Solvent optimization on Taxol extraction from *Taxus baccata* L., using HPLC and LC-MS

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Received 25 Feb 2009; Revised 13 July 2009; Accept 28 July 2009

ABSTRACT

Background and the purpose of the study: Taxol, a natural antitumor agent, was first isolated from the extract of the bark of *Taxus brevifolia* Nutt., which is potentially a limited source for Taxol. In the search of an alternative source, optimum and cost benefit extracting solvents, various solvents with different percentage were utilized to extract Taxol from needles of *Taxus baccata*.

Methods: One g of the dried needles of *Taxus baccata*, collected from Torkaman and Noor cities of Iran, was extracted with pure ethanol or acetone and 50% and 20% of ethanol or acetone in water. Solvents were evaporated to dryness and the residues were dissolved in 5 ml of methanol and filtered. To one ml of the filtrate was added 50 µl of cinamyl acetate as the internal standard and 20 µl of the resulting solution was subjected to the HPLC to determine the extraction efficiencies of tested solvents. Five µl of filtrate was also subjected to the LC-MS using water/acetonitrile (10/90) as mobile phase and applying positive electrospray ionization (ESI) to identify the authenticity of Taxol.

Results: Results of this study indicated that Taxol extraction efficiency was enhanced as the percentage of ethanol or acetone was increased. HPLC analysis showed that Taxol could be quantified by UV detection using standard curve. The standard curve covering the concentration ranges of 7.8 – 500 µg/ml was linear (r^2= 0.9992) and CV% ranged from 0.52 to 15.36. LC-MS analysis using ESI in positive-ion mode confirmed the authenticity of Taxol (m/z 854; M+H), as well as some adduct ions such as M+Na (m/z 876), M+K (m/z 892) and M+CH3CN+H2O (m/z 913).

Conclusions: The results suggest that 100% acetone is the best solvent for the extraction of Taxol from *Taxus baccata* needles.

Keywords: Taxol, *Taxus baccata*, Solvent Extraction, HPLC, LC-MS

INTRODUCTION

The diterpenoid anticancer drug Taxol (generic name paclitaxel, ure 1) was first isolated from the bark extract of yew trees, *Taxus brevifolia* Nutt. Taxol is an important drug for the treatment of different kind of cancers, as well as AIDS-related Kaposi's sarcoma (1, 2). Its unique antitumor activity by microtubule-stabilizing effect, has been the subject of many investigation (3). For treatment of one patient about 2 g of paclitaxel is required which can be obtained from the bark of 3-10 trees (4). Although Taxol is extracted in higher concentration from the bark but bark harvesting destroys the tree and seriously threatens the very slow-growing yew tree population (5) and has proved unsuitable for long term or large scale production of Taxol. On the other hand various surveys have reported the Taxol content of Taxus spp in the range of 0.001- 0.06% w/w of dried bark (6, 7). The recovery of Taxol extracted from various parts of mature trees has been reported to be in the following order: bark > needles > roots > branches > seeds > wood (5). To save this valuable population of plants two approaches are considered: 1) using renewable part of the plants such as needles; 2) applying efficient method of extraction to enhance Taxol separation as much as possible. Regarding the first approach, needles of *Taxus baccata* has been replaced with the bark by many researchers to obtain docetaxel instead of paclitaxel (5, 8, 9). Considering second approach, different solvents or their combinations (7), using ultrasound (10), microwave energy (11) and other techniques have been introduced.

Purification of Taxol from needles is more difficult because it contains waxes, chlorophyll and many other endogenous compounds (12). To overcome this problem a wide range of non-polar to polar

