Biological Fingerprinting Analysis of Interaction Between Taxoids in Taxus and Microtubule Protein by Microdialysis Coupled with High-performance Liquid Chromatography/Mass Spectrometry for Screening Antimicrotubule Agents

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Abstract Some natural products, such as traditional Chinese medicines (TCMs), contain compounds with anticancer activity and have attracted a great interest in recent years as alternative anticancer therapies. A quick and convenient assay for screening antimicrotubule compounds in which in vitro microdialysis/high-performance liquid chromatography (HPLC) is used to monitor the binding of the compounds extracted from TCM Taxus cuspidata Siebold & Zucc(Taxus) to microtubules is reported. It was observed that the extract of Taxus contains at least five compounds which have affinity interaction with microtubules by biological fingerprinting analysis, and they were identified as the taxoids of taxol, baccatin III, 10-deacetyl baccatin III(10-DAB), cephalomannine and 7-epi-10-deacetyl taxol (7-epi-10-DAT) based on the comparison of their high-performance liquid chromatographic/mass spectrometric and UV spectra with those of the standard samples, both assembly-promoting and disassembly-inhibiting characteristics of those compounds were evaluated. It was observed that baccatin III and 10-DAB bound to microtubules and the binding degrees were influenced by GTP. Competitive binding behavior of taxol with other four taxoids to microtubules was also investigated.

Keywords Microdialysis/high-performance liquid chromatography(HPLC); Biological fingerprinting analysis; Taxoids; Microtubule; Taxus

1 Introduction

Some natural products, such as traditional Chinese medicines (TCMs), are currently being promoted for clinical use in cancer therapy. Nevertheless, it is conceivable that they represent a rich source of biologically active compounds and present an example of molecular diversity, with recognized potential in drug discovery and development. Many natural products, for example certain herbal medicines could have potent anti-cancer properties¹,². It is possible to isolate a number of homologues and obtain structure-activity information by means of the analytical chemistry of medicine or combinatorial approaches.³ Taxus is an herb that has been used in China for several hundred years for the treatment of gastroenteritis, rheumatism leucocytokiaemia and carcinoma. It is usually prepared as a herbal extract from the needles and leaves of the yew tree, Taxus cuspidata Siebold & Zucc. Extracts of Taxus have been shown to contain essential components, like taxol(pacilitaxel) and the other taxoids which are regarded as well-known and potent anti-cancer components.⁴

In recent years, many taxane-type diterpenoids have been found in various yew trees⁵,⁶ and some of them exhibit interesting biological activities⁷,⁸. Taxus cuspidata is a low trailing shrub or a tall tree ubiquitous in the northeastern region of People’s Republic of China, Korea, Japan and Quebec regions of Canada. The composition has been extensively invested⁹–¹¹. Although more than 200 taxanes have been isolated from the needles, stem, bark, and seeds of T. cuspidata, there are still new taxoids waiting isolation and struc-
tural elucidation. The isolation of new and minor taxanes is important in the investigation of the plant biosynthesis and metabolism of these compounds\cite{12}. Taxoids are diterpenoid natural products and usually interact with the microtubule system. Microtubules are central to a number of cellular processes including the formation of the mitotic spindle composed of a backbone of tubulin dimmers. Their importance in mitosis and cell division makes microtubules an important target for anticancer drugs. In taxoids, the most clinically useful product with this characteristic is taxol, which can promote tubulin dimmers assembly to microtubules even under otherwise unfavorable conditions and also inhibits depolymerization\cite{13}. This mechanism is different from those of other antimicrotubule agents, such as vincristine or podophyllotoxin, which is bound to the protein tubulin in the mitotic spindle, preventing polymerization and assembly into microtubules\cite{14}. Now, taxol has emerged as one of the most active anticancer agents in clinic for the chemotherapy of ovarian, breast, and non-small-cell lung cancer\cite{15-17}.

