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Immunoaffinity chromatography for the sample pretreatment of *Taxus* plant and cell extracts prior to analysis of taxanes by high-performance liquid chromatography

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Abstract

The application of immunoaffinity chromatography for the purification of *Taxus* plant and cell extracts prior to the HPLC analysis is described. Polyclonal antibodies raised against 10-deacetylbaccatin III (10-DAB III), paclitaxel's main precursor in plant, were characterised by enzymed-linked immunosorbent assay. Immunoglobulins from selected antisera were immobilised on CNBr-activated Sepharose 4B. The immunoaffinity column was used for the purification of plant and plant cell culture extracts prior to their analysis by HPLC. Immunoaffinity chromatography enabled the selective concentration of taxoids and enhanced sample clean-up. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: *Taxus* spp.; Immunoaffinity chromatography; Sample handling; Plant materials; Taxol; Paclitaxel; Deacetylbaccatin III; Taxanes

1. Introduction

Paclitaxel (Taxol) and docetaxel (Taxotere) are potent anticancer drugs derived from the *Taxus* tree. Paclitaxel is a natural diterpenoid whereas docetaxel is a semisynthetic derivative, produced from 10-deacetylbaccatin III (10-DAB III), paclitaxels main precursor in plant [1–3]. Paclitaxel and other taxane analogues occur in plant in very low quantities within a complex mixture, and their quantitative determination is accomplished almost exclusively by RP-HPLC [4]. Prior to HPLC, complicated sample preparation schemes have been deployed, often requiring more than 24 h for the processing of one sample. Bioassays have also been reported, but they were prone to cross-reactivity against paclitaxel analogues and especially cephalomannine [5–12]. Enzyme-linked immunosorbent assay (ELISA) and related methods have thus been used mainly for a semi-quantitative determination of paclitaxel and 10-DAB III in biological fluids and plant extracts, enabling a rapid and sensitive screening for taxanes in various samples. Tubulin polymerisation assays resulted in detection limits at μ*M* range [5,6],

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whereas ELISA provided much lower detection limits (0.3 n*M*) [7–11]. Suye et al. [12] reported on a receptor protein-based bioassay for the quantitative determination of paclitaxel. Paclitaxel occurring in the sample competed with horseradish peroxidase-labeled paclitaxel for tubulin binding sites. The assay was performed in microtitre plates similar to ELISA, and resulted to a linear range of 0.001–1 n*M*. The authors reported no interference with other taxanes (cephalomannine, baccatin III). The method was applied for the determination of paclitaxel in human plasma.

Until recently, *Taxus* bark has been the sole source for the supply of paclitaxel to be used clinically. However, other plant parts and plant cell tissue cultures extracts have been studied as alternative sources. From the authors previous work on the biosynthesis on taxanes in cell and cell tissue culture, it was shown that 10-DAB III is excreted in the cell culture medium [13,14]. Furthermore, the HPLC determination of taxanes [15] and solid-phase extraction (SPE) for the pre-treatment of *Taxus* plant and cell culture samples [16,17] resulted in fast and robust schemes for either screening or accurate determination of taxanes in plant and cell cultures. In contrast it was observed that liquid–liquid extraction of cell suspension medium lead to formation of emulsions which are difficult to break and require centrifugation, a time consuming and cumbersome step when processing large volumes. The major goal of the developed SPE method [16] was the straightforward application of the aqueous medium on the SPE column, lowering considerably the time and the cost of processed sample. However the complexity of the matrix and the low paclitaxel content in the extracts could hinder the quantification of the analytes.

Recently there has been increased awareness on the use of molecular recognition mechanisms in separations. Immunoaffinity chromatography (IAC) is based on the reversible and selective interaction of an antigen with its antibodies (IgGs mostly) which are immobilised on an appropriate support. IAC combines the high selectivity of the bioassays with the high-resolution power of separation techniques. The method can be applied either off-line, like a conventional SPE process, or on-line by a column-switching approach utilising a precolumn. Immunoaffinity chromatography has been used for the sample clean-up of aflatoxins, ochratoxin, triazine herbicides, oxytocin, veterinary drug residues, cannabinoids and many other target compounds in various types of samples [18–34].