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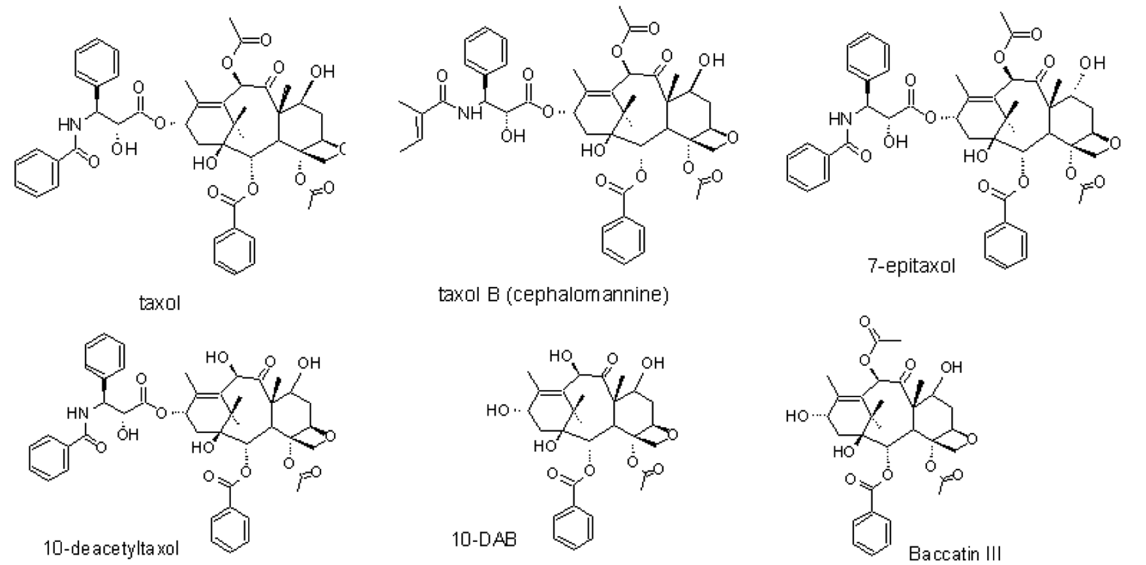

Figure 1. Chemical structure of Taxol and its related taxans.

solvents or their combinations have been examined. Various solvent systems have been compared for their efficacy in the extraction of Taxol and other taxans from the contaminating residue. Ketchum et al (7) used only potable solvents in combination with solid phase extraction and avoided chlorinated hydrocarbon solvents. This system which can be used for fresh needles prevents degradation of taxoids during the drying process. Using this method, Taxol extraction yield was 0.02 to 0.04 % of the dry weight of the needles. Young Park and co-workers (6) reported the content of Taxol in the Korean yew tree as 64 mg/kg of dry powder by using methanol as extracting solvent. In this study the methanolic extract was partitioned between methylene chloride and water to concentrate the Taxol prior to HPLC analysis.

Structural similarities between taxans is another problem in obtaining pure Taxol. For instance single extraction with methanol resulted in difficulties in the determination of cephalomanine, Taxol and baccatin III in the bark and needles of taxus species by HPLC (13). To remove waxes and other lipophilic compounds, hexane has been used as an additional step before using extracting solvent (14). Literature survey revealed that HPLC-MS methods, positive and negative ionization modes have been used to determine the quantity of Taxol in the Taxus species at the picogram level (5, 15) where selectivity and sensitivity of this efficient method, could be enhanced by using single ion monitoring (16).

The range of solvents available for the extraction of material from medicinal plant is not large. Mixtures of organic solvent such as alcohols with water are used to produce certain effects. Of the numerous ketones only acetone and occasionally methyl ethyl ketone are used for extraction. Aromatic or chlorinated hydrocarbon because of their flammability, explosiveness and their toxicity are now used only with great hesitation (17). The influence of ethanol with different polarity and pure methanol on Taxol and related taxoids extraction from needles of three Taxus species have been described (18). The best solvent was 80% ethanol, by which high yields of all the taxoids could be extracted (18). In order to find an optimum and cost benefit solvent extraction system for taxoids from *Taxus baccata* needles grown in northern part of Iran, in this study the use of two relatively cheap and safe solvents, ethanol and acetone, were investigated. HPLC and LC-MS were employed for quantification and confirmation of the isolated compound.

MATERIAL AND METHODS

Reagents and solutions

Taxol were obtained from Calbiochem-norabiochem (San Diego USA). Acetonitrile and methanol (HPLC grade) were from Merck, Germany. Water (HPLC grade) was prepared by Direct Q^{TM}, waters (USA), obtained from the local market. All other reagents and solutions were either HPLC or analytical grades.