Microdialysis is a technique based on the use of an artificial capillary vessel developed as a sampling tool in neurobiology researches. The membrane of microdialysis probe allows the determination of the concentrations of unbound small molecules after a dialysis membrane has been placed in the tissue of drug-protein mixed solution. This sampling technique is easy to be automated and can be on-line hyphenated with analytical techniques, such as HPLC, mass spectrometry(MS), capillary electrophoresis(CE)\cite{18}. Microdialysis has become a widely used technique in physiology, pharmacology, and analytical chemistry for the recovery of exogenous substance. Recently, a concept of biological fingerprinting analysis was proposed to characterize the binding property between the multiple compounds in natural products or TCMs and larger biomolecules by biochromatography and biochromatography coupled with microdialysis/ultracentrifugation\cite{19-23}.

Here is reported the application of microdialysis sampling coupled with HPLC to study the binding characteristics of the compounds in the extract of Taxus with microtubules through biological fingerprinting analysis. As a complex sample, the extract of Taxus contains many components; this method allows a rapid determination of the change between concentrations of control and interaction samples in small solution volumes. In the experiment, multiple bioactive components interacted with microtubules can be simultaneously discerned. It has paved the way for screening and analyzing the anticancer compounds in TCMs, and may be extended to the study of the interaction of the extract of TCMs with target protein. Biological fingerprinting chromatogram can provide the insight into the interactions of multi-components in Taxus extract with microtubules at a molecular level, and the competitive interaction of the taxol with other active compounds in Taxus with microtubules was also studied.

2 Experimental

The needles and leaves of Taxus and the standards of taxol, baccatin III, 10-deacetylbaccatin III(10-DAB), cephalomannine and 7-epi-10-deacetyltaxol(7-epi-10-DAT) were purchased from Xi’an Guanyu Biotechnology Co. Ltd(Xi’an, China). Guanosine-5’-triphosphate(GTP) was purchased from Hangzhou Meiya Biotechnology Co. Ltd.(Hangzhou, China). The bicinchoninic acid(BCA) protein assay kit was purchased from Pierce Chemical(Rockford, IL, USA). Acetonitrile was of HPLC grade, Distilled water was further purified by Milli-Q system(Millipore, Milford, MA, USA). Other chemicals were of analytical grade.

2.1 Preparation of Taxus Extract

Powered plant material(2 g) was extracted twice with 20 mL of 90% ethanol by soaking with agitation for 16 h. The combined extract(50 mL) was evaporated to dryness, and the residue was partitioned three times between water(4 mL) and methylene chloride(8 mL). The organic fraction was evaporated and the residue was extracted with 20 mL of hexane. The hexane portion was discarded. The residue was extracted with methylene chloride(15 mL). The methylene chloride extracts were evaporated and dissolved in ethanol to produce a solution with a concentration of 20 mg/mL.

2.2 Preparation of Microtubule Protein

Fresh pig brain microtubule protein was prepared via two cycles of temperature-dependent assembly/disassembly by means of a Beckman L8-70M ultracentrifuge(Fullerton, CA, USA). In the first cycle, the homogenate of the brain tissue in 0.1 mol/L
MES[2-(N-Morpholino)ethanesulfonic acid] buffer, pH 6.5, containing 1 mmol/L EGTA(ethylene glycol-bis-N,N,N',N'-tetraacetic acid) and 0.5 mmol/L MgCl$_2$ was centrifuged at 100000 $g$ for 45 min at 0 °C in a Ti-60 rotor. The second supernatant was diluted with MES buffer containing 8 mol/L glycerol, for a final GTP concentration of 1 mmol/L and glycerol concentration of 4 mol/L. Tubulin dimmers assembled for 30 min at 37 °C were collected by centrifugation at 100000 $g$ for 45 min at 25 °C in a Beckman Ti-60 rotor. In the second cycle, the same step in the first cycle was repeated to get purified protein[24]. The protein obtained was stored at −80 °C in 0.1 mol/L MES buffer. Prior to use, the microtubule protein was gently thawed, centrifuged at 5000 $g$ for 10 min at 4 °C, and then purified by desalting and removing glycerol with the Sephadex G-25 columns. The concentrations of tubulin were determined by the BCA method. The increase and the decrease in the turbidity of microtubule polymerization and depolymerization were monitored by the UV absorption spectra on a Jasco-550 spectrophotometer(JASCO, Kyoto, Japan) interfaced with a computer. The temperature in the cell was maintained at 37 °C in a water bath.

