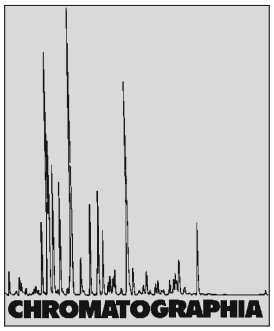
Monitoring of Paclitaxel, Taxine B and 10-Deacethylbaccatin III in *Taxus baccata* L. by Nano LC–FTMS and NMR Spectroscopy



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Abstract

In this work, an aqueous extraction method has been combined with a preparative LC for isolation of 10-deacetylbaccatin III (10-DAB) from leaves of *Taxus baccata* L. The extracted aqueous solution showed two major peaks in LC. Semi-preparative-LC was used for isolation of these peaks. Nano liquid chromatography-Fourier transform mass spectrometry and nuclear magnetic resonance showed that these peaks belong to taxine B and 10-DAB in the extracted aqueous solution. The residual sample of aqueous extraction solution was extracted again by MAE. The amount of paclitaxel in *Taxus baccata* L. was compared with and without aqueous extraction and results showed that paclitaxel was stable and had no significant difference in concentrations during this aqueous extraction process.

Keywords

Column liquid chromatography
Nano liquid chromatography-Fourier transform mass spectrometry
Nuclear magnetic resonance
10-Deacthybaccatin III
Taxine B and paclitaxel

Introduction

The affair of paclitaxel (Taxol) and its analogs is one of the most stimulating subjects in cancer therapeutic studies. Its powerful effect on breast, ovarian and lung cancer has been proven, but paclitaxel is extremely rare and expensive [1–3]. Paclitaxel is naturally present in small amounts in the bark of the species of *Taxus* genus which are very slow growing plants. To tackle the problems encountered with paclitaxel production, huge efforts have been devoted to develop a more sustainable source of paclitaxel including total and semi-synthetic approaches, biotechnological and bioprocess engineering methodologies [4–6]. One of the common ways to obtain paclitaxel is its semi-synthesis from a precursor, named 10-deacetylbaccatin III (10-DAB) which is present in larger amounts in the same plants and is mainly located in the needles. Also, 10-DAB has been used in the semi-synthesis of Taxotere which is twice as active of the of paclitaxel as an anti-tumor agent [7].

A previous study [8] had reported an aqueous extraction method for the isolation of 10-DAB from plant material based on the different polarities of paclitaxel and 10-DAB in which a purity in the range of 75–90% was obtained for 10-DAB. The initial aim of this study was to develop Margraff's method and combine it with preparative LC in order to obtain a higher purity for 10-DAB (>98%). Furthermore, as reported in our previous article the concentration of paclitaxel was evaluated in this sample before and after the aqueous extractions using microwave-assisted extraction (MAE) [9–11].

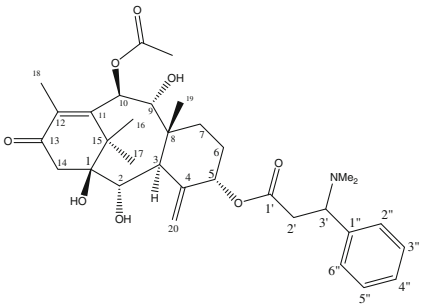

Fig. 1. Chemical structure of taxine B

By using Margraff's patent no. satisfactory experimental results for 10-DAB from the leaves of *Taxus baccata* L. could be obtained and LC showed two peaks. The isolation process by semi-preparative LC was followed by nano-liquid chromatography–Fourier transform mass spectrometry (nano-LC–FTMS) and nuclear magnetic resonance (NMR). The results indicated that taxine B (Fig. 1) was present at high concentration levels (<50%) in the aqueous extraction solution along with 10-DAB. Taxine B and some related alkaloids occur in relatively high concentrations, also using 10-DAB as a precursor for semi-synthetic production of paclitaxel. Accordingly this method produces taxine B, a valuable starting material for semi-synthetic studies toward 7-deoxytaxol and related taxoids [4, 12].

