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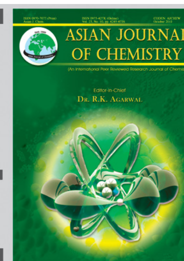

HPLC Method for Determination of Paclitaxel in Rabbit Plasma

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A simple rapid stability-indicating isocratic assay has been developed and validated for the determination of paclitaxel in rabbit plasma. The forced degradation of paclitaxel in solution form under stress conditions including acid and base hydrolysis, oxidation and heat were also studied. The assay is performed using a μ Bondapak-C_{18} (150 mm × 4.6 mm i.d) with a mobile phase consisted of acetonitrile and 20 mM phosphate buffer (pH 5) (50:50 % v/v), the flow rate was 1 mL min and UV detection at 229 nm. The method was found to be specific for paclitaxel in the presence of degradation products, no interfering peaks were observed with an overall analytical run time was 10 min. The percentage recovery were found to be 98.92-103.95 % and 94.4 -103.4 % for interday and intraday accuracy, respectively. Inter-day precision (reproducibility) was found to be 0.23-3.6 % RSD, while intraday precision (repeatability) was found to be 0.47-3.7 % RSD for the samples studied. The calibration curve was found to be linear with the equation y = 0.7576x + 0.1189, with a correlation coefficient of 0.9996 (R^2) over the concentration range 0.1-40 μg/mL. The limit of quantitation was the lowest concentration. Degradation of paclitaxel in solution form was found to be more degradable to alkaline hydrolysis than other stress conditions. The study showed that the fully validated HPLC method is simple and rapid and can be used without requiring any preliminary treatment of the sample.

Key Words: HPLC, Paclitaxel, Stability-indicating assay, Degradation, Validation.

INTRODUCTION

Paclitaxel, tax-11-en-9-one,5β,20-epoxy-l, 2β,4,7β,10β, 13α-hexahydroxy-4,10-diacetate-2-benzoate-13-(α-phenyl-hippurate) (Fig. 1), is a natural alkaloid isolated from the bark of the pacific yew tree, *Taxus brevifolia*[1], currently used to treat a variety of tumors, including ovarian, breast and non-small-cell lung cancers. The importance of paclitaxel was not recognized until the late 1970s, since, it is difficult to obtain and also due to its low solubility it has a formulation problem. Horwitz *et al.*[2-4] discovered that paclitaxel has a unique mechanism of action and interest was further stimulated when impressive activity was demonstrated in NCI tumor screening and it was first approved by the US FDA in 1992. The mechanism of action was established as the stabilization of cellular microtubules and blocking of chromosome segregation[5], and the β-tubulin functional domains and the domains containing paclitaxel-binding sites were identified[6].

The ICH guideline Q1A on stability testing of new drug substances and products[7] emphasizes that the testing of those features, which are susceptible to change during storage and are likely to influence quality, safety and/or efficacy must be done by validated stability-indicating testing methods. It is also mentioned that forced decomposition studies (stress testing) at temperatures in 10 ºC increments above the accelerated temperatures, extremes of pH and under oxidative and photolytic conditions should be carried out on the drug substance so as to establish the inherent stability characteristics and degradation pathways to support the suitability of the proposed analytical procedures.

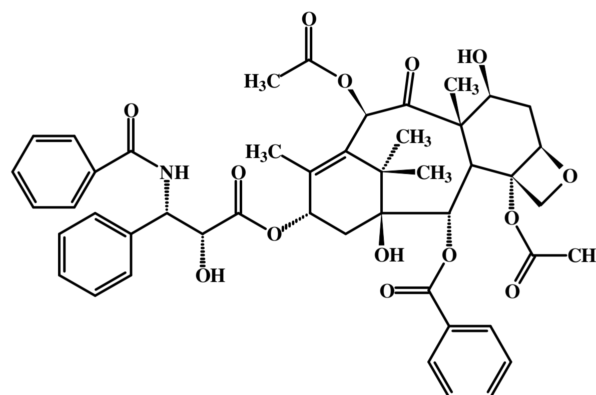

Fig. 1. Structure of paclitaxel

A number of assay methods have been published for the determination of paclitaxel in biological fluids, including

capillary electrophoresis[8-10], liquid chromatography-mass spectrometry (LC-MS)[11-26] and high-performance liquid chromatography (HPLC)[27-38]. Of these, the HPLC methods have been most frequently used in the pharmacokinetic studies of paclitaxel because of their simplicity, sensitivity and selectivity. These HPLC methods utilize either liquid-liquid extraction (LLE)[31], solvent extraction[30] or solid-phase extraction (SPE)[32-38]. To our best of knowledge, no validated stability- indicating analytical method for determination of paclitaxel dissolved in acetonitrile has been reported in the literature. Therefore, an isocratic stability-indicating HPLC method for the rapid quantitative determination of paclitaxel in presence of degradation products in solution form was developed. This manuscript reports the forced degradation of paclitaxel under stress conditions including acid and base hydrolysis, oxidation and heat. In present study, a simple and sensitive HPLC method was developed for paclitaxel in rabbit plasma with a lower limit of quantitation (LLOQ) of 100 ng/mL.

