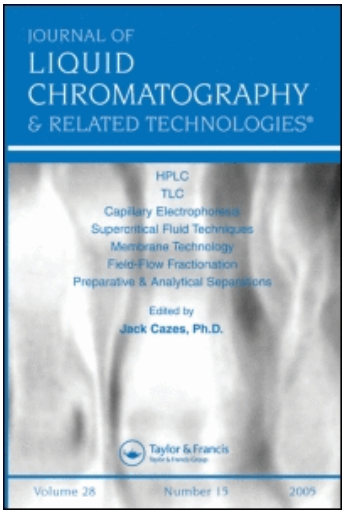

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RECOVERY OF TAXOL FROM THE EXTRACT OF *TAXUS CUSPIDATE* CALLUS CULTURES WITH Al_2O_3 CHROMATOGRAPHY

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ABSTRACT

In this study, we reported a chromatographic protocol using C_{18} silica gel phase and basic Al_2O_3 phase to research the creating and recovery of taxol from the extract of *Taxus cuspidate* callus cultures. With the cleaning up by basic Al_2O_3 chromatography, the content of taxol determined in our experiment was increased greatly. The main source of increasing taxol concentration was the isomerization of 7-epi-taxol to taxol; taxol could also be decomposed to baccatin III and 10-deacetylbaccatin III in the Al_2O_3 column. At the optimized conditions, the total recovery of taxol from the extract was more than 160%.

INTRODUCTION

Taxol, the only secondary plant metabolite initially separated from *Taxus* spp. by Wani et al.,[1] has significant anti-cancer activity and is known to promote the assembly of microtubules and inhibit the tubulin disassembly process. Currently, the main commercial source of taxol is the bark of *Taxus* spp, which grows slowly and yields relatively low amounts of taxol. The total synthesis, and semi-synthesis from natural taxoids, as well as the plant tissue culture of taxol, has been successful.[2] Specifically, the plant tissue culture can provide a stable supply of taxol and related taxanes, and be viewed as a potential alterna-

tive to plant extract. Since taxol occurs in small amounts in the callus, and the plant tissue is slow growing, it is important that any separation method should be effective in removing all the taxol from the callus cultures, and the method must not cause any degradation of the taxol. Hence, chromatographic methods have been developed to detect and isolate taxol from the above sources on an analytical and preparative basis.[2-4] As the presence of many interfering compounds, especially cephalomannine and 7-epi-taxol that coelute with taxol, the isolation procedures are complicated, and suffer from low recovery and selectivity of taxol.

In the extract of *Taxus*, there have been some combined taxanes and taxanes containing taxol core, such as 7-xyloside taxol[5] and 7-epi-taxol.[6] Carver et al.[7] indicated an increase in the taxol recovered from the extract of *Taxus brevifolia* with ion-exchange resin treatment. However, there appear to be little data with details on which kind of taxanes could convert to free taxol. In our previous experiment, we also found an increase in the content of taxol from the extract of *Taxus cuspidate* callus cultures in the Al_2O_3chromatography separation process.

Therefore, the objective of this study was to develop chromatographic protocol using C_{18} silica gel phase and Al_2O_3 phase to research the increase and recovery of taxol from the crude methanol extract of *Taxus cuspidate* callus cultures. The study showed that the main source of the increase in taxol was the isomerization of 7-epi-taxol to taxol, and the conversion rate of the isomeric reaction in the Al_2O_3 column was more than 60% at the optimized conditions.

EXPERIMENTAL

Materials

Acetonitile, methanol (HPLC-reagent grade) and Chloroform (analytical grade) were purchased from Beijing Chemical Reagent Company. Taxol was obtained from Sigma (Poole, UK). Cephalomannine and 7-epi-taxol was obtained from Chinese Academy of Medical Science (Beijing, China). Water was deionised and bi-distilled. The rotary evaporator was the product of Kelong Company (Beijing, China). Al_2O_3 media (50-70 μm) was supplied by Shanghai Chemical Works (Shanghai, China).

Methods

Sample Preparation

Callus cultures were established from *Taxus cuspidate* as described by Xu et al.[8] The six to eight week old, slow-growing callus samples were harvested

and freeze-dried. Callus was extracted with methanol at ambient temperature for 2 days. The resulting solution was filtered through 2 filter papers (the pore size of paper was 0.4-0.6 μm), and the methanol solution was evaporated to dryness on a rotary evaporator at 40-50°C. The residue was redissolved in chloroform for the subsequent separation-steps.