Experimental

Apparatus

A national domestic microwave oven (Matsushita Electric Industrial Co. Japan) was used. Its power could be used in six different levels varying from 10 to 100% of total power. Analytical LC consisted of a Knauer WellChrom (Berlin, Germany) system with LC pump (K-1001)' diode array detector (K-2800) and Eurospher 100-5 C18 (5 μm, 250 mm × 4.6 mm with precolumn) were used. Semi-preparative LC system included a WellChrom preparative pump (K-1800), UV detector (K-2501) and a Büchi fraction collector B-684 (Flawil, Switzerland) were applied. Eurospher-100 C_{18} (5 μm, 120 mm × 16 mm) was used for preparative LC system. For nano-LC–MS analysis, we utilized the Dionex/LC packings (Idstein, Germany) ultimate binary nano LC pump/autosampler system. 1 μL of a 1:10 dilution of the sample was pre-focused on a trap column (Dionex, C18 PepMap, I.D. 300 μm, length 5 mm) and separated on a fused-silica C18 PepMap100 capillary column (Dionex, 3 μm, 100 Å; I.D. 75 μm; length 150 mm). The flow rate was 0.2 μL min^{-1}. Mass spectra were acquired by a Finnigan LTQ FT ultra hybrid instrument (Thermo Fisher Scientific, Bremen, Germany) consisting of a linear quadropole ion trap and a Fourier transform ion cyclotron resonance mass spectrometer with a 6 Tesla magnet. Nanospray ionization interface was used for sample analyses (spray voltage 1.0–2.0 kV, capillary temperature 250 °C, capillary voltage 10 V and tube lens 100 V). Mass resolution was set to 100,000 and allowed to record a high number of spectra during a chromatography peak. Mass accuracies were found to be better than 1.0 ppm in most cases. The parameter settings for the collision-induced dissociation of MS–MS experiments were as follows: Activation energy (normalized) 30; activation duration 30 ms; activation Q 0.25. We used Xcalibur 2.0 SR2 software (copyright Thermo Electron Corporation 1998–2006).

Microwave-Assisted Extraction

An aliquot of 1.5 g ground needles was transferred into a disposable test tube and placed in a container which was transparent to the microwave energy. The collection was placed in the microwave oven and extracted with 90% aqueous methanol for 5 min. The conditions for MAE were adjusted with respect to our previous study on determination of taxoids in which the needles were extracted by MAE and the condition optimized by experimental design [9, 10].

Chemicals

Standard of 10-deacetylbaccatin III and paclitaxel (taxol) were from Sigma (St Louis, USA). LC grade acetonitrile was from Caledon Laboratories (Ont., Canada). Methanol, dichloromethane, and *n*-hexane, all 99.5% purity, were from Merck (Darmstadt, Germany). LC grade water was used throughout the analysis.

Plant Material

Fresh intact clipping of *Taxus baccata* L. were started from the Botanical garden of the University of Tehran, Iran, in August 2006. After being dried for 5 days at room temperature, the leaves were separated from the stems and both were ground to a particle size of 1-3 mm and stored in refrigerator until analysis.

Liquid Liquid Extraction

Liquid liquid extraction (LLE) was used for 10-deacetylbaccatin III [8]: 50 g dried and powdered leaves of the plant were put in 250 mL 50 °C water for 1 h. The sample was filtered and aqueous fraction was extracted by 90 mL of ethylacetate for three times. Diluted sodium carbonate (0.1 M) was used two times for this solution then sodium sulfate was added for removing trace contaminant of water. The solvent was then evaporated under reduced pressure and the residue dissolved in acetonitrile at 70 °C.

LLE for paclitaxel carried out according to Glowniak and Mroczek [13]. An aliquot of 10 mL of methanol extraction was mixed with 10 mL water and extracted with *n*-hexane (2 × 10 mL). The hexane extract which contained lipids, waxes, and pigments, was discarded and the aqueous layer was extracted with dichloromethane (5 × 10 mL). In this stage polar compounds like carbohydrates remained in aqueous phase and taxoids were extracted to organic phase. Dichloromethane extracts were combined, evaporated under reduced pressure and the residue was dissolved in 50 mL acetonitrile.

