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Original Article

The Effect of Temperature, pH, and Different Solubilizing Agents on Stability of Taxol

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Abstract

Inabilities to attain adequate aqueous solubility and stability have made the preparation of a clinically suitable formulation of taxol difficult. Addition of nicotinamide (ND), 2-hydroxypropyl-β-cyclodextrin (HPßCD), polyethylene glycol, (PEG), bile salts (BS, 50:50 sodium cholate: deoxycholate), and cremophor EL can improve the solubility of taxol. Studies were undertaken to compare the stability of taxol in HPßCD (20%), ND (20%), PEG (20%), BS (20%), and cremophor EL (cremophor: ethanol 0.5% of each) solutions at 25, 37, 50, and 60 ^{o}C. Taxol concentration was measured by HPLC method. The rate of degradation proceeded in a Log-linear fashion and increased progressively with the elevation of temperature in all solutions except in HPβCD which taxol had negligible degradation. In the absence of added agents, taxol exhibited the lowest and highest stability at pH 1.2 and 4.5, respectively. Taxol was most stable in HPβCD followed by PEG and then ND. The stability of taxol increased linearly with the HPβCD concentration (0-5% w/v). Due to the observed improved stability and solubility, HPβCD may be considered for pharmaceutical studies as a suitable stabilizer for formulation of taxol.

Keywords: Bile salts; PEG; Hydroxypropyl-β-cyclodextrin; Inclusion complex; HPLC; Nicotinamide; Taxol; Stability; Solubilizing agents.
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1. Introduction

Clinical usage of taxol has been limited by its low solubility, low stability, and toxic side effects [1-4]. Taxol is susceptible to degradation processes such as mild basic and acidic hydrolysis, oxetane ring cleavage, and epimerization which results in the formation of baccatin III as the major product and several biologically active and inactive minor compounds [5-6]. In hope of increasing taxol solubility and therapeutic efficacy, various taxol formulations have been devel-

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oped, especially in solutions of hydroxypropyl-ß-cyclodextrin (HPßCD), nicotinamide, polyethylene glycol 400 (PEG), bile salts (50:50 sodium cholate:deoxycholate), and cremophor EL (CR, cremophor:ethanol 50:50). Despite the clinical use of taxol in treatment of cancer, very little information on the stability of taxol is available. An increasing interest in developing a new formulation of taxol has heightened the need to understand the basic physicochemical properties of taxol in order to facilitate the formulation of liquid product. As a part of continuing effort in our laboratory to further understanding of the physicochemical properties of taxol, the solution stability of taxol was followed as a function of temperature, pH, ionic strength, buffer components or solubilizing agents.

2. Materials and methods

2.1. Materials

Taxol was purchased from Calbiochem-Novabiochem Co. (USA), and was used as supplied. Hydroxypropyl-β-cyclodextrin (molecular weight=1380) was obtained from Aldrich Chemical Company (Milwaukee, WI, USA), and was used without further purification. Nicotinamide, polyethylene glycol 400, and bile salts (50:50, sodium cholate: deoxycholate), were supplied from Sigma Chemical Company (St. Louis, MO, USA). All other materials were reagent grade. Water was deionized and passed through a Milli-Q apparatus (Millipore).

2.2. Stability determination

The stability studies were carried out by adding a stock solution of taxol in cremophor EL (CR, cremophor:ethanol 50:50) to: 1) water; 2) aqueous solutions of citrate buffers (pH values of 1.2 and 2.5) and phosphate buffers (pH values 4.5, 6.5, 7.4, and 8) ionic strength of all was adjusted with sodium chloride to 0.5; 3) aqueous solutions of HPβCD (concentration ranged from 0.5 to 20% w/v); 4) nicotinamide (20% w/v); 5) PEG (20% w/v); and 6) bile salts (20% w/v). Before the addition of taxol, all solutions were equilibrated at the desired temperature (20, 37, 50, and 60 ^{o}C) in a water bath and after the addition of taxol they were mixed thoroughly. The effect of buffer concentration (0.025, 0.05, and 0.1 M) on the degradation of taxol was studied in phosphate and citrate buffers. The influence of different ionic strengths (0.1, 0.25, and 0.5) on the degradation of taxol was studied in 0.025 M phosphate buffer (pH 5.5) at 37 ^{o}C. The initial taxol concentration was 10 µg/ml. The CR concentration in the final reaction mixture was less than 0.5%. Screw-capped vials containing the test solutions were placed at the desired temperature. Aliquots of samples were withdrawn at appropriate time intervals and were processed as described in the assay under analysis of samples.