Plant material

Fresh needles and young stems of *Taxus baccata* L. were supplied by Shahid Fozveh Research Center, Isfahan, Iran in winter of 2006. Voucher specimens (No: 1692) were deposited at the department of Pharmacognosy, School of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran.

Sample preparations
Plant material was dried under controlled condition at room temperature, and then was grounded. One gram of the powdered material was added to 5 ml of hexane. The mixture was kept for 24 hrs at room temperature and then filtered in order to remove waxes, lipids and unwanted non-polar compounds. Extracts from defatted powders were prepared using 5 ml of acetone (100%, 50% and 20%) or ethanol (96%, 50% and 20%) in water as solvents. Plant materials were mixed with solvents and shaked at room temperature for 24 hrs and the extracts were filtered. Combined extracts from the three consecutive extractions were vacuum-evaporated. In order to remove the polyphenols and tannins; the residues were dispersed in 5 ml of 15% lead acetate solution by vortex and centrifuged for 10 min at 5000 rpm, then the extracts were evaporated to dryness. The residues were dispersed in 5 ml of distilled water and extracted (3x) with 5 ml of ethyl acetate. The combined extracts were evaporated and the residue was dissolved in 5 ml of methanol. Then 1 ml of this methanol solution was filtered through a 0.45-mm Millipore and to the filtrate was added internal standard (IS; 50 µl cinnamyl acetate, 0.2 µg/ml) prior to HPLC injection. Twenty microliter of this solution was injected to the HPLC column.

Chromatographic conditions
A reversed phase HPLC method was developed to quantitate extracted levels of Taxol. The apparatus was a Waters HPLC system (USA), consisting of a model 600 pump and controller waters solvent delivery pump, 7125-rheodyne injector, a computerized system controller (with the Millennium software), a UV-486 detector. Chromatographic separation was performed using a Nova-Pack C_{18} (3.9×150 mm, waters Association) reverse phase HPLC column. The mobile phase consisted of acetonitrile-water (40-60). The mobile phase was eluted at a flow rate of 1 ml/min, and effluent was monitored at 227 nm. Quantization was achieved by measurement of the peak area ratios of the compound to the internal standard.

Calibration procedure
In order to prepare standard solutions of Taxol, to 1 ml of Taxol at concentrations of 7.8, 15.6, 31.25, 62.5, 125, 250 and 500 µg /ml in methanol was added 50 µl of methanolic solution of the internal standard (I.S) at fix concentration of 0.2 µg /ml. The calibration curve was plotted using peak ratios of Taxol/I.S. versus Taxol concentrations. Final sample concentrations were calculated by determination of the peak area ratio of Taxol related to I.S. and comparing the ratio with the standard curve obtained after analysis of calibration samples.

Precision
a) Within-day variability
The within–day variability of the assay was determined by repeated (n=3) analysis of samples at concentrations of 7.8 - 500 µg /ml on the same day.

b) Between – day variability
The between-day variability of the assay was determined by repeated analysis of samples at concentrations of 7.8 - 500 µg /ml on 3 consecutive days (n=9).

LC-MS sample preparation
The dried extracts were dissolved in methanol (0.5ml) and applied on preparative TLC (GF254), using pure Taxol for checking the Rf. Taxol layer was scratched, dissolved in methanol and centrifuged for 10 min at 5000 rpm. Five microliter of supernatant were injected to a Shimadzu 2010EV LC-MS system (Shimadzu, Japan) coupled with a UV and quadruple detector, using a computerized system controller (with the LC solution software). Water/acetonitrile (10/90) was used as mobile phase at a flow rate of 0.2 ml/min and mass spectra were acquired in the positive ion mode (ESI+). The instrument was set to scan from 200 to 1000 mass units. Selected ion monitoring (SIM) mode was also applied for diagnostic the molecular ion (M+H) of Taxol. Pure nitrogen (99.995%) was used as drying gas and nebulizer with a flow rate of 15 L/min and 1.5 L/min, respectively.

RESULTS

Solvent selection
Two different solvents with different percentage (Ethanol: 96%, 50%, 20% and acetone: 100%, 50%, 20%) were used for extraction of Taxol from *Taxus baccata*, as described in sample preparation. The Taxol extraction yields are presented in Table 1.