The objective of this work was to develop an IAC procedure for the sample pretreatment of 10-DAB III in needles samples and cell tissue culture extracts. Rabbit antibodies were prepared against bovine serum albumin (BSA)–10-DAB III and BSA–paclitaxel conjugates. The IgG fractions were isolated by precipitation with ammonium sulphate and the antibodies were immobilised on Sepharose (Amersham Pharmacia Biotech). The immunoaffinity columns were used successfully for the purification of extracts from *Taxus* needles and cell culture extracts.

2. Experimental

2.1. Materials and methods

All organic solvents used were of analytical grade and water was of Milli-Q (Millipore) quality. Solvents used for HPLC analysis were of HPLC grade and were used as obtained. Aqueous buffers used as HPLC mobile phases were filtered through a 0.45 μm filter prior to use. Phosphate buffers were prepared as follows: 192 g Na_2HPO_4 and 35.1 g KH_2PO_4 were dissolved in 1600 ml water and the pH was adjusted to 7.4 with the addition of 1 *M* NaOH. Finally the volume was brought to 2 l by the addition of water.

2-*N*-Morpholineethanosulfonic acid (MES) and 1-ethyl-3-dimethylaminopropylcarbodiimide (EDC) were from Pierce (Rockford, IL, USA). Goat anti-rabbit horseradish peroxidase (GAR–HRP) was from Dako (Glostrup, Denmark). TMB Peroxidase substrate solution was from Kirkegaard & Perry Labs. (Gaithersburg, MD, USA). Ovalbumin (OVA), BSA and 10-DAB III were from Sigma (St. Louis, MO, USA). CNBr-activated Sepharose 4B was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Dialysis membranes (12.000–14.000) were from Medicell (London, UK). Paclitaxel was a kind gift from Dr. Erik van Rozendaal and Yew Tree Pharmaceuticals (Haarlem, Netherlands).

2.2. Synthesis, conjugation and antisera production

Succinyl paclitaxel and succinyl 10-DAB III were prepared as reported previously [10,11]. Prior to the synthesis, the reagents were dried overnight under vacuum. The reagent mixture, succinic anhydride (150 mg) and dimethylaminopyridine (10 mg), was dissolved in dried pyridine (2 ml) and added to 10-DAB III (150 mg) or paclitaxel (50 mg). The mixture was incubated at room temperature for 2.5 h. The reaction mixture was evaporated (under N_2) and dried overnight in high vacuum. The reaction products were purified by preparative TLC on silica gel with $CHCl_3$–MeOH (95:5) as mobile phase. The identity of the succinyl derivatives was confirmed by mass spectrometry and ^{1}H NMR spectrometry.

For immunisation, the succinyl derivatives were conjugated to BSA by the mixed anhydride method [35]. A 10 mg sample of the succinilated taxane (10-DAB III or paclitaxel) were dissolved in dimethyl sulfoxide (DMSO) (1 ml) and after the addition of tri-*n*-butylamine (50 μl), the mixture was cooled to 4°C. After 30 min, isobutylchloroformate (25 μl) was added and the mixture was left at 4°C for another 30 min. Then, the mixture was added drop wise to 3 ml of an aqueous solution of BSA (25 mg, pH 9.5 also cooled at 4°C). After mixing, the pH was adjusted to 7 by the addition of 1 *M* $NaHCO_3$ and the mixture was incubated overnight at 4°C. Finally, the solution was dialyzed against water for 3 days.

For the preparation of OVA–2′-succinyl 10-DAB III and OVA–2′-succinyl paclitaxel, an adaptation of the carbodiimide method was followed [10]. Succinilated taxane (5 mg) was dissolved in 0.1 ml DMSO and mixed with 0.5 ml water containing 10 mg of OVA. To this solution, 0.4 ml of the conjugation buffer (0.1 *M* MES, 0.9 *M* NaCl at pH 4.7) were added dropwise. Next 10 mg EDC were added dropwise and the mixture was left overnight at 4°C. The product was dialyzed against water for 3 days.