2.3 Interaction of Taxus Extracts with Microtubule Protein

Taxus extract was dissolved in ethanol. An appropriate volume of this stock solution was put in a 5 mL open vial, and filtered through a 0.45 µm membrane and evaporated by a Thermo Savant SpeedVac Concentrator(Thermo, MA, USA). Then the dry extract of Taxus was dissolved in 1 mL of DMSO (dimethyl sulfoxide). Before addition of it to microtubule protein solution, the extract solution was diluted 10 times by 0.1 mol/L MES buffer and 100 µL of the diluted extract was added to 900 µL of 10 mg/mL microtubule protein solution(in 0.1 mol/L MES buffer of pH 6.5, and 1 mmol/L GTP) to produce the interaction solutions.

2.4 In vitro Microdialysis Sampling

The in vitro microdialysis system comprised of a 74900 Micro-injection pump from Cole-Parmer Corporation(Chicago, IL, USA) and a 10 mm homemade microdialysis probe using the regenerated cellulose, hollow fibers membrane(0.200 mm i.d., 0.216 mm o.d.) from Spectrum Medical Industries(LA, CA, USA) with molecular weight cut-off at 18000 Da. The perfusion solution was a 0.1 mol/L MES buffer of pH 6.5 containing 1 mmol/L EGTA, 0.5 mmol/L MgCl$_2$ and 1 mmol/L GTP, perfusion rate was 1 µL/min. The Taxus extract-microtubule protein mixture solution and extract solution without microtubule protein were incubated at 37 °C in a water-bath for 30 min, and then the microdialysis probe was put into those solutions to take samples from those solutions. After 15 min’s washing of microdialysis probe with the perfusion solution at a flow rate of 4 µL/min, the dialysate was collected for 60 min at a perfusion flow rate of 1 µL/min. The collected dialysate was used for HPLC analysis.

2.5 HPLC Analysis

HPLC analysis was performed on a Waters HPLC system consisting of two 515 pumps(Milford, MA, USA), a Rheodyne-type injector valve with a 20 µL loop, a Waters 2487 dual wavelength UV absorbance detector(Milford, MA, USA), and the analytical data were based on WDL-95 chromatographic workstation(National Chromatographic R&A Center, Dalian, China). The HPLC analysis conditions were that the dimensions of the column were 250 mm ×4.6 mm i.d. packed with 5 µm Kromasil-ODS (Kromasil, Sweden). The mobile phases were acetonitrile(A) and water(B). A gradient elution was performed by adopting 10% A for 5 min, a linear gradient elution from 10% to 25% A for 20 min, then a second gradient elution from 25% to 95% A for 50 min, the final composition was maintained for 10 min. The flow rate was set at 1 mL/min. The detection wavelength was set at 227 nm.

The atmospheric pressure chemical ionization(APCI)-MS(Shimadzu, Kyot o, Japan) conditions for detection of analytes were that nitrogen gas flow rate was 25 L/min, APCI temperature was 400 °C, CDL temperature was 230 °C, block temperature was 200 °C and detector voltage was 1.8 kV. The mass data were expressed based on the control software LC-MS Solution Version 2.02(Shimadzu, Kyoto, Japan).

3 Results and Discussion

3.1 Identification of Active Components in Taxus

In the experiment, microdialysis sampling allowed the determination of the concentrations of un-
bound drugs after a microdialysis probe had been placed in the Taxus extract-tubulin mixed solution. The compounds with binding activity to microtubules were confined out of the membrane of microdialysis probe and collected in the dialysate. Fig. 1 shows the biological fingerprinting chromatograms at a detection wavelength of 227 nm for the extract of Taxus and standards of five taxoids. After HPLC separation, the peak areas of the compounds with binding activity to microtubules decreased in the biological fingerprinting chromatogram; the representative chromatograms reveal that there are at least five components in the dialysate of Taxus extract to interact with microtubule protein.