Identification of Taxol by HPLC and LC-MS
Figure 2 shows a chromatogram of Taxol and cinamyl acetate as internal standard in standard solution. The typical HPLC chromatograms of Taxol obtained from the needles extracts of *Taxus baccata* using different solvents are presented in Figure 3. The structures of the major peaks were assigned by comparison of retention times, UV spectral data and spiking with known standard Taxol and cinamyl acetate as internal standard. The results indicate that the retention time (nearly16 min) of Taxol in different solvent solution was consistent with its presence in the standard solution. The peak of internal standard and Taxol were completely resolved and there were no interfering peak. The compound corresponds to the second peaks in the chromatogram was analyzed by LC-MS in ESI

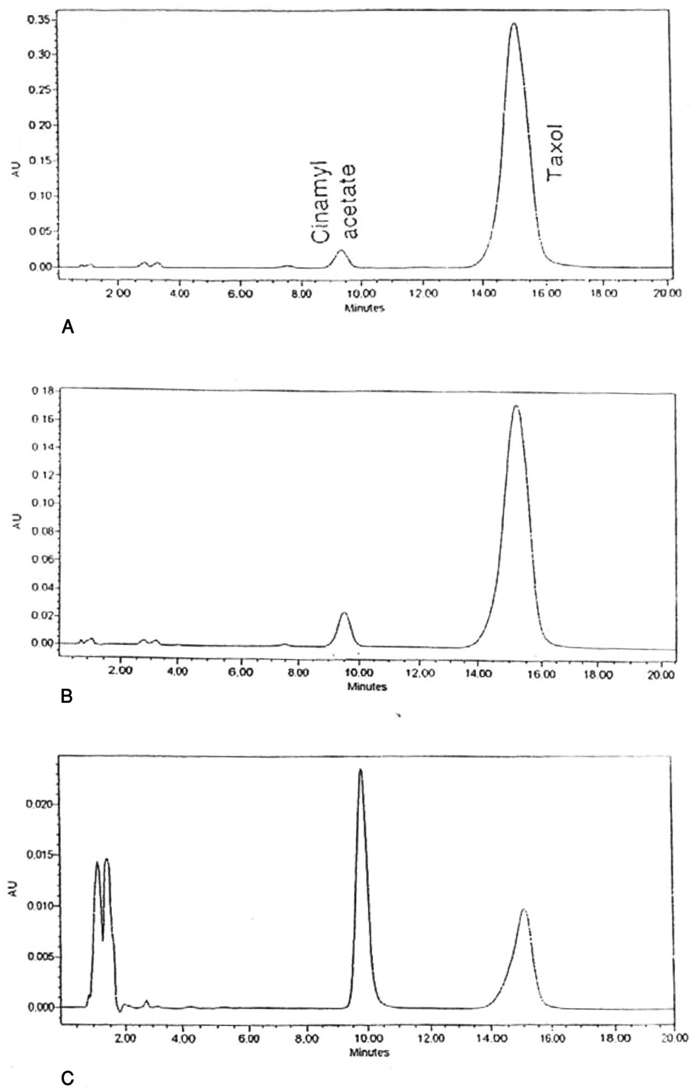

Figure 2. Representative chromatogram of Taxol and cinamyl acetate in standard solution with different concentrations. (A) 250 µg/ml; (B) 125 µg/ml; and (C) 15.6 µg/ml. For chromatographic condition see materials and methods.

positive mode. The MS spectrum showed baseline resolution of Taxol using conditions suitable for LC-MS. Under these conditions the predominant response for all analytes were sodium adduct (M+ Na; m/z 876) and m/z 854 (M+H), other peaks were m/z 892 (M+K), m/z 913 (M+ACN+H2O) and m/z 381 (Base peak) (Figure 4).

Linearity

Calibration standards containing 7.8 to 500 µg /ml were prepared from working solutions of Taxol. The calibration curve was constructed by plotting the peak area ratio of Taxol to I.S. against the Taxol concentration. The calibration curves showed a good linearity within the examined concentration range (r^2=0.9992).

Accuracy and precision

The within-day coefficients of variation (CV %) of Taxol was between 0.52 and 15.36% and the between-day coefficients of variation were between 0.26 and 8.5% for all compounds (Table 2). These results, therefore, validate the calibration curves which were used for each set of samples.