The two BSA-conjugated taxanes were administered each to three rabbits for immunisation. The conjugate dissolved in 0.9% NaCl was emulsified with an equal volume of Freud's complete adjuvant to give a final concentration of 1 mg/ml. This mixture was given in three intradermic injections. Boosting injections of Freud's incomplete adjuvant were made at 2-week intervals. Blood was collected 1 week after the third booster injection. After removal of the whole cells by centrifugation, the antisera were collected and stored at −20°C. The protein concentration of the purified antisera and protein conjugates were determined with the BCA assay (Pierce) with BSA as the standard.

3. ELISA

An ELISA was developed to study the binding characteristics of the antisera. Microtitre plates were coated with 150 ng/well of OVA–taxane conjugate (OVA–10-DAB III or OVA–paclitaxel) in 200 μl of coating buffer (0.1 *M* carbonate buffer, pH 9) and incubated overnight at room temperature. Plates were washed and then blocked with 0.1% casein in phosphate-buffered saline (PBS) (200 μl/well) for 2 h at room temperature. After washing, 100 μl/well of the serially diluted antiserum in dilution buffer [PBS containing 0.01% (w/v) Tween 80], were added to the plates. After incubation for 2 h and washing, 100 μl of GAR–HRP, diluted 1/10 000 in the dilution buffer, was added to each well. After incubation for 30 min at room temperature, the plates were washed and 100 μl of peroxidase substrate solution (TMB) was added to each well. After 15 min, the reaction was stopped by addition of 100 μl of 1 *M* H_3PO_4 and the absorbance of each well was measured at 450 nm in an Argus 400 microtitre plate reader (Canberra Packard, Downers Grove, IL, USA).

For competitive assays methanolic solutions of paclitaxel and 10-DAB III were seven serially diluted in PBS and mixed (1:1, v/v) with an appropriate dilution of the antiserum. The mixture was incubated for 30 min at room temperature, and 100 μl of each mixture was added to each pre-coated well and the above described assay was followed.

3.1. Preparation of IAC columns

IgGs were collected from the antisera after precipitation with $(NH_4)_2SO_4$. The antisera (10 ml) were mixed with 20 ml PBS, 30 ml of saturated $(NH_4)_2SO_4$ were slowly added and the mixture was incubated at room temperature for 30 min. Next, the

mixture was centrifuged at 8000 rpm for 10 min, the supernatant was removed and the pellet (IgGs) was reconstituted in PBS. The concentration of IgGs in this solution was determined with the BCA assay, in order to find the amount of Sepharose to be used.

Preparation of the affinity media was performed following the manufacturer's instructions (Amersham Pharmacia Biotech). To 1.5 g of CNBr-activated Sepharose 4B, 5 ml of 1 m*M* HCl was added and the swollen gel was poured into a glass column. The gel was washed with 300 ml of 1 m*M* HCl. The IgG (1 mg) was dissolved in 8 ml of the coupling buffer (0.1 *M* $NaHCO_3$ containing 0.5 *M* NaCl) and the resulting solution was gently stirred overnight at 4°C with the Sepharose gel. To remove excess IgG, the slurry was washed with five gel volumes of the coupling buffer. Protein determinations showed that IgG was not present in these fractions. Remaining active groups on the Sepharose base material were blocked with 0.1 *M* Tris–HCl buffer (pH 8) and the gel was washed with four cycles of alternate pH: 0.1 *M* acetate buffer (pH 4) and 0.1 *M* Tris–HCl buffer (pH 8), both containing 0.5 *M* NaCl. Finally, the gel was re-suspended in 0.1 *M* Tris–HCl buffer (pH 8) and the gel was cautiously transferred to an empty polypropylene SPE cartridge. The upper frit was placed and a light vacuum (10 p.s.i.; 1 p.s.i.= 6894.76 Pa) was applied to assist the formation of a constant bed of the gel. The same protocol, without the IgG, was followed in order to obtain a "control" Sepharose column.

3.2. Chromatographic conditions

Chromatographic analyses were performed in two HPLC systems: A Spectra-Physics system, consisting of an SP8800 pump, a Spectra Chrom 100 UV detector operating at 227 nm, an SP 4290 integrator and a Rheodyne injection valve equipped with a 10 μl loop. Separations were performed on a Taxsil-3, 250×4.6 mm, 5 μm column (kind gift from Metachem Technologies, Torrance, CA, USA).