Fig. 1 Biological fingerprinting chromatograms for the extract of Taxus
Chromatograms for 20 μL microdialysate from the mixed solution of extract with blank buffer and the mixed solution of extract with microtubules proteins are indicated as black(A) and grey(B) curves, respectively. (C) the comparison chromatogram. (D) the chromatogram of standards.

The binding degrees of the components could be expressed by the decreased percentage of peak area calculated via the following equation.

\[ \text{Binding degree} = \left( \frac{A_c - A_s}{A_c} \right) \times 100\% \] (1)

where, \( A_c \) and \( A_s \) are the peak areas of a compound in solution before and after the interaction with microtubule protein in the chromatograms. The binding degrees of the compounds in Taxus extract are listed in Table 1.

Table 1 Binding degrees of the Taxol and other four taxoids in Taxus extract to microtubules and relative recoveries of them by microdialysis sampling

<table>
<thead>
<tr>
<th>Compound</th>
<th>Binding degree (% , 37 °C)</th>
<th>Relative recovery (%)</th>
<th>RSD (% , n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taxol</td>
<td>57.0</td>
<td>62.6</td>
<td>2.1</td>
</tr>
<tr>
<td>Baccatin III</td>
<td>12.1</td>
<td>81.0</td>
<td>1.4</td>
</tr>
<tr>
<td>10-DAB</td>
<td>10.5</td>
<td>83.2</td>
<td>1.7</td>
</tr>
<tr>
<td>Cephalomannine</td>
<td>12.8</td>
<td>60.5</td>
<td>1.9</td>
</tr>
<tr>
<td>7-epi-10-DAT</td>
<td>16.2</td>
<td>77.8</td>
<td>2.3</td>
</tr>
</tbody>
</table>

The more the percentage decreases, the stronger the interaction of the compound with microtubule protein is in the experiment. The components have been identified based on the comparison of retention time and UV spectra, together with their molecular formulas and molecular weight determined by HPLC-MS(APCI) with those of the standard samples. Taxol and the other taxoids were reported as main anticancer compounds present in Taxus and taxol was regarded as a potent antimicrotubule compound. It was observed that the molecular ion signal of peak A in APCI-MS spectrum is at 854.1 Da and its maximum UV absorption wavelength is 229 nm. By comparison of the above-mentioned data with those of the standard compound of taxol, peak A which has a big decrease in area in the chromatogram of the extract of Taxus after the interaction of it with microtubule protein could be confirmed as taxol. The molecular ion signal of peak B is at 810.3 Da and its maximum UV absorption wavelength is 228 nm. Comparison of above-mentioned results with those of standard sample shows peak B could be identified as 7-epi-10-deacetyltaxol(7-epi-10-DAT). Peak C, the retention time and UV absorption chromatogram of which are the same as those of cephalomannine standard, its molecular ion signal is at 832.2 Da, can be identified
as cephalomannine. Also based on the comparison of retention time by HPLC-UV and MS spectra of the standard sample, the peak D with molecular ion signal at 587.1 Da and the maximum UV absorption wavelength at 232 nm was confirmed as baccatin III. Peak E, the last peak, which has a decrease in area in the chromatogram of Taxus extract to interact with microtubule is identified as 10-deacetylbaccatin III (10-DAB), for its molecular ion signal is at 543.2 Da; the maximum UV absorption wavelength at 232 nm and the retention time are equal to those of 10-DAB standard compound. Typical APCI-MS and UV spectra for the peaks A—E are shown in Fig.2 and Fig.3. The molecular structures of the compounds identified for peaks A—E are shown in Fig.4. These taxoids were also reported to have different degree interactions with microtubule protein previously\cite{25,26}. The calibration curves for the quantitative determination of taxol and the other four taxoids have been measured, and the obtained results are presented in Table 2.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Calibration curve</th>
<th>slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taxol</td>
<td>$A = 16723 + 1.678 \times 10^7 c$</td>
<td>0.9997</td>
</tr>
<tr>
<td>Baccatin III</td>
<td>$A = 196353 + 6.279 \times 10^6 c$</td>
<td>0.9998</td>
</tr>
<tr>
<td>10-DAB</td>
<td>$A = 127800 + 5.693 \times 10^6 c$</td>
<td>0.9997</td>
</tr>
<tr>
<td>Cephalomannine</td>
<td>$A = 735000 + 8.059 \times 10^5 c$</td>
<td>0.9999</td>
</tr>
<tr>
<td>7-epi-10-DAT</td>
<td>$A = 16551 + 1.662 \times 10^5 c$</td>
<td>0.9999</td>
</tr>
</tbody>
</table>