LC Method Development

For determining 10-DAB and taxine B, C18 column with water/acetonitrile

(70:30 *v*/*v*) at flow rate of 1 mL min^{-1} and detection wavelength of 227 nm, was used, and for paclitaxel, the same flow rate and detection wavelength were used with Eurospher-100 C18 column and water/acetonitrile (51:49 *v*/*v*).

Semi-Preparative Separation

Semi-preparative separation was carried out at the same analytical scale, but with a considerable difference in the flow rate. Flow rate was calculated from the equation:

$$\frac{V_1}{V_2} = \frac{r_1^2}{r_2^2}$$

where V_1 and V_2 are flow rates of the mobile phase and r_1 and r_2 are inner diameters of the columns in analytical and preparative scales, respectively [14]. This equation predicts 16 mL min^{-1} for flow rate in preparative chromatography and C18 column (120 × 16 mm) was used. But, the flow rate for separation of 10-DAB and taxine B was 6 mL min^{-1} which was adopted through this study. Also, 4 mL injection volume was the highest amount of sample that could have been injected to coincide both mass and volume overload.

Nuclear Magnetic Resonance

Fraction containing taxine B was collected from semi-preparative column, lyophilized and dissolved in $CDCl_3$ and subjected to 500 MHz NMR spectroscopy. All NMR spectra were recorded on Brucker DRX500 Avance and running XWINNMR2.6 software using 500 uL sample. The chemical shifts (delta) are reported in parts per million (ppm).

Results and Discussion

Extraction of *Taxus baccata* L. was carried out with aqueous solution according to Margraff's report [8] in order to obtain 10-DAB and then purified by semi-preparative LC to obtain higher purity of 10-DAB III. The aqueous extraction was analyzed by analytical LC as shown in Fig. 2. The first peak has

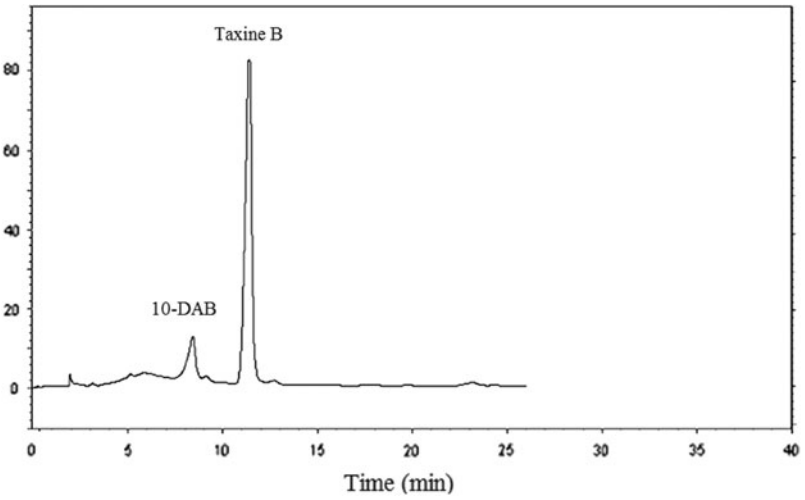

Fig. 2. Analytical LC of an extracted sample using Margraff method [8]. Separation conditions involved a mobile phase composition of water:acetonitrile (70:30 *v*/*v*). The flow rate was 1.0 mL min^{-1}, with an injection volume of 20 µL. Detection: UV at 227 nm