A Waters (Milford, MA, USA) system, consisting of a 600E pump, a Waters 440 UV detector working with a 254 nm UV filter, a Waters Wisp 712 autosampler (10 μl injection volume), and a Shimadzu CR6A (Kyoto, Japan) integrator. Separations were performed on an Alltech Altima C_{18} column 250×4.6 mm, 5 μm (Alltech, Deerfield, IL, USA).

3.3. Plant sample pre-treatment

Plant samples were treated as reported previously [16]. Needles (5 g) were ground in a blender and the material was homogenised with 20 ml methanol. The methanolic extract was defatted with 10 ml *n*-hexane. An aliquot (200 μl) of the methanolic fraction was mixed with 1.8 ml of water and applied to a C_{18} cartridge (Supelco, Bellefonte, PA, USA). The cartridge was subsequently washed with 2 ml portions of water, 20 and 40% of MeOH in water. The taxanes were eluted with 2 ml of 80% MeOH in water.

3.4. Immunoaffinity procedure

The affinity column was rinsed twice with 2.5 ml water. Methanolic extract (200 μl) was mixed with 2 ml of PBS (pH 7.4) and was passed through the column by gravity only. After 5 min, the column was dried by vacuum. To wash away any substances remaining in the column, or substances attached to the column by non-specific interactions, the column was rinsed with 5 ml water followed by 2 ml of PBS–MeOH (3:1, v/v). The taxanes were eluted with 2 ml of an appropriate solvent (see Results and discussion). The obtained fraction was evaporated to dryness at 55°C (in a water bath), under a stream of N_2. The residue was dissolved in 100 μl of the mobile phase and 10 μl was injected into the HPLC system.

After usage, the immunoaffinity column was rinsed with 5 ml water and 5 ml PBS. When not used for a period longer than a day, the column was stored at 4°C, with PBS containing 0.02% (w/v) of sodium azide, to prevent mould and bacterial growth.

4. Results and discussion

4.1. HPLC analysis

A fast, simple and reliable isocratic HPLC method for the separation and quantification of 10-DAB III and paclitaxel was aimed for. Adaptation of existing

methods (other columns and new instrumentation), already developed by the authors [15], was sufficient. Using the Alltima column with a mobile phase consisting of methanol–acetonitrile–0.1 *M* aq. ammonium acetate (44:22:34, v/v/v) and a flow-rate of 0.8 ml/min, the two diterpenes were separated in sharp peaks within reasonable times (retention time of 5.7 min for 10-DAB III and 33.3 min for paclitaxel). Similar retention times (5.4 min for 10-DAB III and 21.4 min for paclitaxel) were obtained with the Taxsil-3 column, using a mixture of acetonitrile–0.05 *M* aq. ammonium acetate (45:55, v/v) as mobile phase and a flow-rate of 0.8 ml/min. Detection limits (defined as twice the noise) obtained with the Taxsil-3 column were 8 and 15 ng/ml for 10-DAB III and paclitaxel, respectively.

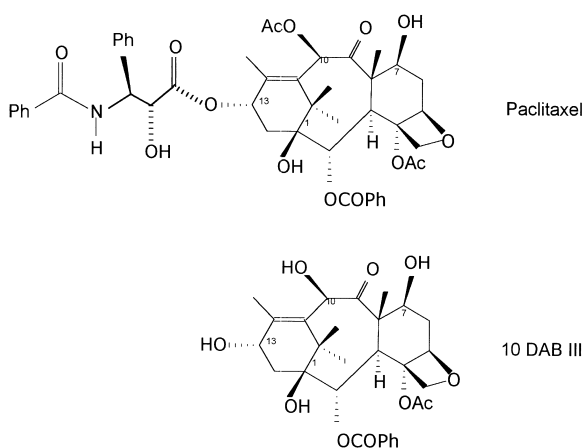

Fig. 1. Molecular structures of paclitaxel and 10-DAB III.

4.2. Antibody validation

In ELISA, the paclitaxel antibodies from the three animals showed interaction with the OVA–paclitaxel conjugate with medium titres (1:4000 to 1:6000). However, no competition with free paclitaxel or 10-DAB III was observed and the antibodies showed no binding to OVA. Thus, the antibodies recognised the paclitaxel–carrier protein conjugate (paclitaxel–OVA) much stronger than the free paclitaxel and, unfortunately, they were useless for the development of an ELISA for the determination of taxanes in real samples. Nevertheless, the anti-taxol sera were used for the preparation of an immunoaffinity column where there is no competition with a paclitaxel–carrier protein conjugate. However, with this anti-paclitaxel immunoaffinity column binding of taxanes was not observed and the use of the anti-paclitaxel column was omitted.