Al_2O_3 Adsorption Chromatography

The column size was 15 mm x 250 mm. The media of adsorption chromatography was basic Al_2O_3, and the particle size of the alumina support was 50-70 μm. The activation of the column was carried out using chloroform. The buffer for feed loading was chloroform/methanol (98.5:1.5, v/v). Feed flow rate was 2.0 mL/min. After feed loading, the column was washed with the buffer chloroform/methanol (99:1, v/v) to remove unadsorbed impurities. The isocratic elution was performed with chloroform/methanol (95:5, v/v) at 3.5 mL/min. Eluents were dried down and resuspended in methanol for HPLC analysis.

Reversed-Phase Chromatography

The support of reversed-phase chromatography was C_{18}-silica gel (80 μm, Sigma, Poole, UK). The column (15 mm x 260 mm) was laboratory packed with theoretical plates, more than 5,000 m^{-1}, determined by blue dextran. After washing with acetonitrile and equilibrating with acetonitrile / water (40:60, v/v), the taxol sample, from the adsorption chromatography with the content of 29% (based upon the solid product from the adsorption chromatography), was loaded. The mobile phase for the first elution was acetonitrile / water (40:60, v/v), and for the final elution was acetonitrile / water (50:50, v/v). The elution rate was 1.0 mL/min and the UV detected at 227 nm. Eluents were dried down with a rotary evaporator at 40-50°; the solid product was resuspended in methanol for HPLC analysis.

HPLC Analysis

The HPLC system was Beckman FL-750, equipped with a Gold system software. It consisted of Programmable solvent (Module 126), 7725 injector, and Diode array detector (Module 168). Taxol was separated by HPLC using a C_{18}-silica column (4.5 mm x 250 mm) (Beckman, California, U.S.A.). A 20 μL sample was injected, and the eluent monitored at 227 nm. The mobile phase for isocratic elution was methanol/acetonitrile/water (25:35:40, by vol.) at 1.4 mL/min. The sample solution and the mobile phase were filtered through 0.2 μm PVDF filters. The retention time of taxol is about 32 min. Taxol was quantitated by comparing the average peak area of the sample to that of the standard (Sigma). It was shown that there is a linear relationship (r=0.9996) between the peak area and the amount of taxol over the range of 0.04-0.96 mg/mL.

RESULTS AND DISCUSSION

In our callus cultures, non-polar components, but also highly polar impurities were found. In order to obtain high purity taxol with chromatography methods, the initial separation of these impurities were essential. An adsorptive method, such as silica gel adsorption and Al_2O_3 adsorption, was found to be very useful to initially clean up the very crude samples in natural product preparative isolation. Figure 1 shows the profile of Al_2O_3 chromatography to separate taxol from the extract of *Taxus cuspidate* callus cultures. It showed that taxol could be freed from many related compounds in the extract, and the extraction of the highly hydrophobic components, such as oils, waxes, etc. in the plant tissue cultures was reduced effectively. Taxol of greater than 29% purity could be obtained after the step of Al_2O_3 chromatography. Furthermore, the recovery of taxol surprisingly showed to be more than 100%, even up to 170%.

To check the recovery, the spiked sample containing definite amounts of the taxol standard was analyzed with the same HPLC procedure; by comparing the peak response of taxol in sample and spiked sample, the recovery rates was obtained. In other words, the recovery of 170% was not caused by the bias of taxol analysis. Therefore, it was most likely that there was new taxol being produced during Al_2O_3 chromatography.

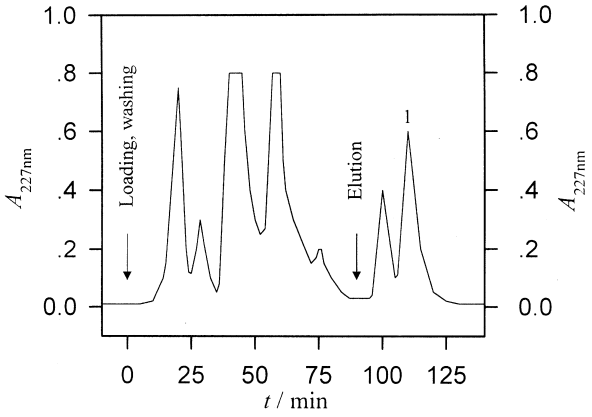

Figure 1. The chromatogram of Al_2O_3 chromatography. Chromatographic conditions: solvent and Al_2O_3 being dried, 15mmx260mm column, sample loading: 200mg, washing buffer: CH_3OH / $CHCl_3$(1:99, *v/v*), eluent: CH_3OH / $CHCl_3$(4:96, *v/v*), elution rate: 2.5 mL/min, peak 1 = taxol.