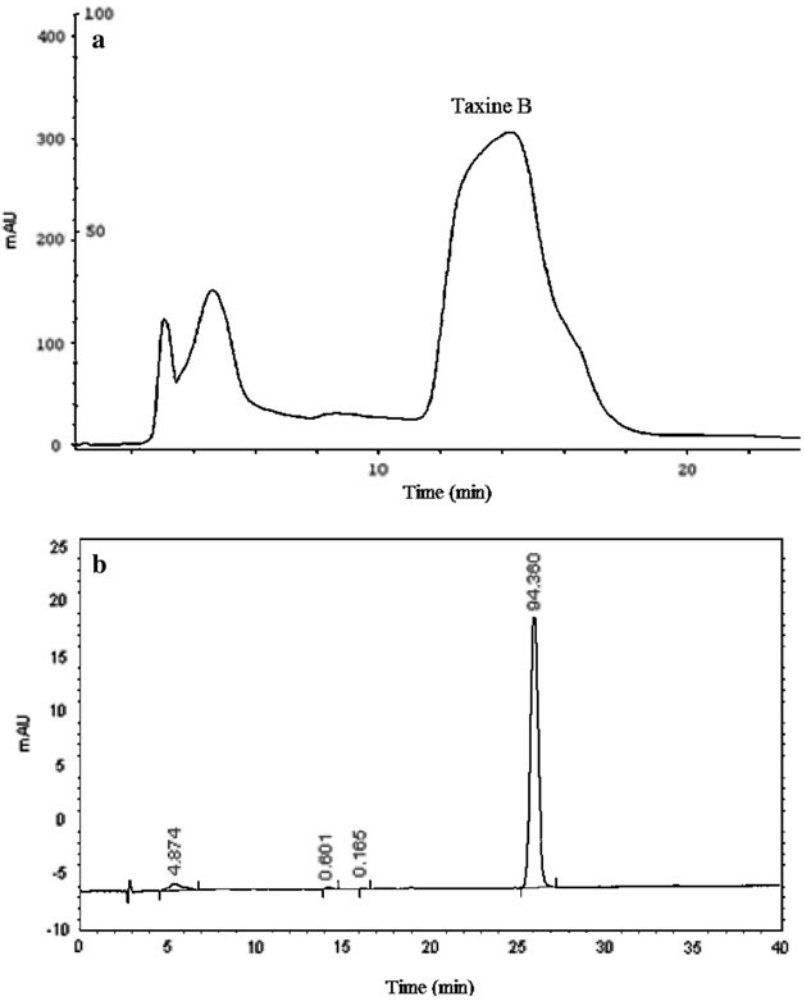

Fig. 3. a Semi-preparative LC of an aqueous extraction of taxanes. Separation condition involved a mobile phase composed of water:acetonitrile (70:30 *v*/*v*), flow rate: 6 mL min^{-1}, injection volume: 4 mL and **b** analytical LC of second peak of semi-preparative separation related to taxine B fraction (the separation conditions were the same as Fig. 2)