One of the anti 10-DAB III sera showed a high titre (1:160 000) in the ELISA. As shown in Fig. 1, the 10-DAB III molecule encompasses the central taxane ring and, when compared to paclitaxel, 10-DAB III is a smaller and more polar molecule. Thus, it was anticipated that the anti-10-DAB III sera would exhibit cross-reactivity with other more complex taxanes. As reported previously for several taxanes [10,11], some cross-reactivity with paclitaxel was indeed observed. The specificity of polyclonal antibodies has proven to be dependent on the way of conjugation of the hapten to the carrier protein. Grothaus et al. [7] reported that paclitaxel conjugated at the C-7 atom, elicited antibodies specific for the C-13 side chain. These antibodies did not cross-react with 10-DAB III (which lacks the C-13 side chain, but cross-reacted with other taxanes bearing a modified C-13 side chain (cephalomannine). Guo et al. [9] studied the succinilation of 10-DAB III at both the C-7 and the C-10 atom. Antibodies raised against C-7-DAB III conjugates provided a less sensitive ELISA compared to antibodies raised against C-10-DAB III conjugates. Cross-reactivity is a major problem in ELISA and other quantitative analytical assays. In contrast, for extraction or isolation procedures, cross reactivity is not a problem, provided that a separation technique is employed subsequently. In fact the use of polyclonal antibodies that recognise selectively a group of related compounds, may offer an elegant mode to isolate and purify these compounds from complex matrices. Therefore, cross reactivity is sometimes considered an advantage rather than a problem for screening purposes [34].

4.3. Immunoaffinity chromatography

Initial studies to find conditions for the elution of antibody-bound 10-DAB III were performed in microtitre plates. The 96-well plate format facilitated a high through-put multivariate optimisation. Therefore, within the described ELISA protocol, 50 μl of different organic solvents and chaotropic mixtures were mixed with 50 μl of diluted antibodies in the

microtitre plate and the ELISA protocol was followed. The tested solvents were: 6 *M* guanidine–HCl (pH 1.5), 0.1 *M* glycine–HCl (pH 1.5), 0.1 *M* glycine–NaOH (pH 10), 1 and 2 *M* acetic acid, 0.75 and 1 *M* NaCl and varying percentages of organic solvents (methanol, acetonitrile, dioxane, acetone, ethanol and ethylene glycol) in water and acetic acid solutions.

During this experiment, the binding of antibodies to the microtitre plate in the presence of these solvents was compared with the binding of antibodies in PBS (total binding). A strong reduction of absorbance indicated a potential solvent to be used as elution solvent in IAC.The best result (strong reduction of absorbance) was obtained with a mixture of MeOH–1 *M* acetic acid (4:1, v/v) and it was anticipated that this mixture would also prove to be a potent solvent for the elution of 10-DAB III bound to the immunosorbent. This was confirmed as this solvent resulted in a quantitative elution of the bound 10-DAB III from the immunosorbent. Further experiments were made with varying percentages of methanol (20, 40, 60 and 80%) in water. Increasing the percentage of methanol resulted in a better elution of 10-DAB III from the IAC column. However, the best performing MeOH–water mixture (80% MeOH) did not reach the yields obtained with the selected MeOH–acetic acid mixture. These results indicate that the binding of taxanes to the antibodies is a combination of hydrophobic and ionic interactions. The recovery of 10-DAB III from the IAC column was studied over a concentration range representative of the needs of a taxane analysis HPLC protocol [4,15]. A fixed volume (200 μl) of different solutions of 10-DAB III with concentrations ranging from 0.4 to 32 μg/ml was loaded on the IAC and the collected fractions were analysed by HPLC. Recoveries found were adequate (69–82%) as shown in Table 1.