2.3. Method of analysis of samples

Analyses were carried using an HPLC system consisting of (Waters Lc module 1. 600E pump, 715 auto-injector, and 486 UV detector), with a partisil ODS column (phenomenex® 100 mm ,4.6 mm ID, 5 µm particle size) protected by a partisil ODS guard column (phenomenex® 30 mm, 4.6 mm ID, 5 µm particle size), using a mobile phase of 43% acetonitrile in water containing 0.05% v/v glacial acetic acid (pH 4.5) at a flow rate of 1.7 ml/min. The eluate was monitored at 227 nm. Samples of 1 ml plus 0.1 ml of internal standard (fenoprofen 3.5 µg/ml in acetonitrile 47% in water) were extracted with 3.5 ml of *t*-butyl methyl ether and vortex-mixed for 1 min. The mixture was then centrifuged for 10 min at 1100 g after which 3 ml of the organic layer were removed and evaporated to dryness under vacuum at low temperature. The residue was reconstituted in 0.2 ml of 47% aqueous acetonitrile and 50 µl of it was injected in the HPLC system.

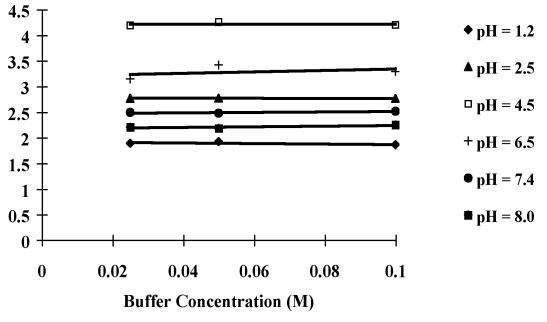


Figure 1. Effect of pH (1.2-8.0) and buffer concentration on the degradation rate constant of taxol at 37 ºC and μ = 0.5. Solid line is the regression line through the data points.

Standard solutions of taxol were made when appropriate, extracted and injected before, during, and after a series of samples were injected. Data are presented as the means of three experiments.

3. Results and discussion

3.1. Determination of the first-order rate constants

At a constant temperature and ionic strength, the loss of taxol in the presence of aqueous buffers and solubilizing agents was best described by first-order kinetics. Apparent-first-order rate constants (k_{obs}) were calculated from the linear first-order plots based on:

$$\text{Log } [C] = \text{Log } [C_o] - (k \cdot t / 2.303)$$

where the concentration is [C], the initial concentration is $[C_o]$, and time is t. Rate constants (Table 1) were obtained from linear regressions.

3.2. Effect of buffer concentration

Figure 1 displays the effect of increasing buffer concentration (0.025-0.1 M) on the degradation rate constant of taxol at 37 ºC at pH values of 1.2, 2.5, (citrate buffers) 4.5, 6.5, 7.4, and 8.0 (phosphate buffers). Since the slope of this line was almost zero, the buffer concentration effect was interpreted to be negligible on the degradation kinetics of taxol in that range. The higher value of the degradation rate constant in the 0.1 M solution of phosphate buffer at pH 8 may be due to day-to-day variation in the assay for this particular sample. Therefore, the k_{obs} in citrate and phosphate buffers at 0.025 M

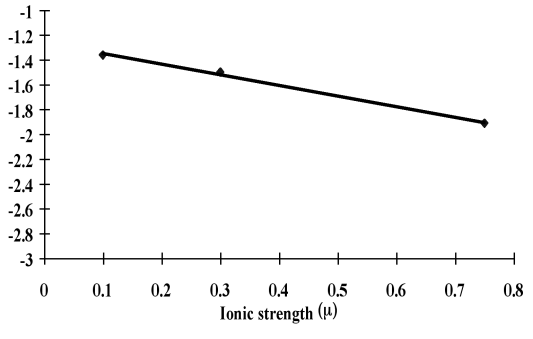


Figure 2. Effect of ionic strength on the degradation rate constant of taxol in 0.05 M phosphate buffer at 37 ºC and pH 4.5. Ionic strength was adjusted with NaCl. Solid line is the regression line through the data points.

