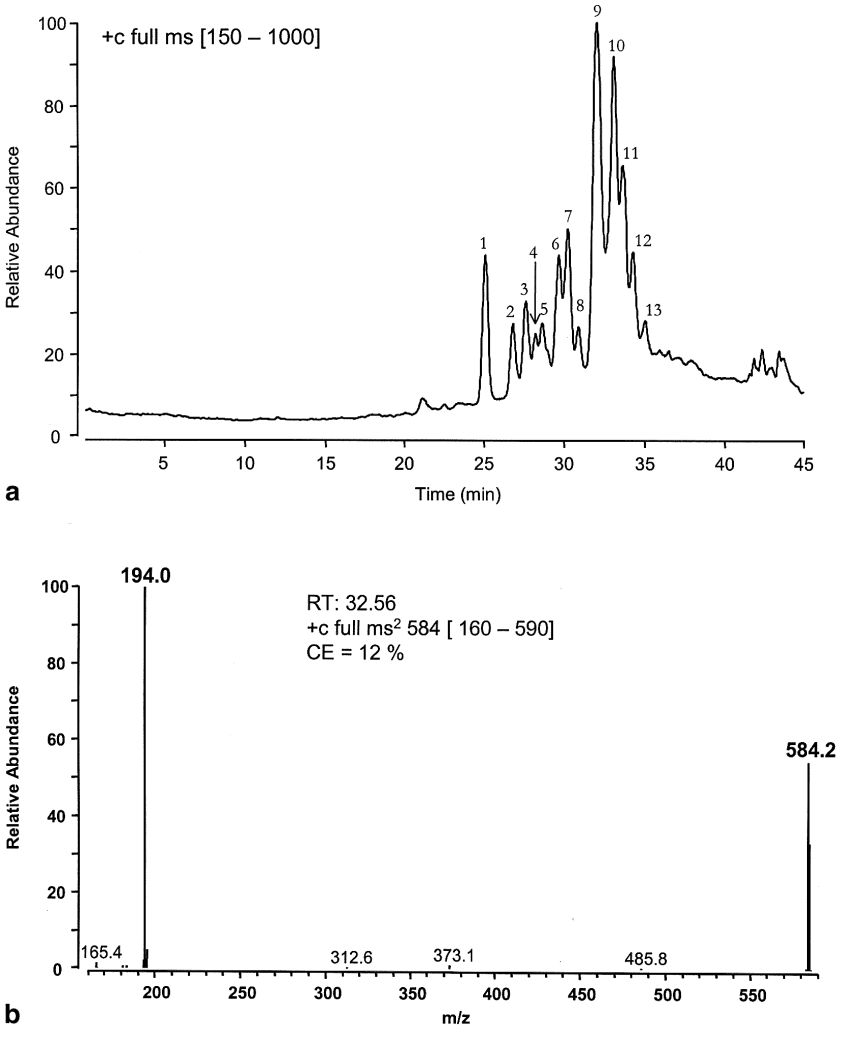


Fig. 2 **a** Chromatogram of the taxine fraction, recorded in the full scan mode. The taxine fraction was the result of an alkaline extraction of the fresh leaves of *Taxus baccata L.* Peaks no. 9 and 10 show taxine B and isotaxine B, the main alkaloids of the taxine fraction. The numeration of the peaks is congruent with Fig. 1 and Table 1. **b** Collision-induced dissociation spectrum of taxine B/isotaxine B, m/z 584 ($[M+H]^+$ of taxine B/isotaxine B) was collected. The collision gas was helium, the collision energy 12% and the ion isolation width 2 Da. The fragment ion with m/z 194 can be deduced from protonated 3-(dimethylamino-3-phenylpropionic acid, the nitrogen-containing acid which is esterified with the diterpenoid moiety of the taxine alkaloids [14]

Results and discussion

The alkaline extraction from 2.5 g of fresh leaves resulted in 20.6 mg (=0.8%) of the taxine fraction which corresponds to the taxine content of 0.5–1% as reported by Jeniskens et al. [7].