Table 1
Recovery of the immunoaffinity extraction (mean value of four assays)

10-DAB III (μg/ml)		Recovery (%)
Added	Found	
0.4	0.32±0.025	80
0.8	0.61±0.050	75
2	1.39±0.082	69
4	3.28±0.229	82
8	6.24±2.624	78
16	12.05±0.843	75
32	19.20±1.595	60

In order to demonstrate that the binding of 10-DAB III to the IAC column was due to the immobilised antibody and not due to non-specific interactions, the same extraction protocol was applied to the control column. Solutions of paclitaxel (44 μg/ml) and 10-DAB III (32 μg/ml) were transferred to both columns and the results are given in Table 2 (mean values from three determinations). For 10-DAB III, close to 60% of the added amount was recovered in the final elution from the immunoaffinity column. With the control column, the major portion was washed away either during the loading or during the washing steps. As can be seen in Table 2, the total recovery, i.e. the sum of the recoveries observed in all the fractions did not reach 100% and ranged from 53 to 68%. The reason is not fully understood. To investigate whether the taxanes were quantitatively recovered from the IAC column or not, the column was further eluted with the elution solvent (three times with 2 ml of MeOH–1 *M* acetic acid, 4:1) and more lipophilic solvents (2 ml chloroform, 2 ml dichloromethane). However, traces of the taxanes were not found in these fractions. Therefore, it was assumed that the the lower recoveries were caused by the decomposition of the analytes during the prolonged evaporation of the aqueous elution solvent.

As mentioned above, the anti 10-DAB III anti-sera cross-reacted with paclitaxel in ELISA. Therefore, retention of this compound on the IAC was expected and observed with standard solutions (see Table 2). However, the performance of the IAC column was not substantially different compared to the control column. Therefore, it was assumed that the binding of paclitaxel was governed by non-specific interactions to both the IAC and the control column.

Sample clean-up is a major problem in the analysis of taxanes in complex samples such as in plant and cell tissue cultures. In most cases, the problem deals with the quantitative determination of 10-DAB III, which is the most polar taxane. Thus, in reversed-phase HPLC systems, 10-DAB III is typically eluted early and close to interfering peaks. From earlier results it was observed that SPE, although a

Table 2
Mean recovery of paclitaxel (8.8 μg) and 10-DAB III (6.4 μg) on the anti-10-DAB III column (IAC) and the control Sepharose column

Fractions	Recovery (%)			
	Paclitaxel		10-DAB III	
	IAC	Control	IAC	Control
Loading	3.4	0	0	16.5
Wash water	1.9	2.2	0	27.3
Wash PBS–MeOH (3:1)	23.4	34.2	7.4	10.3
Elute MeOH–1 *M* acetic acid (4:1)	24.2	18.2	59.9	5.1