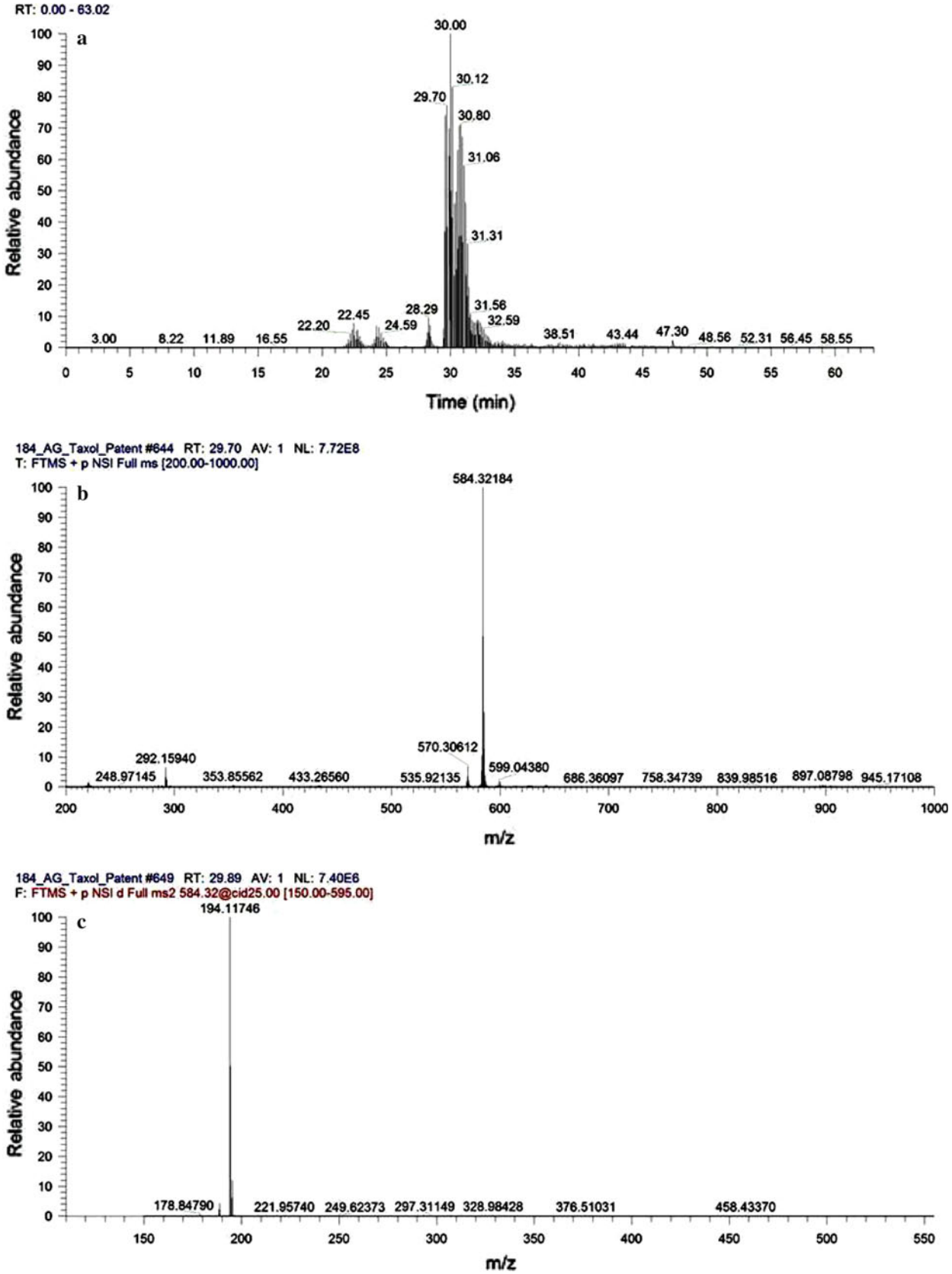

Fig. 4. **a** TIC of nano-LC–FTMS of taxine B, **b** mass spectrum of taxine B, and **c** MS–MS of m/z 584.32

been confirmed to be 10-DAB by spiking it with 10-DAB standard. The amount percentage (based on UV absorption) of 10-DAB in the extracted sample was lower than 25%. According to Magraff's report [8], the purity of 10-DAB was supposed to be 75–90% in aqueous extraction solution. However, our results showed that the intensity of the second peak was higher than the 10-DAB peak (Fig. 2).

The second peak was thought to be related to the decomposition of 10-DAB during the extraction process. Several parameters including different temperatures, volume of aqueous solvents, time of extractions among other parameters were applied, yet the LC results showed that these parameters had no significant effect on the intensity of the second peak and an independent compound was extracted by aqueous extraction solution.

The two major peaks of the aqueous extract were isolated by semi-preparative LC. Figure 3a shows the semi-preparative chromatogram of aqueous extraction and Fig. 3b shows the analytical LC analysis of the second collected peak of Fig. 3a.

The analytical LC results demonstrate that the collected peak from semi-preparative LC has at least 94% purity. Thus the components of aqueous extraction from *Taxus baccata* L. must be purified by preparative LC in order to obtain suitable purity for each compound.

Nano-LC–FTMS was used for analysis of aqueous extraction sample. Figure 4a shows the total ion current (TIC) of collected peak of semi-preparative LC injected into nano-LC–FTMS. The spectrum of FTMS results is shown in Fig. 4b and displays a base peak at m/z 584.32184 after searching in mass spectra of taxanes [15], this mass could be related to $[M + H]^+$ of taxine B. The accuracy is usually employed for evaluation of data in FTMS over the ppm differences between the theoretically calculated and observed m/z. The calculation shows 0.086 ppm accuracy which has a good agreement with theoretical data. For higher confirmation, MS–MS data shown in Fig. 4c displays the fragmentation of taxine B in MS–MS condition. The peak at m/z 194.12 is the base peak of MS–MS fragmentation of