In the full scan LC-MS chromatogram of the taxine fraction (Fig. 2a) several peaks with their corresponding mass spectra could be identified. The retention times and the mass to charge ratios (m/z) of the base peaks are listed in Table 1. Due to the positive ionisation mode these base peaks were assigned to the protonated molecule or fragment masses ($[M+H]^+$). We could allocate most of the peaks by their mass spectra to molecular structures as reported elsewhere (Fig. 3) [7]. Based on the mass spectra, a differentiation between the isomers was not possible because fragmentation experiments by collision-induced dissociation (CID) of a parent ion resulted in identical fragment ions, e.g. the fragment m/z 194 after fragmentation of substances no. 9 and 10 (see Table 1 and Fig. 2a), which can be deduced from protonated 3-dimethylamino-3-phenylpropionic acid, the nitrogen-containing acid which is esterified with the diterpenoid moiety of the taxine alkaloids [19]. This is the reason for the assignment of the substances 9 and 10 (Table 1 and Fig. 2a) to the sum of taxine B and isotaxine B with Mr=583 each and an expected m/z value of 584 for $[M+H]^+$ each. Figure 2b shows the CID spectrum of one isomer of taxine B/isotaxine B, recorded in the MS-MS mode collecting m/z 584 and applying a collision energy of 12 %. Increasing the collision energy to 15% resulted to an entire fragmentation of m/z 584 to the product ion m/z 194 in both isomers.

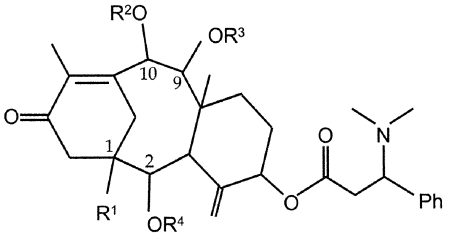
In addition to the main alkaloids several further components could be identified, e.g. taxine A (Table 1 and Fig. 2a no. 6) and 3 peaks with m/z 584 (Table 1 and Fig. 2a no. 1, 2 and 7), one of them also a major component (no. 1). Also in recent publications additional substances with m/z 584 other than taxine B and isotaxine B were reported [19]. Substance no. 1 may possibly be due to a structure reported by Graf et al. [22] with an acetyl group in the C2 position of the diterpene moiety and which was also mentioned by Kite et al. [19].

The major alkaloids from the taxine fraction of Taxus leaves which are responsible for the toxic action are taxine B and isotaxine B [7, 8, 9, 23]. To enhance the sensi-

Table 1 Retention times and the corresponding m/z values of the peaks in the chromatogram of the taxine fraction of taxus leaves, which was injected at a concentration of 1 mg/mL

Substance no.	Retention time	m/z
1	25.07	584
2	26.72	584
3	27.61	542
4	28.08	526
5	28.64	568
6	29.69	642
7	30.26	584
8	31.04	626
9	**32.06**	**584**
10	**33.08**	**584**
11	33.60	568
12	34.29	626
13	35.06	612

The values for taxine B + isotaxine B (no. 9+10) are in bold type. The chromatogram was recorded in the full scan mode collecting m/z 150–1000.
Corresponding chromatogram see Fig. 2.



taxine B isotaxine B

Mr	541	567	567	583	583	625	625	651	667
R^1	OH	H	H	OH	OH	OH	OH	H	OH
R^2	H	Ac	H	Ac	H	Ac	H	Ac	Ac
R^3	H	H	Ac	H	Ac	H	Ac	Ac	Ac
R^4	H	H	H	H	H	Ac	Ac	Ac	Ac

Fig. 3 Taxine B, isotaxine B and seven further related alkaloids from the taxine fraction which were described by Jenniskens et al. [7]

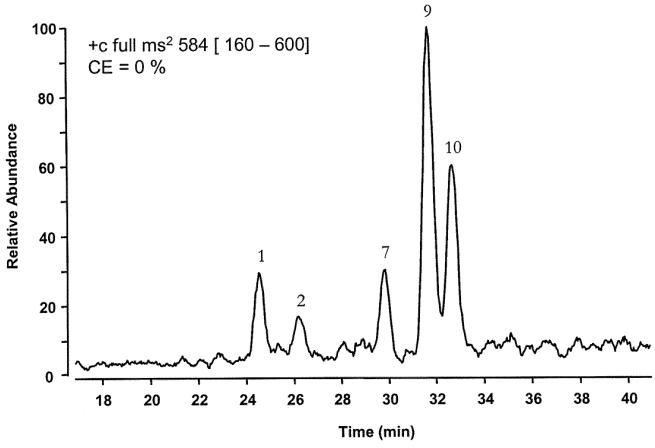


Fig. 4 Chromatogram of a blood sample spiked with 100 ng taxine/g, recorded in the MS-MS mode collecting m/z 584, $[M+H]^+$ of taxine B and isotaxine B. The numeration of the peaks is congruent with Fig. 1 and Table 1