(which is similar to zero concentration of buffer) were assumed to be the degradation rate constant at that specific pH.

3.3. Effect of ionic strength

At constant temperature (37 ºC) and buffer concentration (0.05 M), the effect of

Table 1. Kinetic parameters of degradation of taxol in water at different pH at 37 ºC.

Parameters	pH = 1.2	pH = 2.5	pH = 4.5	pH = 6.5	pH = 7.4	pH = 8	Water
k_{obs} (h^{-1}) ?10^3	16.9	1.90	1.10	2.70	2.60	15.4	0.14
$t_{1/2}$ (h)	41.0	357	647	259	270	44.9	487
$t_{90\%}$ (h)	6.20	54.3	98.4	39.3	41.0	6.80	74.0
E_a (Kcal/mol)	12.7	6.20	2.40	7.50	20.30	5.40	4.20
ΔG^o (Kcal/mol)	2.49	3.79	4.20	3.61	3.73	2.67	4.01
ΔS^o (cal/mol/deg)	43.6	16.2	-3.20	13.2	11.7	55.9	19.9

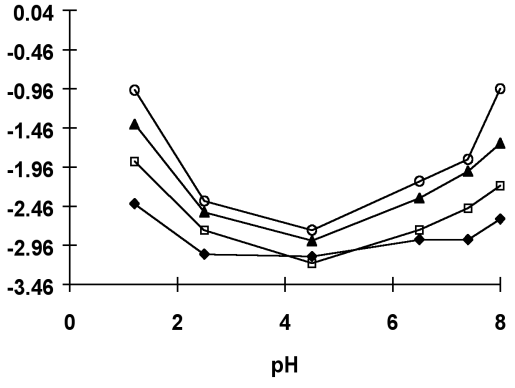


Figure 3. pH-rate profile for the degradation of taxol at different temperatures at an ionic strength of 0.5. Data are presented as the mean of three experiments.

ionic strength on the degradation of taxol at pH 4.5 was studied and the rate constant in infinitely dilute solution (k_o) in which $\mu = 0$, was calculated based on:

$$\text{Log } k_{obs} = \text{Log } k_o + C\mu$$

in which $C\mu$ is a constant obtained from experimental data [7]. Figure 2 shows the effect of ionic strength on the degradation of taxol in (0.05 M) phosphate buffer (pH 4.5) at 37 ^oC. Since the slope, C, of a plot of Log k_{obs} versus the ionic strength was -0.858, the ionic strength was interpreted to be an important factor on the degradation kinetic of taxol. These data suggest that the degradation rate of taxol decreases with increasing ionic strength of the buffers. The y-intercept of that plot represents the rate constant in infinitely dilute solution (k_o) which was equal to 0.054 hr^{-1}.

3.4. pH-rate profile

The influence of the pH on the degradation of taxol is demonstrated in Figure 3. Figure 1 indicates that buffer concentration has a negligible effect on the degradation kinetics of taxol. Therefore, Log of the k_{obs} at 0.025 M buffer concentration at 37 ^oC was plotted as a function of pH. The profile shows a specific acid catalytic region at pH below 2.5, a plateau of pH-independent degradation between pH 3 and 6, and a sharp increase in degradation rate at pH above 7. In the plateau region (pH 4.5), the activation energy of (E_a) was determined to be 2.4 Kcal/mol (Table 1). Although the exact intramolecular mechanism of degradation of taxol is still unclear, the small negative entropy of activation (-0.003 Kcal/mol/deg) may be a characteristic of unimolecular reaction, (bimolecular reactions usually result in a much larger negative entropy, [8]. Therefore, it was concluded that the instability of taxol at pH 4.5 is due to a unimolecular reaction occurred through possible epimerization of taxol to 7-epitaxol [5].

3.5. Effect of buffer component

Figure 4 illustrates the degradation of taxol in phosphate, acetate, and citrate buffers with the same pH, ionic strength, and concentration at 37 ^oC. It seems that decomposition of taxol in citrate is 4 and 7 times faster than acetate and phosphate buffers, respectively.