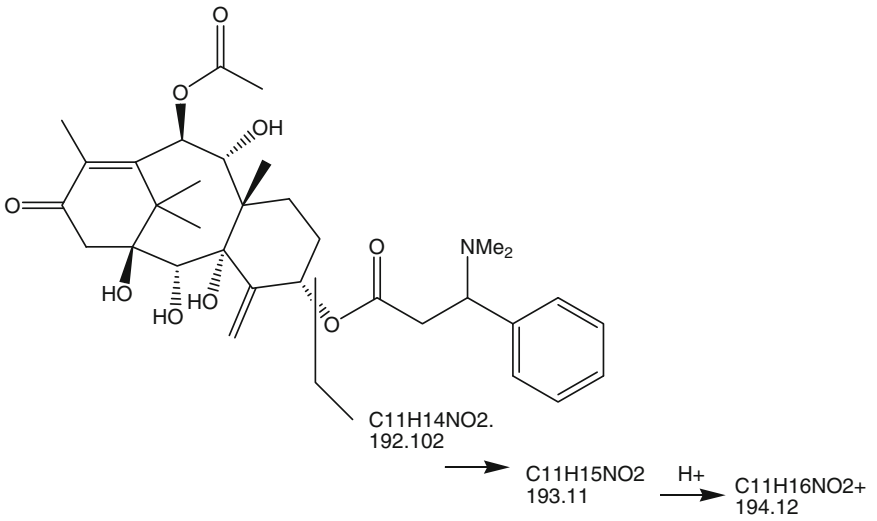

Fig. 5. Fragmentation of taxine B

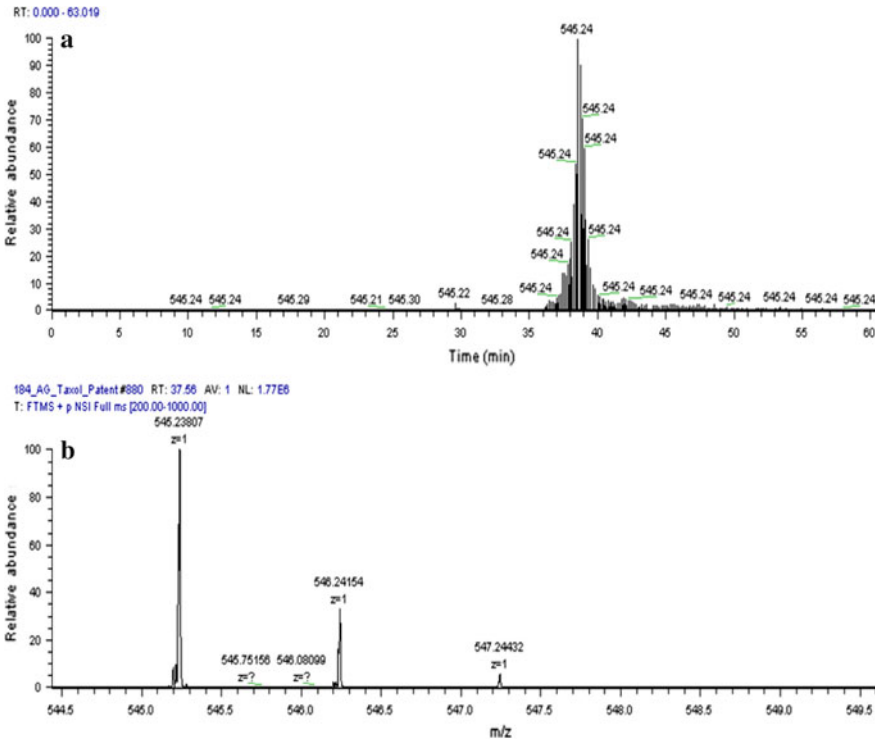

Fig. 6. Nano-FTMS results of 10-DAB III

m/z 584.32. The schematic fragmentation of taxine B is shown in Fig. 5. The TIC peak has a small valley in top of the peak in Fig. 4a. This phenomenon may be related to rearrangement of taxine B to isotaxine B [16].

The first peak in the semi-preparative LC was injected into the nano-LC–FTMS. The FTMS peak and its expansion is shown in Fig. 6 and a peak appeared at m/z 545.23807. The calculated ppm for 10-DAB was −0.11 which represents a good confirmation for 10-DAB.