The components in the extract of our callus cultures were too complicated to obtain each pure component and to study the possibility of conversion to taxol in the Al_2O_3 column. However, according to the polarity of taxanes, the extract of callus cultures could be fractured by C_{18}-silica gel reversed-phase chromatography. As shown in Figure 2, the eluent 1 was acetonitrile/water (40:60, *v/v*), the eluent 2 was acetonitrile/water (50:50, *v/v*), and the extract could be fractured to 4 fractions.

Data from the literature[9] and HPLC analysis indicated: fraction A contained higher polarity taxanes, such as baccatin III, 10-deacetylebaccatin III, fraction B contained median polarity taxanes and sugar bound taxanes, including 10-deacetyletaxol, 10-deacetyle cephalomannine, taxol-7-xyloside, and 10-deacetyletaxol-7-xyloside, fraction C contained cephalomannine, and fraction D was taxol and lower polarity taxanes.

These four fractions were treated with Al_2O_3 column, respectively, with the results indicated in Table 1. The concentration of taxol in the extract of callus cultures was about 0.68% (g/g). In fraction B and fraction D, the content of taxol was increased. It was possible that the sugar bound taxanes in fraction B was converted to free taxol as reported by Carver et al.[7] and Durzan et al.[10] However, the main source of increasing taxol was from fraction D.

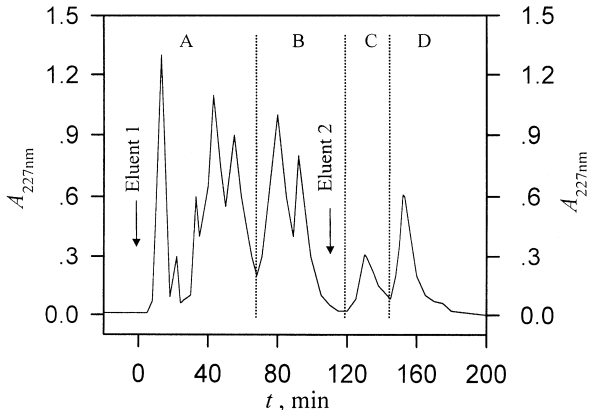

Figure 2. Sample fraction with C_{18}-silica gel reversed-phased chromatography. Chromatographic conditions: 1.6 mmx300 mm column, sample loading: 200mg, eluent 1: acetonitrile / water (40:60, *v/v*), eluent 2: acetonitrile / water (50:50, *v/v*), elution rate: 2.5 mL/min. fraction A: higher polarity taxanes, fraction B: median polarity taxanes and sugar bound taxanes, fraction C: cephalomannine, fraction D: taxol and lower polarity taxanes.

Table 1

The Fractions from Figure 2, Treated by Al_2O_3 Chromatography

Fractions	Taxol in Fractions (mg)	Taxol after Al_2O_3 Chromatogr. (mg)
A	0	0
B	0	0.10
C	0	0
D	1.36	2.15

Figure 3 showed the assay profile of sample fraction D separated by C_{18}-silica HPLC. There were two main components (peak 1 and peak 2 in Figure 3) in fraction D. The HPLC analysis indicated that peak 1 and peak 3 were taxol and cephalomannine separately.[2] Seldom did the literature mention peak 2, which was deduced to be 7-epi-taxol or other higher polarity taxane, as that being eluted with or after taxol by reversed-phase chromatography.[11] To iden-

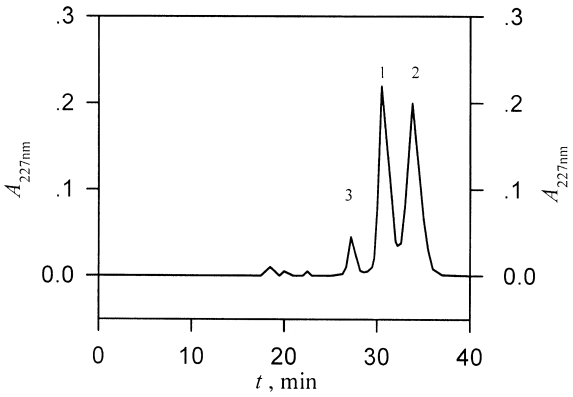

Figure 3. The chromatogram of HPLC to detection fraction D. Chromatographic conditions: 4.6mmx250mm C_{18}-silica gel HPLC column, elution rate: 1.0 mL/min, the mobile phase was methanol / acetonitrile / water (25:35:40, by vol.), peak 1 = taxol, peak 2 = 7-epi-taxol, peak 3 = cephalomannine.