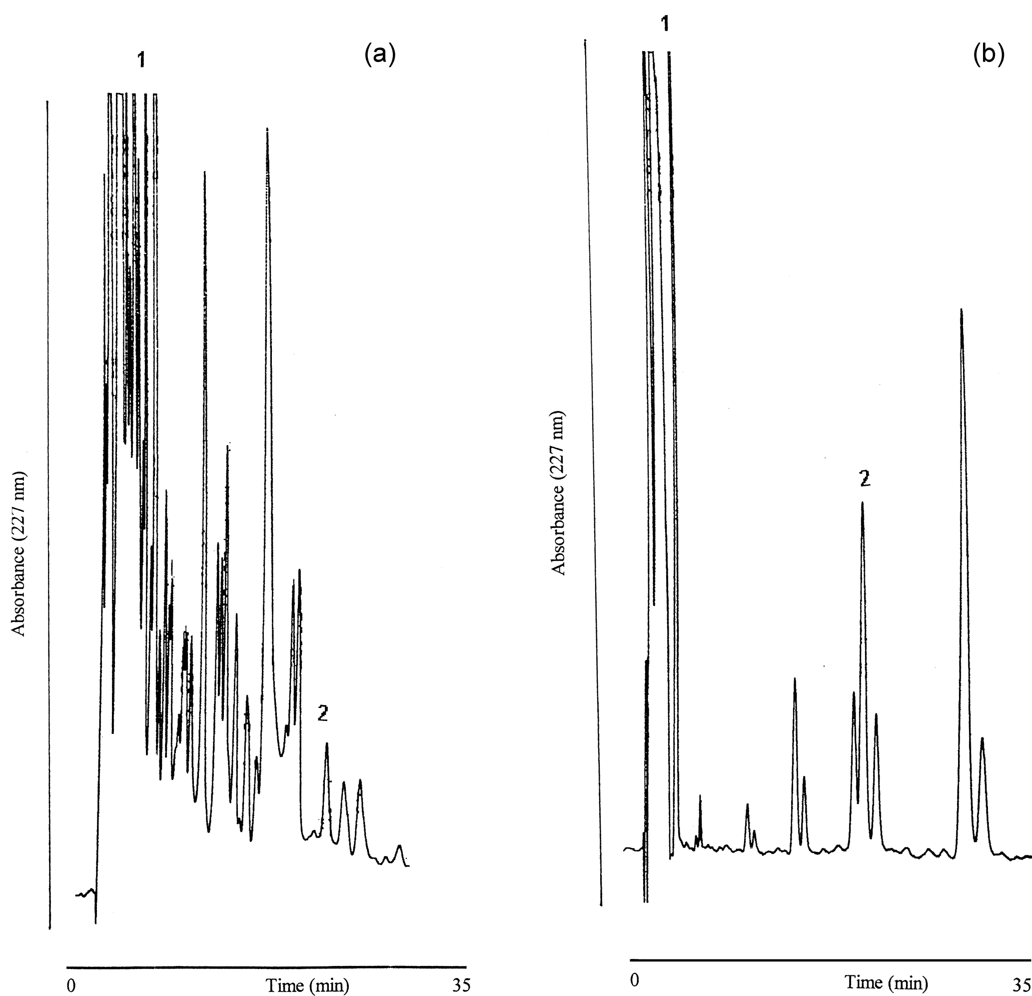

Fig. 2. Enhanced sample purification after immunoaffinity extraction. HPLC analysis of *Taxus* baccata needles extract following SPE (A) and immunoaffinity extraction (B). Peaks correspond to (1) 10-DAB III: 5.38 min and (2) paclitaxel: 21.97 min. Analysis on the Taxsil-3 column. Chromatographic conditions as in the Experimental section (Spectra-Physics system).

powerful tool for the analysis of a wide range of *Taxus* samples [16], when applied to certain types of calli extracts, failed to provide adequate clean-up prior to the HPLC analysis. Hence in a few cases the peak of 10-DAB III was covered by a large complex of co-eluting peaks, disabling quantitative or sometimes even qualitative aspects. However, application of ELISA revealed the presence of 10-DAB III in high amounts in these extracts [14]. Clean-up of *Taxus* plant and cell culture extracts was substantially enhanced by the use of an IAC column. For *Taxus* needles SPE on C_{18} followed by HPLC analysis resulted in obscured chromatograms (Fig. 2A). In contrast IAC provided much clearer chromatograms (Fig. 2B).

5. Conclusions

The objective of this research was the development of an IAC protocol for the analysis of *Taxus* needles and cell tissue culture extracts. It is demonstrated that IAC can offer enhanced clean-up of *Taxus* needles and thus facilitate the quantitative determination of 10-DAB III in samples that proved difficult and cumbersome to analyse after pretreatment with conventional SPE. The recovery after IAC is approx. 80% within the working concentration range of a conventional HPLC assay.

Due to their matrix complexity, *Taxus* needles were regarded as an attractive, but non-realistic alternative source for paclitaxel production. Complex sample preparation required hindered any interest for their exploitation. A further obstacle was the extremely low amount of paclitaxel in the sample. Employment of molecular recognition mechanisms may pave the way in order to overcome such limitations. The developed method may prove of interest in the light of the increased interest in both 10-DAB III and renewable taxane sources such as needles, and cell culture. 10-DAB III exists in needles and plant cell culture in at least ten times larger quantities than paclitaxel. Hence, semi-synthesis from 10-DAB III is expected to be the pipeline for the production of paclitaxel and docetaxel. Development of methods with enhanced selectivity for the isolation of 10-DAB III from plant material may be of value in the evolution of the above approaches. Future research in this area could be on the development of affinity matrices (immunoaffinity media and molecularly imprinted polymers) to be used for in situ trapping and isolation of taxanes of high value, from cell tissue culture or other renewable sources.

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