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Preparation and Characterization of Paclitaxel from Plant Cell Culture

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Abstract−The solvent treatment of paclitaxel is a convenient method for controlling the morphologies of paclitaxel. Amorphous paclitaxel was simply made by dissolving paclitaxel in methylene chloride/methanol (98/2, v/v) and in relatively non-polar solvents (t-butyl methyl ether, pentane, acetonitrile/hexane (1/2, v/v), methylene chloride, chloroform). On the other hand, dihydrated paclitaxel (paclitaxel·$2H_2O$) was made by dissolving paclitaxel in a special polar solvent containing a small amount of water. However, when we used only methanol, we got mixed morphologies of paclitaxel made of both the dihydrated and amorphous forms. Their physicochemical properties were investigated by X-ray powder diffraction (XRPD), differential scanning calorimetry (DSC), and thermo gravimetric analysis (TGA). The initial water content of amorphous paclitaxel and dihydrated paclitaxel was determined for 0.65 wt% and 5.85 wt%, respectively. The hygroscopic property of dihydrated paclitaxel was very changeable in all given humidity (15, 60, 95 RH%) during storage. Dissolution profiles for paclitaxel showed that amorphous paclitaxel measured the highest solubility in water and its solubility held most stable during the measurements. The residual solvent could be reduced to the maximum allowed value (600 ppm for methylene chloride, 3,000 ppm for methanol) of guidance for the International Conference on Harmonization (ICH) by spray drying.

Key words: Amorphous Paclitaxel, Dihydrated Paclitaxel, X-ray Powder Diffraction, Thermo Gravimetric Analysis, Physicochemical Property, Spray Drying

INTRODUCTUON

Paclitaxel, which is one of the most potent anticancer drugs, is relatively complex organic drug having several solid structures [Wani et al., 1971; Kwon et al., 1998]. The control of solid drug properties is one of the important issues in the pharmaceutical industry [Chiou and Riegelman, 1971; Martin et al., 1983]. Many drugs have different applications according to characteristics of their different solid states. Sometimes, the solubility of a drug in water or other solvents is largely dependent on its solid properties [Mathew et al., 1992; Sharma et al., 1995]. Moreover, the stability of the drug may have a close relationship with the interconversion of the drug among morphologically different states. Therefore, the control of solid states and their characterization of a drug have practical as well as academic importance. Liggins et al. [1997] reported the solid-state physicochemical properties of the different paclitaxel. According to the studies, there are four different states of solid paclitaxel (anhydrous I, II, dihydrated, and amorphous forms), and these can be characterized by using solid-state analytical tools, such as X-ray powder diffractometer (XRPD) and differential scanning calorimeter (DSC). In the paper, they described the methods to control the morphologies of paclitaxel, in which they used some severe experimental conditions. These methods, however, are not suitable for industrial application because they are not practical for large-scale production. Moreover, the large amount of heat used in some of their schemes can cause degradation of the drug. Therefore, a systematic study is needed to find a new way to control the morphologies of paclitaxel, considering the importance of the drug.

In this study, we tried to control the morphology of paclitaxel and prepare morphologically different paclitaxels using several common solvents not exposing the system to high temperature. The resulting morphologies were identified by comparing X-ray powder diffraction (XRPD) patterns as well as differential scanning calorimetry (DSC) data with previous ones [Angell et al., 1991; Liggins et al., 1997]. The additional powerful and efficient method, spray drying, was used to remove the residual solvents in paclitaxel.

MATERIALS AND METHODS

1. Paclitaxel Preparation

We produced paclitaxel from the plant cell cultures of *Taxus chinensis* in a large-scale process [Choi et al., 2001]. The biomass was extracted with methanol and the methanol phase was further extracted with methylene chloride. Paclitaxel and relatively polar taxoides were precipitated by addition of hexane while the relatively non-polar taxoides were soluble in the solvent [Gi et al., 2000; Kim et al., 2002]. The solid compounds were purified by reverse- and normal-phase chromatography [Kim et al., 2001]. Three samples (sample A, B, and C) were formed from the solutions of paclitaxel by using evaporation of solvent-mixtures, methanol/water (90/10, v/v), methanol/water (98/2, v/v), and methylene chloride/methanol (98/2, v/v), respectively. These samples were further dried to constant weight at 60 °C in a vacuum oven equipped with an external pump. Samples A, B, and C were characterized and their morphologies were determined. Several other solvents were used to make amorphous paclitaxel including methanol, methylene chloride, chloroform, t-butyl methyl ether, and pentane. All solvents used in this

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study were HPLC grade. Paclitaxel was dried to constant weight at ambient temperature in a vacuum oven equipped with an external pump.