Table 2

The Elemental Analysis of Peaks From Figure 3

Samples	C (%)	H (%)	O (%)	N (%)
Peak 1	66.10	6.03	26.24	1.63
Peak 2	66.09	6.05	25.21	1.65
Peak 3	65.03	6.42	26.95	1.66

tify peak 2, fractions of peak 1, peak 2, and peak 3 were collected separately for elements assay (determined by Institute of Chemistry, CAS, Beijing, China) as shown in Table 2. Calculating the data of Table 2, the molecular formula of peak1, peak 2, and peak 3 can be estimated: peak 1 ($C_{47}H_{51}NO_{14}$); peak 2 ($C_{47}H_{51}NO_{14}$); peak 3 ($C_{45}H_{53}NO_{14}$). Therefore, we were able to confirm that peak 1 was taxol, peak 2 was 7-epi-taxol, and peak 3 was cephalomannine. Furthermore, the ^{13}CNMR spectrum of the products of peak 1 and peak 2 (determined by Institute of Chemistry, CAS, Beijing, China) were identical to those of taxol and 7-epi-taxxol reported in the literature by Kingston.[12]

The structures of taxanes such as taxol, 7-epi-taxol, and cephalomannine were shown in Figure 4.[13] It indicated that the tiny difference betwen taxol and 7-epi-taxol was isomerization at C-7 in the structure. It could be said, that there existed the isomeric reaction of 7-epi-taxol to taxol during the Al_2O_3 chromatography to clean up the extract, and the main increasing taxol came from this reaction.

The influence of reaction time on recovery of 7-epi-taxol converting to taxol was demonstrated in Figure 5. The reaction time could be controlled by the retention time of the samples in the chromatographic column. The increase in recovery of taxol was maximum at 45 min, and the recovery of taxol was about 160%; further increasing reaction time, showed a decreasing trend. It could be said that there were other reactions existing, in addition to the reaction of 7-epi-taxol converting to taxol, and it was possible that taxol could be decomposed in the Al_2O_3 column.

Baccatin III and 10-deacetylbaccatin III were found after taxol was treated in the Al_2O_3 column. In order to investigate the effect of temperatures on the stability of taxol in the Al_2O_3 column, comparative experiments were performed. As demonstrated in Figure 6, there was basically no difference in the decomposition of taxol below 25°C; with the increasing of the temperature, the

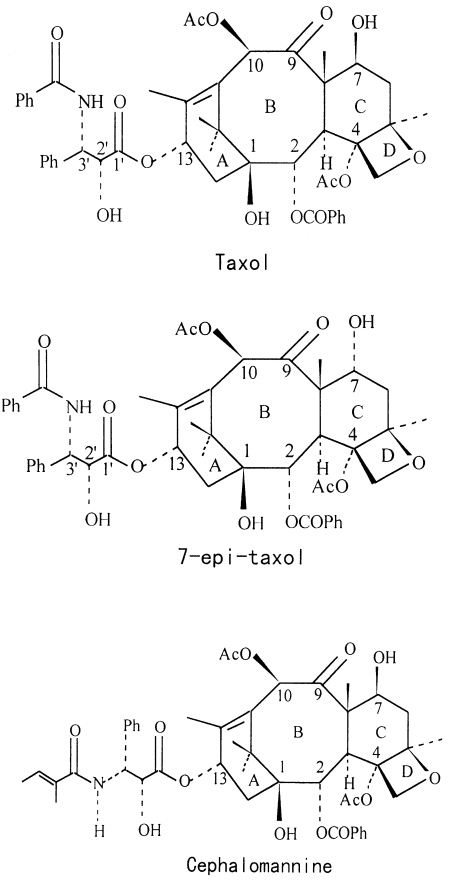

Figure 4. The structures of taxanes.

decomposition of taxol increased significantly. At 60°, more than 85% of taxol was decomposed. So, it was necessary that conversion of 7-epi-taxol to taxol and the separation process should be operated at lower temperature.