* $c$ is the concentration of Taxol and taxoids(µmol/L) and $A$ for the corresponding peak areas.

Fig.2  Mass spectra using positive ion mode for detection of peak A(A) and negative ion mode for detection of peaks B(B), C(C), D(D), and E(E)

Background of all MS spectra was subtracted.
3.2 Recovery of Microdialysis

In the microdialysis sampling, the equilibrium is not established penetrating the probe membrane under the influences of perfusion rate and temperature. The concentration of sample collected by microdialysis probe is a fraction of the actual concentration of the outside solution. So, the recovery of the probe membrane is a very important parameter for microdialysis experiment, which can also be called the dialysate extraction factor. It can be expressed as in the following equation:

$$R = \left( \frac{A_c}{A_a} \right) \times 100\%$$  \hspace{1cm} (2)

where, $R$ is the peak area recovery, $A_c$ is the peak area of the compound in the dialysate and $A_a$ is the peak area in the chromatogram. Thus, the concentration of compounds in dialysate and solution after interaction with microtubule protein can be calculated by Eq.(1) and calibration curve. Recoveries of taxoids in Taxus extracts as listed in Table 1, were affected by factors, such as the chemo-physical properties of microdialysis probe membrane, perfusion temperature and perfusion flow rate.\(^{[27]}\) Usually the stronger the hydrophobicity of a compound (longer retention time in RP-HPLC) and the lower the recovery, it would be expected that the hydrophobicities of taxol, cephalomannine and 7-epi-10-DAT are higher than those of baccatin III and 10-DAB according to the recoveries of those compounds listed in Table 1, which also agrees with their elution order in RP-HPLC.

3.3 Interaction of Taxol and the Other Four Taxoids with Microtubule Protein

As a target protein, microtubule protein is very important in the process of mitosis, during which the duplicated chromosomes of a cell are separated into two identical sets before the cleavage of the cell into two daughter cells. Polymerization and depolymerization of microtubules and the dynamics are the targets of antimicrotubule drugs (with various tubulin-binding sites) that have been used with great success in the treatment of cancer.\(^{[28]}\) Most of these anticancer drugs are antimicrotubule agents and inhibit cell proliferation by acting on the polymerization dynamics of spindle microtubules, the rapid dynamics of which are essential to proper spindle function. Microtubule-targeted antimicrotubule drugs are usually classified
into two groups. One group inhibits microtubule polymerization at high concentrations, such as vincal alkaloids, colchicine. The second group of the drugs, including taxol, taxotere(docetaxel), is synthesized from the naturally occurring compound, 10-DAB), can stimulate microtubules polymerization. They bind to β-tubulin subunits and inhibit microtubule dynamics, kinetically stabilize the microtubules without changing the microtubule-polymer mass, thereby blocking cell cycle progression during mitosis at the metaphase/anaphase transition and activating cell death. Taxol is the first and most potent compound of the taxoids which can be found in many kinds of yew trees. Till today, more than 350 taxoids have been identified and characterized in the Taxus species, and many of them are found to be potent cytotoxics.

The biological fingerprinting analysis shows that the binding degree of taxol to microtubules is much higher than those of the other taxoids and the interaction of taxoids with microtubules is not regarded as non-specific binding for the inhibited polymerization of microtubules by taxoids. Structure activity relationship studies have provided an insight into the structural determinants. It is now believed that the A-ring side chain at C13 with a C2'―OH, the C2 benzoyl group and an intact oxetane ring at C4-C5 of taxol’s taxane ring are essential for both the cytotoxicity and stabilization of microtubules.