DISCUSSION

In order to extract the Taxol from *Taxus baccata*, the first extraction method is the solid–liquid extraction of the dried and powdered plants where by using various solvents of different polarity, different extracts can be obtained.

As presented in Table 1, solubility of Taxol is governed by the type and polarity of extracting solvents where pure acetone showed better characteristics as a solvent for Taxol extraction. However, as it is seen in Figure 5, order of solvents for extraction of Taxol in higher yields was: Acetone 100% > Acetone 50% > Ethanol 50% > Ethanol

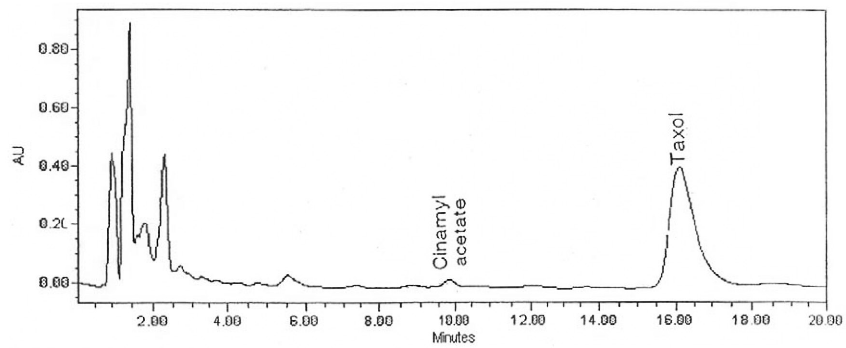

Figure 3. Typical HPLC chromatogram of Taxol extracted from *Taxus baccata* needles using 100% acetone, in the presence of cinamyl acetate as internal standard. For chromatographic condition see materials and methods.

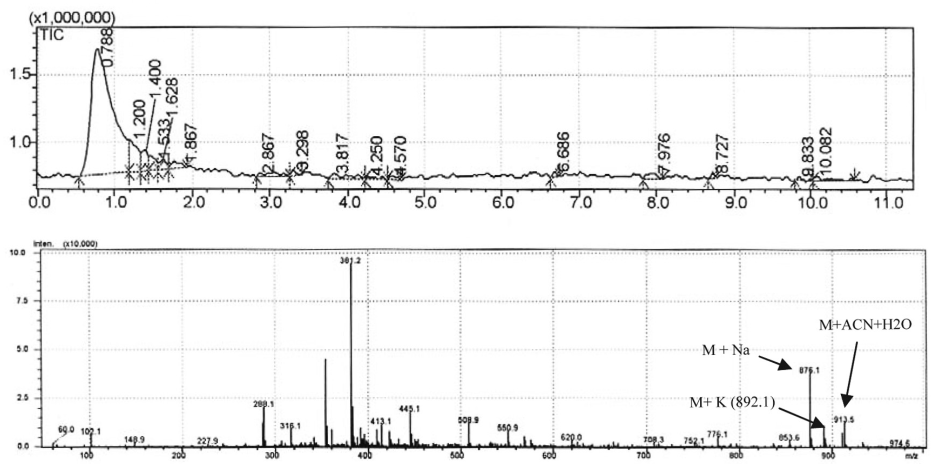

Figure 4. MS chromatogram and MS spectrum of Taxol extracted from *Taxus baccata* by 100% acetone; Water/acetonitrile (10/90) was used as mobile phase using ESI in positive mode. m/z 854 (M+H); m/z 876 (M+ Na); m/z 892 (M+K), m/z 913 (M+ACN+H2O) and m/z 381 (Base peak) are detected by MS detector.

96% > Acetone 20% > Ethanol 20%. The solubility depends on the number, type and position of the -OH in the Taxol molecule. There is no uniform or completely satisfactory procedure suitable for extraction of Taxol from plant materials. Methanol, ethanol, acetone, water, ethyl acetate and, to a lesser extent, chloroform, dichloromethane and their combinations are frequently used for the extraction of Taxol (19-20).