3.6. Effect of temperature

Figure 5 shows the Arrhenius plot for the degradation of taxol in water. The logarithm of the rate constant at the lowest buffer concentration studied (0.025 M) was plotted against the reciprocal of the temperature in Kelvin degrees to determine the temperature dependency of buffer catalytic constant of taxol [9]. The data from the lowest buffer

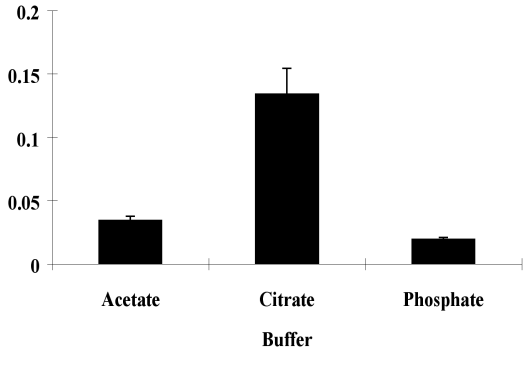
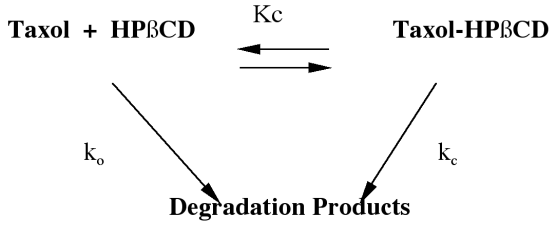


Figure 4. Effect of buffer component on the degradation rate constant of taxol in different buffer at 37 ^oC (pH = 4.5, C = 0.05 M, and $\mu = 0.5$). Data are presented as the mean ± standard error of mean (n=3).

Table 2. Calculated kinetic parameters of degradation of taxol in water and in the presence of different solubilizing agents at 25 °C

Parameter	PEG 400	HPβCD	Nicotinamide	Bile Salts	Water
$k_{(obs)}$ (h^{-1})	$2.88?10^{-4}$	$4.02?10^{-5}$	$1.33?10^{-3}$	0.09	$7.44?10^{-4}$
$t_{1/2}$ (day)	100	719	21.7	0.31	38.8
$t_{90\%}$ (day)	15.3	109	3.29	0.05	5.96
E_a (Kcal/mol)	11.1	10.1	8.35	2.24	6.17
ΔG^o (Kcal/mol)	4.83	5.99	3.90	2.77	4.27
ΔS^o (cal/mol/deg)	-46.5	-53.8	-39.0	-11.3	-38.6

concentrations were used in all of the estimations, in order to predict a shelf life in the presence of a weak buffer. A linear regression was performed on the data using the 0.025 M buffer concentration and the energies of activation were calculated from the slopes. The value of the observed degradation rate constant was extrapolated to 25 °C and the shelf life of 74 hrs or more was predicted by these estimates, at a pH value of 6.5. All calculations were made based on the assumption that the energy of activation does not change with temperature. The energy of activation in pH range of 4.5 to 6.5 was calculated to be between 3.28 to 7.79 Kcal/mol, and it demanded a detailed investigation of various approaches to stabilize the cremophor formulation of taxol.



Scheme 1: Relationship between the total HPßCD concentration and the observed rate constant for degradation of taxol.
k_o represents the apparent-first-order rate constant for degradation of free taxol (h^{-1}),
k_c represents the apparent-first-order rate constant for degradation of taxol in the complex (h^{-1})
K_c represents the stability constant for the complex, assuming 1:1 complex formation (M^{-1}).

3.7. Solubilizing and stabilizing agents

3.7.1. Cyclodextrins

Cyclodextrins can substantially increase the stability of compounds through their ability to form inclusion complexes with many drugs. Improvement of stability of prostaglandin E1[10], psoralen [11], nitrazepamm [12], mitomycin [13,14], tauromustine [8], and 2', 3'-dideoxyinosine [14], by cyclodextrin complexation have been reported. The observed rates of degradation of these compounds were significantly reduced by complexation with 2-HPβCD and γCD, depending on the structure of the compound. These studies suggest that cyclodextrin complexation may be a useful approach to stabilize taxol without loss of its pharmacological activity. Taxol was found to form an A_L type inclusion complex with HPβCD in solution. The effects of temperature and concentration of HPβCD on the stability of taxol in HPβCD solutions were studied. The apparent-first-order rate constants (k_{obs}) were determined from the disappearance of the drug by linear regression of the logarithm of the concentration of the drug remaining versus time. Degradation of taxol in HPβCD followed a first-order kinetics and the introduction of up to 20% (w/v) HPβCD to the reaction medium did not affect this kinetic behavior, as linear relationship ($P<0.05$) was in all cases obtained between the logarithms of the concentration of the drug remaining and time (Figure 6). Degradation of taxol, both within the different media, or water, increased progressively