2. X-ray Powder Diffraction

The X-ray powder diffraction (XRPD) patterns for samples A, B, and C were obtained by using an X-ray diffractometer (D/WAX-3B, Rigaku, Japan). The measurements were performed in the 5 to 40° 2θ range at a rate of 2° 2θ/min using CuKα radiation (45 kV, 40 mA) as X-ray source. The amount of each sample was about 50 mg.

3. Thermal Analysis

Differential scanning calorimetry (DSC) measurements of prepared samples were carried out with the model of DSC-7, Perkin Elmer (MA, USA) calibrated with indium. Approximately 5 mg of the sample was placed on the aluminum pans for each measurement. The cell was purged with nitrogen flowing at 40 ml/min. All measurements were made in the range of 25 °C to 300 °C with a scan rate of 20 °C/min. Thermo gravimetric analysis (TGA) was carried out with the model of TGA-7, Perkin Elmer (MA, USA). Approximately 5 mg of the sample was weighed and scanned to 700 °C at a rate of 20 °C/min. The cell was purged with nitrogen flowing at 40 ml/min.

4. Karl Fischer Titration

Water content was determined with a Karl Fischer Titrator (658 KF, Wetrohm, Swiss). In order to remove the residual moisture in the solvent, it was carried out with KF-reagent and the end point was set-up. The titrant was standardized with 20 µl injections of distilled water. The amount of each sample was about 20 mg.

5. Dissolution Test

Solubility measurements of prepared samples were carried out with HPLC (HP 1100, Hewlett-Packard, CA, USA) using HP ODS hypersil column (2.1×100 mm, 5 µm). The elution was performed in the isocratic condition of 2 mmol/*l* ammonium acetate in acetonitrile/water (60/40, v/v). Approximately 1 mg of each paclitaxel was weighed, put into 50 ml volumetric flask, filled with water, and incubated at 37 °C with shaking for 20 h. Approximately 2 ml of each sample filtered with 0.4 µm filter.

6. Removal of Residual Solvent

Organic solvent of sample C was removed by using a spray drier (Mini spray drier B191, Büchi, Germany). Approximately 3 g of solid paclitaxel was resolved in 50 ml of methylene chloride/methanol (98/2, v/v) and put through a peristaltic pump into spray drier with a velocity of 5 ml/min at 70 °C. A gas chromatography system (HP 5890, Hewlett-Packard, CA, USA) with DB-5 column (0.32 mm ID×30 m, 0.25 µm film) was used for quantitative analysis of residual solvents. The condition was as follows: FID with a temperature program of 40 °C to 100 °C (10 °C/min), hold for 2 min, up to 300 °C (30 °C/min) fold for 15 min, flow rate 3 ml/min with helium.

RESULTS AND DISCUSSION

1. Effect of Solvents on the Morphologies of Paclitaxel

To identify the morphologies of the prepared samples, XRPD and DSC data of the samples were compared with those of the previous study [Angell et al., 1991; Liggins et al., 1997]. Fig. 1 shows the XRPD patterns of samples A, B, and C. In comparison with

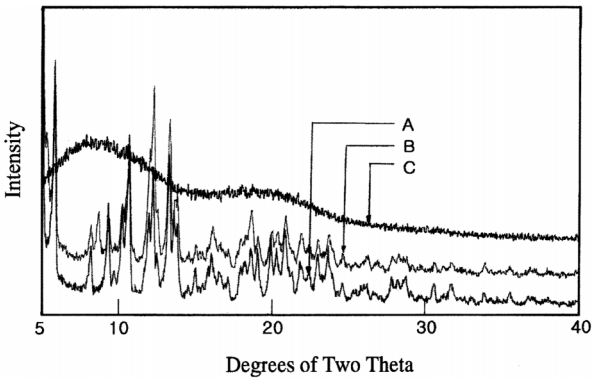

Fig. 1. XRPD patterns of (A) dihydrated paclitaxel from methanol/H_2O (90/10, v/v), (B) dihydrated paclitaxel from methanol/H_2O (98/2, v/v), and (C) amorphous paclitaxel from methylene chloride/methanol (98/2, v/v).