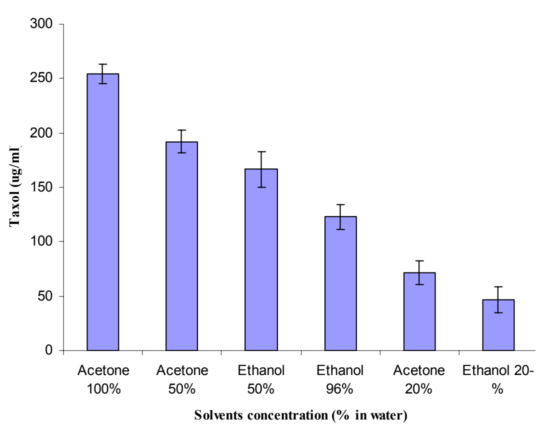

Figure 5. Influence of solvents concentration on extractability of Taxol from the *Taxus baccata* needles in decreasing order. Solvents were used as pure or diluted with water.

Different methods for determination of Taxol have been described (5, 10, 11, 13, 15 and 18). The method presented herein was validated using a linearity range of 7.8 to 500 µg /ml with a limit of detection less than 7.8 µg /ml. The mean within-day CV was 5.66% (0.52-15.36%) and the mean between-day CV was 2.5% (0.26-8.5%). All chromatograms of the standard solutions were free from interferences at the retention times of Taxol or internal standard. (In this study internal standard was added after extraction but for more accurate quantification it is recommended that to add the internal standard at the beginning of sample preparation). The retention times for Taxol and cinamyl acetate were 15.1 and 9.8 min, respectively. In addition to the 15.1 minute peak corresponding to Taxol, there might be other peaks corresponded to the taxoids which may have been extracted from *Taxus baccata* such as peaks observed at earlier retention times (e.g., at 6 min). In this study the

Table 1. Influence of solvents on extractability of Taxol from 1 g of *Taxus baccata* needles, n=3.

Solvents	Taxol conc. (µg/ml) using calibration curve			Mean Taxol conc. (µg/ml)	SD	%CV
	Test 1	Test 2	Test 3			
Acetone 100%	267.6	267.6	228.6	254.6	22.5	8.8
Acetone 50%	202.3	204.2	170.0	192.2	19.2	10.0
Acetone 20%	79.1	72.7	63.7	71.8	7.8	10.8
Ethanol 96%	120.0	137.5	110.6	122.7	13.6	11.1
Ethanol 50%	143.0	160.5	196.0	166.5	27.0	16.2
Ethanol 20%	40.5	48.0	51.0	46.5	5.4	11.7

Table 2. Within-day and between-day variability of the HPLC assay for determination of Taxol concentrations (n=9).

Conc. (ng/ml)	Within-day variations				Between-day variations			
	Mean	SD[a]	%CV[b]	%Error	Mean	SD	%CV	%Error
7.8	9.03	0.20	2.30	15.81	9.08	0.11	1.16	16.40
15.6	18.63	2.21	11.87	19.42	18.20	0.78	4.30	16.70
31.25	33.13	5.09	15.36	6.02	32.00	2.72	8.50	2.30
62.5	64.50	1.37	2.12	3.19	65.20	1.24	1.90	4.42
125	113.90	4.37	3.84	8.85	114.00	0.77	0.68	8.82
250	252.00	9.02	3.58	0.78	252.70	0.66	0.26	1.10
500	499.50	2.60	0.52	0.10	501.00	4.14	0.82	0.20

a; Standard deviation b; Coefficient of variation

possibility of the presents of taxoids can not be excluded since it was not possible to detect them by our HPLC method due to unavailability of taxoids standards. In agreement with these results, other investigators have reported a method of high performance liquid chromatography tandem mass spectrometry for the trace amount of paclitaxel and other six taxoids in three Taxus species (18, 21). In all of presented chromatograms taxoid peaks were observed at earlier retention times than that of Taxol, except 7-epi-10 DAT and none of peaks overlapped with Taxol peak. By this analogy and using Taxol standard in HPLC and LCMS analysis, it may be concluded that the 15.1 minute peak may only corresponds to Taxol. However, some other peaks may coincide with the retention time for Taxol which need further investigation.

In conclusion, it seems that selectivity, ease of handling, economic concern, protection of the environment and safety are major factors to consider mixture of acetone or ethanol with water as solvent of choice for Taxol extraction.

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