The suitable retention time of sample in the column was 45 min. The profile of HPLC, with the sample of 7-epi-taxol/taxol (1.1:1.0, g/g), treated at the

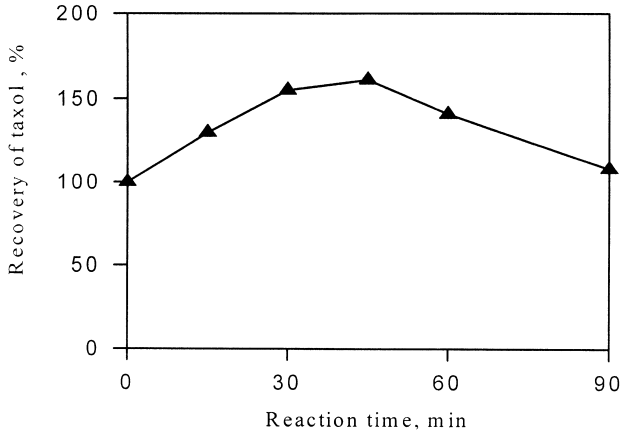

Figure 5. The conversion of 7-epi-taxol to taxol. Experimental conditions: 15mmx260mm Al_2O_3 column, initial sample 7-epi-taxol / taxol (1.1:1.0, g/g), sample loading: 20mg, eluent: CH_3OH / $CHCl_3$ (4:96, *v/v*).

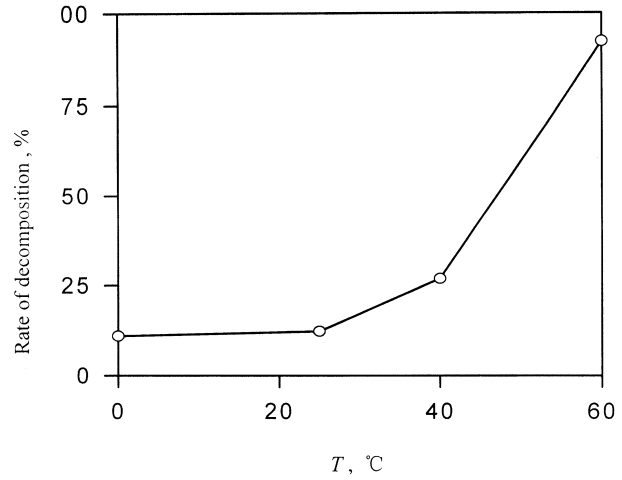

Figure 6. The influence of temperature on the stability of taxol in the Al_2O_3 column. Conditions: 15mmx260mm Al_2O_3 column, reaction time 1.5 h, the mobile phase CH_3OH / $CHCl_3$ (4:96, *v/v*).

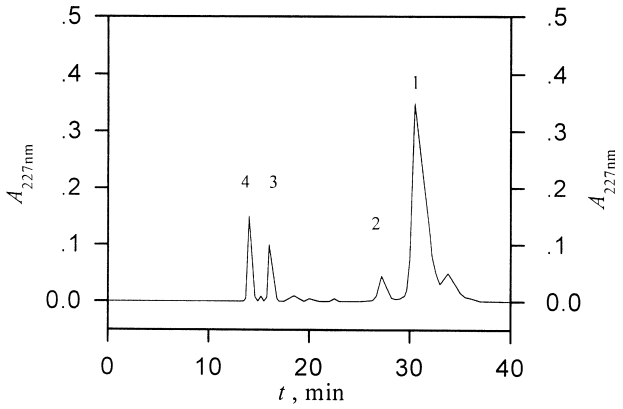

Figure 7. The profile of HPLC with the sample of 7-epi-taxol and taxol treated by the Al_2O_3 column. Chromatographic conditions: 4.6mmx250mm C_{18}-silica gel HPLC column, elution rate: 1.4 mL/min, the mobile phase was methanol / acetonitrile / water (25:35:40, by vol.), peak 1 = taxol, peak 2 = cephalomannine, peak 3 = baccatin III, peak 4 = 10-deacetylbaccatin III.

optimized conditions was shown in Figure 7. Almost all of the 7-epi-taxol was converted to taxol, and small amounts of taxol were decomposed to baccatin and 10-deacetylbaccatin.

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