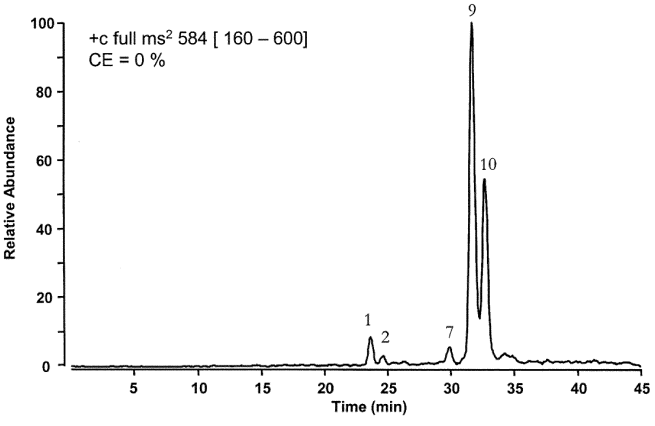


Fig. 5 Chromatogram of the heart blood sample of the deceased recorded in the MS-MS mode collecting m/z 584. The numeration of the peaks is congruent with Fig. 1 and Table 1. The semi-quantitative evaluation revealed a taxine concentration of 11 µg taxine/g, calculated by the sum of taxine B and isotaxine B

tivity for these substances we detected in the MS-MS mode, collecting m/z 584. Figure 4 shows the chromatogram of a blood sample spiked at a concentration of 100 ng taxine/g. Analysing the signal-to-noise ratio, the limit of detection for taxine was estimated as 15 ng/g, the limit of quantitation as 50 ng/g, based on the signals of taxine B and isotaxine B. Recovery experiments with spiked blood samples revealed an average recovery of 81% for the SPE procedure on C18 columns.

Results of the case

In the stomach content taxine alkaloids could be determined in the full scan mode. In the kidney, the liver and the blood sample taxine alkaloids could not be determined in this mode so these samples were analysed in the MS-MS mode collecting m/z 584 without further collision energy (CE=0%). On this way, taxine could be determined in the kidney, the liver and the heart blood and the semi-quantitative analysis of the blood sample (chromatogram see Fig. 5) revealed a concentration of 11 µg taxine/g blood, calculated by the sum of taxine B and isotaxine B. The LC-MS results of the toxicological investigations are summarized in Table 2. The immunohistochemical staining demonstrated early myocardial damages compatible with a cardiotoxic effect lasting for approximately 30 min.

Conclusion

A new semi-quantitative method for the direct determination of Taxus alkaloids in human blood and tissue samples

Table 2 Results of the analysis of samples from the case

Sample	MS Mode	Result
Stomach content	Full scan and MS-MS	Taxine B and isotaxine B (in both modes)
Kidney	MS-MS	Taxine B and isotaxine B
Liver	MS-MS	Taxine B and isotaxine B
Blood (heart)	MS-MS	Taxine B and isotaxine B, equivalent to 11 ng taxine/g, calculated by the sum of taxine B and isotaxine B

In the full scan mode the mass range was m/z 150–1000.
In the MS-MS mode m/z 584 was collected with an ion isolation width of 2 Da.
All MS-MS results were confirmed by their CID spectra obtained by application of 12% collision energy.

was developed because no plant components could be identified inside the stomach. To the best of our knowledge this is the first time that taxine B and isotaxine B could be detected in human blood and tissue samples. A comparison with lethal blood levels in the literature is therefore not possible but the synopsis of findings in this case leaves no doubt that the cause of death was Taxus intoxication. The toxic action of *Taxus baccata* is based on the direct cardiotoxic properties of a wide range of alkaloids. This is supported by our histology investigations.

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