the previous study, it can be easily noticed that XRPD patterns of samples A and B were identical to that of dihydrated paclitaxel. The only difference was the improved S/N ratio of our data which may be attributed to the quantity of samples. For sample C, we could see no meaningful peaks in the XRPD patterns. Therefore, sample C is amorphous state paclitaxel. DSC analysis of the above samples also gave us the same results. Above experimental results suggest that the properties of solvents in the process can be decisive factors in determining the resulting morphologies of solid paclitaxel. This consideration may have some relationship with several other studies of solution structures of paclitaxel in different solvents including interesting topics such as the hydrophobic collapse model [Nicolaou et al., 1994]. To examine more closely the effect of solvent polarities on the morphologies of paclitaxel, we took the same measurements on the samples resulting from evaporating the solvents from the solutions made by several different solvents including methanol/water (90/10, v/v), methanol/water (98/2, v/v) and methylene chloride/methanol (98/2, v/v). In the above solvent treatments, we found that we could get different morphologies of paclitaxel using different solvents. We got dihydrated paclitaxel by dissolving paclitaxel in methanol/water (90/10 and 98/2, v/v) followed by successive evaporating of the solvents. However, dissolving paclitaxel in methylene chloride/methanol (98/2, v/v) and successive evaporation of the solvent resulted in an amorphous paclitaxel. In addition to the above results, we found also that amorphous paclitaxel was made in other relatively non-polar solvents system (e.g., t-butyl methyl ether, pentane, acetonitrile/hexane (1/2, v/v), methylene chloride, chloroform) (data not shown). In previous studies [Liggins et al., 1997; Hancock et al., 1995], amorphous paclitaxel was produced from an anhydrous paclitaxel by heating up to melting temperature of 221 °C and quench cooling. However, these methods are not suitable for industrial application because they are not practical for large-scale production. Moreover, the large amount of heat used in some of their schemes can cause degradation of the drug [Kim and Hong, 2000]. In this study, we found that solvent treatment of paclitaxel was a convenient method to control the morphologies of paclitaxel.

The dihydrated paclitaxel could be confirmed by TGA. The weight loss due to water evaporation is 4.3%, which is about equivalent to 2 mol water-molecule of paclitaxel (Fig. 2). This result showed in

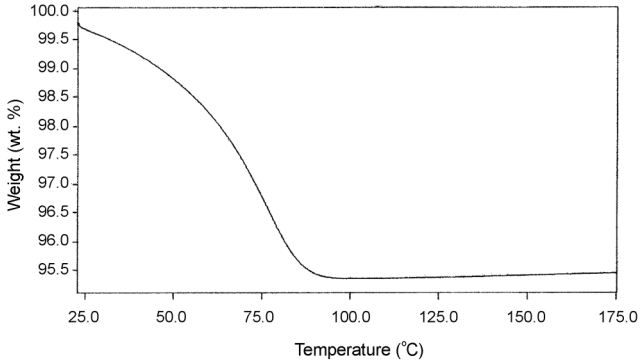

Fig. 2. TGA thermogram of dihydrated paclitaxel (Sample A).

accordance with the previous study [Liggins et al., 1997]. The XRPD, DSC, TGA, and HPLC measurements of amorphous and dihydrated paclitaxel are summarized in Table 1. Amorphous paclitaxel showed no serious weight loss due to water evaporation by measurement of TGA. On the other hand, dihydrated paclitaxel showed solid-solid transition at 160 °C and melting point at 221 °C as well as weight loss of 4.3% at the beginning of TGA measurement. However, when we used only methanol, we got mixed morphologies of paclitaxel made of both the dihydrated and amorphous forms by XRPD analysis (Fig. 3). In this case, the weight loss due to water evaporation was 2.8% by TGA. The intensity of diffraction peak for this sample showed much lower than that in the pattern of dihydrated paclitaxel, which indicated a lower degree of crystallinity. From this result, we can recognize that this sample was composed of two different solid-states, amorphous and dihydrated paclitaxel, because the weight loss of amorphous conducted 0.7% (Table 1).