with elevation of temperature in all solutions of taxol in HPβCD (Figure 7). The kinetic parameters for the degradation of taxol are displayed in Table 2. Comparison of the rate constants shows that all HPßCD solutions tested (concentration of HPßCD: 0-20% w/v), have significant effect on stability of

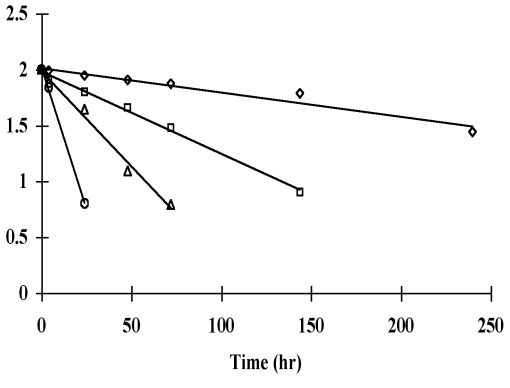


Figure 5. Effect of temperature on decomposition rate of taxol at pH 1.2. Solid lines are regression lines through the data points.

taxol. Increasing the HPβCD concentration in the reaction medium decreased the rate of degradation of taxol (18.5 times slower in 20% w/v HPβCD), and a non-linear relationship was obtained between the apparent-first-order rate constants and HPβCD concentration (Figure 8). This type of dependence of the rate constant on the HPβCD concentration is characteristic of saturation kinetics where the drug degrades at a slower rate within the cyclodextrin complex than outside of it (Scheme 1) [15]. The enthalpy for the complex formation is negative, resulting in a decrease in free energy due to the complexion process. On the other hand, the activation parameters for the degradation result in an increase in the free energy. Thus, when the temperature of the HPßCD containing reaction medium is lowered, both decrease in the degradation rate constant and increase in the stability constant for the inclusion complex will result in stabilization of taxol.

Figure 8 shows the relationship between the Log of degradation rate constant of taxol in HPßCD solution as a function of 1/T. There is a deviation from the Arrhenius plot at higher temperatures which may be due to changes in the association of HPβCD molecules in solution and consequential interaction with taxol.

3.7.2. Nicotinamide

Nicotinamide (3-pyridine carboxamide) is a nontoxic B vitamin (vitamin B_3) that has been shown to enhance the aqueous solubility of many drugs including taxol through complexation. The exact mechanism by which this compound forms complexes is not entirely clear, but it has been suggested that it could be the result of a *Pi*-donor *Pi*-acceptor interaction [16, 17]. Taxol has a carbonyl group and a *Pi*-conjugated system in its molecular structure and it interacts with nicotinamide in a nonlinear fashion as a function of pH and nicotinamide concentration. Our previous results have shown that the solubility of taxol was enhanced with increasing nicotinamide concentration (0.5 to 20% w/v) up to 95-fold, compared to

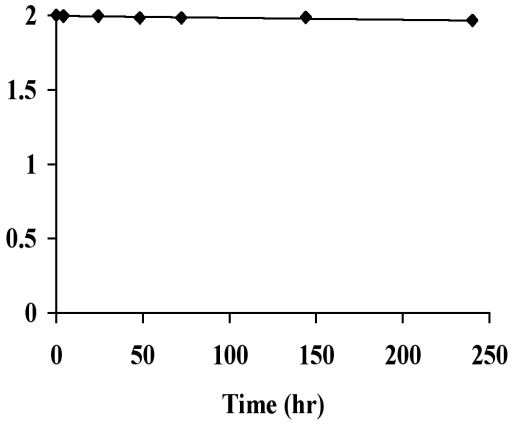


Figure 6. Decomposition of taxol in the presence of HPβCD solution (5% w/v) at 20 °C. Solid line is the regression line through the data points.