3.4 Effect of GTP on the Taxoids Binding to Microtubule Protein

It is necessary to emphasize that microtubules are not simple equilibrium polymers. The polymerization of tubulin dimers requires GTP. They show complex dynamics that use energy provided by the hydrolysis of GTP at the time that tubulin with bound GTP adds to the microtubule ends; these dynamics are crucial to their cellular functions. As there are different states of microtubule protein in the solution, the finding of interacting compounds in Taxus extract with microtubules reveals the entire drug-protein interaction rather than the single-state interaction of the taxoids with microtubules.

Prior to experiment, 900 µL of tubulin dimers’ solution at 10 mg/mL in 0.1 mol/L MES buffer, pH 6.5, which was purified by Sephadex G-25, was mixed with 100 µL of extract of Taxus and incubated in the absence or the presence of 1 mmol/L GTP at 37 °C and followed by microdialysis sampling and HPLC analysis with the same above-mentioned procedure. The biological fingerprinting chromatogram of the extract of Taxus with microtubule protein both in the presence and the absence of GTP were analyzed by HPLC. The obtained results for the effect of GTP on binding degree of active compounds in Taxus extract are shown in Fig.5. Binding degrees of taxol in Taxus to microtubule protein was just 57.4% in the mixed solution and 50.6% at 0.1 mmol/L dissociating tubulin dimers. In vitro, Taxol promotes the assembly of tubulin dimmers into microtubules both in the presence and the absence of GTP. Similar results were observed for cephalomannine and 7-epi-10-DAT, for which the binding degrees were 16.2% to 15.5% and 12.8% to 10.8%.

![Fig.5 Effect of GTP on the binding degrees of taxol and the other four taxoids in Taxus extract to microtubule protein](image)

The decreases of binding degree for compounds baccatin III and 10-DAB in Taxus extract bound to dissociating tubulin dimmers were more sharply than those of taxol, cephalomannine and 7-epi-10-DAT with binding degree changing from 12.1% to 4.6% and 10.5% to 5.2%. The in-day RSD of the binding degree was 0.81%(n=5), which was acceptable for the evaluation of the binding ability of the active agents in Taxus. The binding of baccatin III and 10-DAB to microtubule protein was potently influenced by the different states of microtubule proteins. Possibly, this implies that the taxane ring is primary binding site not only to microtubule protein but also dissociating tubulin dimers and the C13 side chain is also a necessary portion for binding the taxane-based drugs which serve as “anchor” to secure and enhance the binding of the taxane ring.
3.5 Effects of Taxoids on the Polymerization and Depolymerization of Microtubule Proteins

Additional evidence regarding the binding of taxol and the other four taxoids to microtubule comes from microtubule protein assembly experiment. The effects of taxol and single taxoid on the polymerization and depolymerization of microtubules were examined against the CaCl$_2$-induced depolymerization of microtubules. Microtubule proteins were polymerized under normal polymerization conditions in the absence and the presence of taxoids; the temperature was held at 37 °C and the changes in turbidity were monitored at 350 nm by UV spectrometry. For the drug-protein studies, 10 µmol/L of one taxoid dissolved in DMSO was added to 1 mL of buffer solution containing 5 µmol/L microtubule proteins by keeping the final DMSO concentration at 10%. After 15 min incubation of the test mixture, 4 mmol/L CaCl$_2$ was added, and the mixture was further incubated for another 30 min. The obtained results are summarized in Fig.6. The CaCl$_2$-induced depolymerization of microtubules (shown as control) was inhibited by taxol. Amongst the tested taxoids, cephalomannine and 7-epi-10-DAT promoted the polymerization and reduced the depolymerization process, suggesting that cephalomannine and 7-epi-10-DAT have taxol-like activity to microtubule systems, while baccatin III and 10-DAB exhibited little activity of inhibiting microtubules depolymerization. Since these compounds possess an oxetane ring at C$_4$ and C$_5$ on their taxane ring like taxol, the entire taxane ring also plays an important role as a primary binding site of taxoids for interaction with microtubules[38] and possibly implies that C$_{13}$ substituent is the binding group of taxol, cephalomannine and 7-epi-10-DAT to microtubules which provides depolymerization inhibition activity.