The second peak in semi-preparative LC was analyzed by NMR spectroscopy. The observed results and reported data of ^{13}C NMR for taxine B are shown in Table 1. These results confirmed again

Table 1. Comparison of ^{13}C NMR data of taxine B with those of with Wiegerinck et al. [12]

Carbon number	Observed (ppm)	Theoretical (ppm)
1	77.9719	77.9
2	71.5155	71.6
3	46.8133	46.8
4	145.9814	144
5	78.1938	78.2
6	29.079	29.1
7	26.319	26.4
8	45.5071	45.6
9	75.3386	75.3
10	76.7436	76.6
11	–	153.5
12	–	139.1
13	–	199.9
14	44.4023	44.5
15	42.2517	42.3
16	20.3743	20.5
17	34.05	34.2
18	13.8787	13.9
19	17. 8062	17.9
20	117.6621	118.1
1′	–	171.3
2′	38.757	38.3
3′	–	66.5
COMe	–	170.2
MeCO	21.126	21.2
NMe_2	42.2517	42.3
1″	–	138.5
2″ & 6″	128.2383	128.2
3″ & 5″	128.6306	128.7
4″	–	127.6

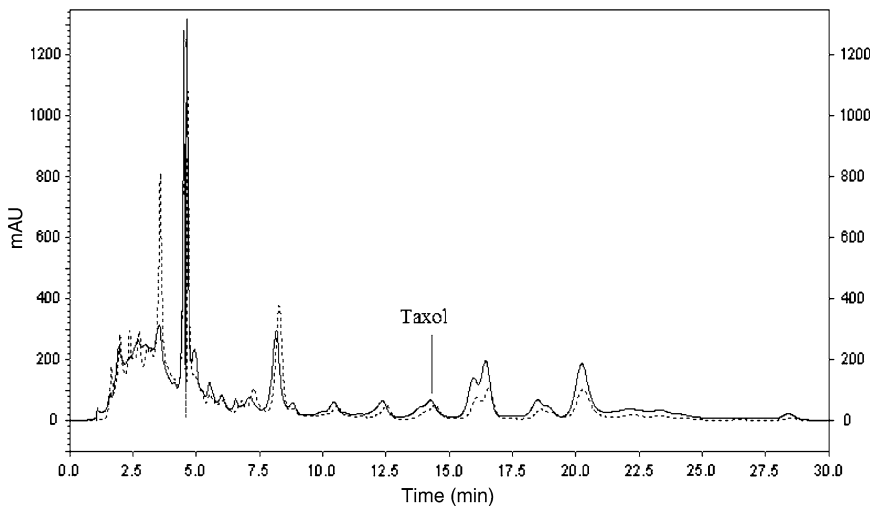

Fig. 7. The analyses of paclitaxel in plant residue after (solid line) and before (dotted line) aqueous extraction by Margraff [8] method (the separation conditions were the same as Fig. 2)

the structure of taxine B in aqueous extraction sample. The values were compared with data of Wiegerinck et al. [12] as shown in Table 1.

We developed an MAE method for extraction and isolation of paclitaxel from *Taxus baccata* L [9, 10]. For evaluation of the influence of aqueous extraction on the amount of paclitaxel, the plant material before and after aqueous extractions, were extracted by MAE and analyzed by analytical LC. Figure 7 shows the results of these analyses and the peak at 14.2 min belongs to paclitaxel. This result shows that the aqueous extraction has no significant effect on the amount of paclitaxel in the residual sample.

Conclusion

The reported method based on aqueous extraction was investigated for isolation of 10-DAB III from *Taxus baccata* L. Successfully aqueous extraction was performed and analytical LC showed two independent compounds. A semi-preparative LC was used for isolation of these compounds. These two compounds were screened by nano LC–FTMS and NMR and identified as taxine B and 10-DAB which were present in the aqueous solution. A microwave-assisted extraction method was developed for extraction and isolation of paclitaxel from *Taxus baccata* L. and applied to this sample and after aqueous extractions. Results showed that the aqueous extraction had no significant effect on the amount of paclitaxel in *Taxus baccata* L.

Therefore, the aqueous extraction method combined with MAE and preparative LC are considered to be efficient for obtaining 10-DAB III, taxine B and paclitaxel with high purity from *Taxus baccata* L.

Acknowledgments

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