water (0.32 mg/L), and at pH 4.5 a maximum solubility of 59 mg/L of taxol was observed in 20% w/v nicotinamide solution

[3]. The effect of temperature on the stability of taxol in nicotinamide solution was studied. The pseudo-first-order rate constants (k_{obs}) was determined from the disappearance of the drug by linear regression of the logarithm of the concentration of the remaining drug versus time. Degradation of taxol increased progressively with the elevation of temperature in all solutions of taxol in nicotinamide (Figure 9). Comparison of the rate constants (Table 2) shows that nicotinamide has significant effect on the stability of taxol. Taxol degraded 1.7 times faster in 20% nicotinamide than in water. Nicotinamide in aqueous solution is almost neutral in reaction, although complexation of nicotinamide and taxol can increase the solubility of taxol, this complexation apparently does not protect taxol from degradation. Figure 9 shows the relationship between Log degradation rate constant of taxol in nicotinamide solution as a function of 1/T. There is a deviation from the Arrhenius plot at higher temperatures which may be due to changes in the association of nicotinamide molecules in solution and consequential interaction with taxol.

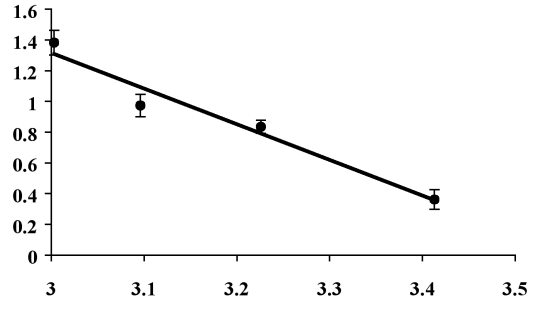


Figure 7. A plot of Log k against 1/T of taxol in HPβCD solution (20% w/v). Solid line is the regression line through the data points. Log k + 5 is plotted on the vertical axis to eliminate the negative values along the axis.

3.7.3. Bile salts

The main products of cholesterol metabolism are bile salts which are, biologically, the most important detergent-like molecules. Bile salts are amphipathic molecules, possessing hydrophilic and hydrophobic regions which enable them to display characteristic behaviors in water. The surface active and self-association properties of bile salts allow them to form micelles which solubilize poorly water-soluble drugs [18]. Bile salts have been reported to increase the apparent solubility of many poorly water-soluble drugs such as diazepam, griseofulvin, NSAIDs, 1,8-dinitropyrene, triamcinolone, betamethasone, and dexamethasone [18-21]. Incorporation into micelles appeared to increase the solubility of these compounds at concentrations above critical micelle concentration of bile salts (13-16 mM or higher).

Our previous results have shown that solubility of taxol increased 45-fold in a nonlinear fashion with increasing bile salt concentration (0.5 to 20% w/v) in the medium [3]. In this study the degradation kinetics of taxol in solutions of bile salts (50:50, sodium cholate: deoxycholate), were investigated. The apparent-first-order rate constants (k_{obs}) were determined from the disappearance of the drug by linear regression of the logarithm of the concentration of the remaining drug versus time. Degradation of taxol increased progressively with the elevation of temperature in all solutions of taxol

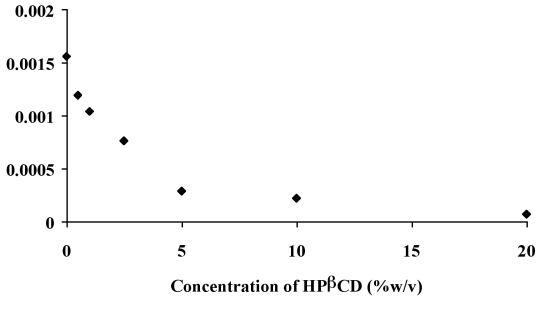


Figure 8. The relationship between k_{obs} and HPβCD concentration at 20 °C.