2. Effect of Paclitaxel Morphologies on Water Solubility and Water Content

The water solubility of amorphous and dihydrated paclitaxel was analyzed by HPLC. For comparison, a dissolution test for anhydrous paclitaxel (Hauser Inc.) was also conducted. Dissolution profiles of dihydrated, amorphous, and anhydrous paclitaxel are shown in Fig. 4. At the beginning, anhydrous paclitaxel showed relative high solubility of 3.8-4.8 μg/ml and decreased to 0.9 μg/ml at 20 h, whereas the solubility of amorphous and hydrated paclitaxel measured 1.0 μg/ml and 0.4 μg/ml, respectively. The dissolution profiles for paclitaxel showed that amorphous paclitaxel determined the highest solubility after 20 h and its solubility held most stable during the measurements in 20 h. In contrast to amorphous form,

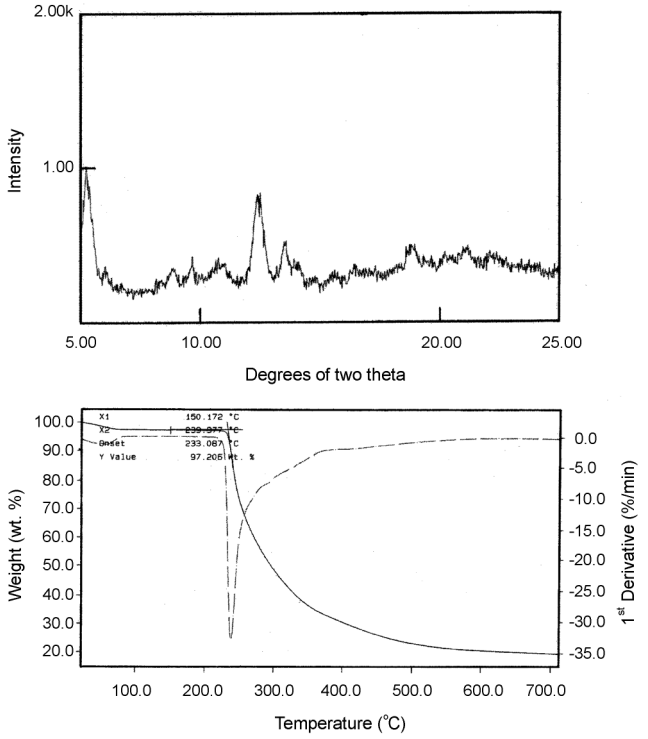

Fig. 3. XRPD pattern (up) and TGA thermogram (down) of the paclitaxel made from absolute methanol.

the water solubility of purchased anhydrous paclitaxel was changeable from 3.8 μg/ml over 4.8 μg/ml to 0.9 μg/ml during the whole analysis.

The hygroscopic property of dihydrated paclitaxel and amorphous paclitaxel was also characterized by analysis of water content. Table 2 shows the initial water content of amorphous and dihydrated paclitaxel for 0.65 wt% and 5.85 wt%, respectively. This means dihydrated paclitaxel absorbed water from the atmosphere, quickly. It could be recognized that dihydrated paclitaxel absorbed more water than amorphous paclitaxel at all given humidity (15, 60, 95 RH%) in storage conditions. So, dihydrated paclitaxel with its initial water content of 5.85 wt% showed very hard to hold under certain water content for formulation of drug because its water contents were extremely changeable during storage.

3. Removal of Residual Solvents by Spray Drying

Residual solvents in pharmaceuticals are defined as organic volatile chemicals that are remaining after manufacturing of drug sub-

Table 1. Summary of XRPD, DSC, TGA, and HPLC measurements for dihydrated and amorphous paclitaxel

Analysis	Item	Dihydrated paclitaxel	Amorphous paclitaxel
XRPD	Diffraction peaks	5.7, 8.5, 9.5, 10.3, 10.6, 12.0, 13.5° 2θ	-
DSC	Water evaporation	50-125 °C	-
	Solid-solid transition	160 °C	-
	Melting point	221 °C	-
	Glass transition	-	152 °C
TGA	Water evaporation temperature	25-80 °C	-
	Water evaporation weight loss (%)	4.3%	0.7%
HPLC	Solubility in water	0.4 μg/ml	1.0 μg/ml