3.6 Competitive Binding of Taxol for the Other Four Taxoids

The antimicrotubule activity of taxol and the other four taxoids is a constituent element of clinical treatment effects of Taxus. Herbal medicines and their prescriptions are composed of groups of active substances, which enter into compatibility and combination according to certain requirements and exert their functions on human body via interaction with multi-targets and various ways. Some components often interact with a co-target in the form of a harmonious network; this phenomenon is common in the interaction of complex components in herbal medicines with the biomolecules target and is a very important character of herbal medicines, such as TCMs different from the western medicines. As there are many other components present in those extracts with ability to bind with microtubule or not, an antagonistic or synergistic effect may occur to the binding behavior of taxol and taxoids to microtubules. It is important to identify the role of taxol, the potent antimicrotubule agent of taxoids plays in the entire bioactivity and toxicity under the existence of the other bioactive components, and to study how the components affect each other during the interaction with the target protein.

In the competitive binding experiment, the total concentrations of extracts of Taxus and microtubule protein were constant by only changing the taxol concentration. Taxol solutions with increased concentrations, used as competing agent, were added into the mixed solutions at 37 °C, then microdialysis sampling was performed and each dialysate was analyzed by HPLC. The binding degrees obtained from the displacement of the taxoids by taxol are shown in Fig.7. The competitive curves display a similar trend for all the other four taxoids with binding activity to microtubules. Baccatin III, 10-DAB, cephalomannine, 7-epi-10-DAT were significantly displaced by taxol, the binding degrees of them were decreased as the taxol was added. It has been reported that taxol interacts with microtubules by binding in a pocket between the ‘M-loop’ (residues 279—287) and the ‘central helix’ located between B7 and H9 areas on the β-tubulin[39]. The binding of taxane ring to microtu-
which have anticancer activity. To taxol, the binding of the C₁₃ side chain may provide an additional anchor to interact stronger with the microtubule. So the obtained results of competitive binding experiment confirm that this group of taxoids including taxol in Taxus extracts binds to the same site of microtubule protein. Their co-interactions with microtubule system may be more efficient and weaken the side effect of taxol during clinical treatments by Taxus extracts.

![Fig.7 Competitive binding of Taxol with other four taxoids in Taxus extract to the microtubule protein](image)

Fig. 7  Competitive binding of Taxol with other four taxoids in Taxus extract to the microtubule protein

a. Baccatin III; b. 10-DAB; c. cephalomannine; d. 7-epi-10DAT.

4 Conclusions

The microdialysis sampling combined with HPLC was applied to biological fingerprinting analysis of Taxus extract with target of microtubule protein. The approach is based on the affinity of antimicrotubule agents for microtubule protein. In this method, potential antitumor candidates are fished out with microtubule protein as targets. Taxol and the other four taxoids baccatin III, 10-DAB, cephalomannine and 7-epi-10-DAT in the extract of Taxus have the activity to bind with microtubule protein. The antimicrotubule properties of taxol and the other four taxoids binding to microtubule protein have also been studied. The compounds of Taxus extract bind with microtubule protein collaboratively and inhibits the depolymerization and polymerization of microtubules. It has been shown via the competitive binding experiment that taxol is the strongest anticancer agent of all the bioactive components in Taxus extract. It is indicated that the biological fingerprinting analysis by microdialysis/HPLC method has the potential application to the analysis and screening for new antimicrotubule agents from natural products libraries, such as TCMs or herbs which have anticancer activity.

References

[26] Andreu J. M., Barasoin I., Biochemistry, 2001, 40, 11975
[35] Carrier M. F., Pantaloni D., Biochemistry, 1983, 22, 4814