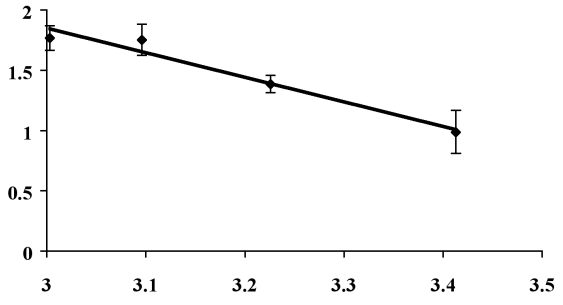


Figure 9. A plot of Log k against 1/T of taxol in nicotinamide solution (20% w/v). Solid line is the regression line through the data points. Log k + 4 is plotted on the vertical axis to eliminate the negative values along the axis.

in bile salts (Figure 10). Comparison of the rate constants (Table 2) shows that bile salts have a significant effect on stability of taxol. Our results revealed that taxol degraded 12.6 times faster in 20% bile salts than in distilled water. The pH of bile salts solutions (con-

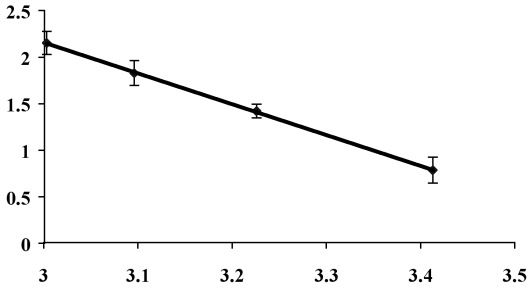


Figure 10. A plot of Log k against 1/T of taxol in bile salts solution (20% w/v). Solid line is the regression line through the data points. Log k + 3 is plotted on the vertical axis to eliminate the negative values along the axis.

centration 0.5-20% w/v) in aqueous solution is alkaline (pH > 8). Although micellization of bile salts can increase the solubility of taxol, this micellization can not protect taxol from alkaline degradation. Therefore, low stability of taxol in bile salts solutions could be due to a high pH of bile salt solutions and the presence of specific base catalytic degradation of taxol at pH above 7.

3.7.4. Polyethylene glycol

Organic co solvents are among the most powerful solubilizing agents for a large number of lipophilic drugs [22]. Our previous results have shown that the solubility of taxol increased dramatically in a linear fashion with increasing polyethylene glycol 400 (PEG) concentrations (0.5 to 100% w/v) in the medium [3]. The influence of PEG on the stability of taxol was studied using a mixture of PEG: water, (20%). Also the effect of temperature on the drug stability of taxol in PEG solution was studied. The apparent-first-order rate constants (k_{obs}) were determined from the disappearance of the drug by linear regression of the logarithm of the concentration of the remaining drug versus time. Degradation of taxol, within both PEG and water was increased progressively with the elevation of temperature in all solutions of taxol (Figure 11). Comparison of the rate constants (Table 2) shows that PEG had a significant effect on the stability of taxol. Taxol degraded 1.7 times slower in 20% PEG than in water. PEG in aqueous solutions is almost neutral in reaction. The presence of PEG not only increased solubility of taxol, but it also partially protected taxol from degradation. Therefore, replacement of water by PEG up to 20% v/v may stabilize taxol against possible hydrolysis. However, the exact mechanism of protection is not clearly understood and further studies are required. Figure 11 shows the relationship between degradation rate constant of taxol in

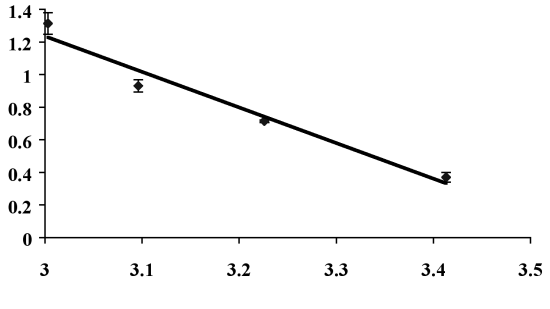


Figure 11. A plot of Log k against 1/T for the thermal stability of taxol in PEG solution (20% w/v). Solid line is the regression line through the data points. Log k + 4 is plotted on the vertical axis to eliminate the negative values along the axis.

PEG solution as a function of 1/T. There is a deviation from the Arrhenius plot at higher temperatures which may be due to changes in the association of PEG molecules in solution and consequential interaction with taxol.

4. Conclusions

The results show that all of the tested solubilizing agents had significant effect on the stability of taxol. However, PEG and HPβCD not only increased the solubility of taxol but they also stabilized taxol against degradation in aqueous solutions. The HPβCD stabilizing effects depended on the concentration of HPβCD and the pH of the median.

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