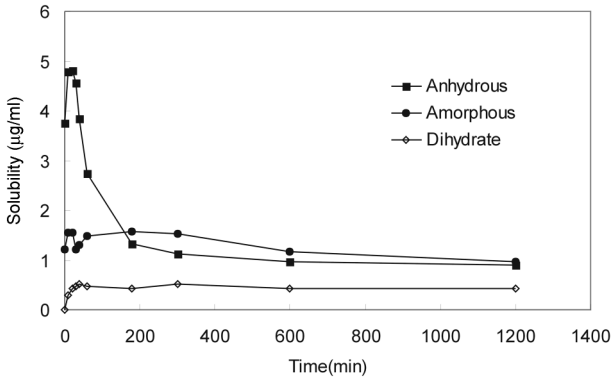

Fig. 4. Dissolution profiles of different solid-state paclitaxels depend on time. Amorphous and dihydrated paclitaxel were made according to the method described in the text. Anhydrous paclitaxel was purchased from Hauser Inc. and used without further purification.

stances or in the preparation of drug products. If there is no therapeutic benefit from residual solvents, all residual solvents should be removed. As one effort to remove residual solvents, amorphous paclitaxel from the solvent mixture of methylene chloride/methanol (98/2, v/v) was sequentially dried at rotary evaporator and in a vacuum drying oven on a temperature of 60 °C for 72 h. The contents of residual solvents, methylene chloride and methanol, after drying were determined over 10,000 ppm and 2,000 ppm, respectively. Actually, in the course of evaporation of solvents, a rigid layer seemed to be formed on the surface of dried paclitaxel and the layer prevented further evaporation of residual solvents. So, evaporating was not considered to be an acceptable method for removing of these solvents for production of pharmaceutical materials. Finally, it was very hard to eliminate the residual solvents below the concentration limit for pharmaceutical drug required by ICH guidance Q3C [1997]. According to ICH guidance Q3C, methylene chloride and methanol are the residual solvents in the manufacturing process of pharmaceutical drugs, they belong to class 2, and their concentration limits showed 600 ppm for methylene chloride and 3,000 ppm for methanol, respectively. The content of methanol for 2,000 ppm in paclitaxel showed under the concentration limits of ICH guidance, but the content of methylene chloride was much higher than the allowed maximum concentration limits. In order to remove the residual solvents of amorphous paclitaxel, paclitaxel was dissolved in methylene chloride/methanol (98/2, v/v) and put into spray drier at 60 °C with a peristaltic pump of a velocity 5 ml/min. Methylene chloride and methanol could be reduced by use of spray drier to 179 ppm and less than 100 ppm, respectively (Table 3). The high initial concentration (ca. 8,000-10,000 ppm) of methylene chloride could be simply reduced to 179 ppm by spray drying and successive drying in vacuum oven.

Table 3. The effect of drying time after spray drying on residual solvents

Sample no.	Methanol (ppm)				Methylene chloride (ppm)			
	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
1	140	88	79	79	8711	497	203	179
2	143	120	57	58	8205	693	193	179
3	206	111	45	63	10769	589	194	179

CONCLUSIONS

In this study, we found that solvent treatment of paclitaxel was a convenient method to control the morphologies of paclitaxel. Amorphous paclitaxel was simply made by dissolving paclitaxel in methylene chloride/methanol (98/2, v/v) and in relatively non-polar solvents (diethyl ether, t-butyl methyl ether, and pentane). On the other hand, dihydrated paclitaxel (paclitaxel·$2H_2O$) was made by dissolving paclitaxel in a special polar solvent containing a small amount of water. Their physicochemical properties were investigated by X-ray powder diffraction (XRPD), differential scanning calorimetry (DSC), and thermo gravimetric analysis (TGA). The hygroscopic property of dihydrated paclitaxel was very changeable in all given humidity (15, 60, 95 RH%) during storage. Dissolution profiles for paclitaxel showed that amorphous paclitaxel measured the highest solubility and its solubility held most stable during the measurements. The residual solvent could be reduced to the maximum allowed value (600 ppm for methylene chloride, 3,000 ppm for methanol) of guidance for the International Conference on Harmonization (ICH) by spray drying.

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Table 2. The change of water contents of amorphous and dihydrated paclitaxel during storage by different humidity

Morphology of paclitaxel	Humidity (RH%)	Water content (wt%)				
		Initial	2 weeks	4 weeks	8 weeks	12 weeks
Amorphous form	15	0.65	0.58	0.90	1.83	2.22
	60		1.62	1.69	2.11	2.39
	95		2.11	2.53	3.66	5.04
Dihydrated form	15	5.85	3.87	4.69	4.70	5.15
	60		5.83	6.13	6.94	7.16
	95		6.14	6.71	7.17	8.46

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