EXPERIMENTAL

Paclitaxel was obtained from David Bull Laboratories, Victoria, Australia. Hydrocortisone acetate was obtained from Fluka AG, Buchs, Switzerland. Methanol and acetonitrile (AnalaR® with 99.8 % purity) were purchased from BDH, Pool, England. Water used in this study was obtained from a Milli-Q water purification system (Millipore, Bedford, MA). All other reagents were of analytical grade and were used without further purification.

HPLC method for determination of paclitaxel

Equipment and chromatographic conditions: The study employed a high pressure liquid chromatography (waters™ 1515 isocratic controller, waters, USA) equipped with a waters 2487 dual λ absorbance detector and an automatic sampling system (waters™ 717). The mobile phase consisted of acetonitrile and 20 mM phosphate buffer (pH 5) (50:50 % v/v) and the flow rate was 1 mL/min. Separation was achieved using a 150 mm × 4.6 mm (i.d.) C_{18}, μ Bondapak™, waters, reversed phase column with an average particle size of 10 μm and the column was kept at ambient temperature. The column effluent was monitored at a set wavelength of 229 nm. The chromatographic data analysis was performed with the Empower™ Program (Waters, USA).

Calibration standards and control solution: Stock solutions containing 1000 and 250 μg/mL of paclitaxel were prepared by dissolving appropriate amounts of the drug in acetonitrile. A stock solution containing 250 μg/mL of the internal standard hydrocortisone acetate was prepared in methanol. These solutions were stored at 4 ºC before use. Working solutions of paclitaxel were prepared as serial dilutions in acetonitrile at concentrations of (0.5, 1.0, 2.5, 5, 10, 25, 50 and 100 μg/mL) with a 50 μg/mL solution of the internal standard being prepared in methanol. Each working solution of the drug included a fixed concentration of the internal standard (10 μg/mL). The stock solutions were used to prepare calibration standards in methanol and the working solutions were used to prepare calibration standards in plasma.

Treatment of plasma samples: The working solutions (100 μL) were separately transferred to clean dry centrifugation tubes. Rabbit plasma (500 μL) was added to each tube. These were mixed using vortex for 2 min before adding 400 μL of acetonitrile. These were mixed again before centrifugation at 5000 rpm for 5 min. The supernatant was separated and loaded into HPLC vials before injecting 50 μL into the HPLC system.

Method validation: The calibration curve of paclitaxel is a plot of the peak area ratio (PAR) of the drug to the internal standard as a function of the drug concentration (C). This gives the following equation: PAR= Slope × C + Intercept. The slope and the intercept were determined from the determined peak area ratio and the nominal concentration of the drug. The unknown paclitaxel concentrations were determined from this equation.

Linearity and range: Calibration curves were constructed in acetonitrile by preparing a series of concentrations of the drug (100, 500, 1000, 2000, 5000, 10000, 20000 and 40000 ng/mL) with the internal standard included at a fixed concentration of 2000 ng/mL. Calibration curves were also constructed in rabbit plasma. These involved replicate analysis of plasma samples spiked with varying concentrations of paclitaxel (100, 200, 500, 1000, 2000 and 5000, 10000 and 20000 ng/mL) and a fixed internal standard (IS) concentration (2000 ng/mL).

Linear regression equation and correlation coefficient (R^2) were employed to statistically evaluate the linearity of the results.

Accuracy and precision: The accuracy of the method was determined by comparing the practical amounts recovered from the control samples with actual values present in the samples (theoretical values).

The precision of the method based on intraday variability was determined by replicate analysis of the calibration standards in the same day. The reproducibility was taken as the interday variability and was determined by replicate analysis of the calibration standards in different days with one replicate being analyzed each day. The relative standard deviation (RSD) values were calculated from the ratios of the standard deviation (SD) to the mean and expressed as percentage.

The selectivity of the method was determined by examining the interference from the endogenous materials in rabbit plasma, from the degradation products of the drug or from the tablet additives.

Limit of detection and lower limit of quantification: From the serial dilutions of paclitaxel stock solutions, the limit oif detection (LOD) and lower limit of quantification (LLOQ) were taken as the lowest concentration that can be accurately (relative error < 20 % for biological samples and < 2 % for the *in vitro* samples) and precisely determined (RSD < 20 % for biological samples and < 2 % for the *in vitro* samples).

It should be noted that paclitaxel is subjected to possible degradation in aqueous liquid preparations. Accordingly, it was necessary to validate the assay method as stability indication.

Method validation as a stability indicating assay: To validate the method as a stability indicating assay the drug was subjected to the following stress conditions:

Base exposure: An appropriate volume (0.25 mL) of the paclitaxel stock solution (1 mg/mL) was taken and exposed to hydrolysis by adding 10 mL of 0.5 N NaOH. This mixture

was incubated in a water bath at 60 ºC for 1 h at the end of which the solution was neutralized by adding 10 mL of 0.5 N HCl. An appropriate volume (1 mL) of internal standard working solution (50 µg/mL) was added before adjusting the volume to 25 mL with water. 30 µL of sample was injected to HPLC system.

Acid exposure: An appropriate volume (0.25 mL) of the paclitaxel stock solution (1 mg/mL) was taken and exposed to hydrolysis by adding 10 mL of 0.5 N HCl. This mixture was incubated in a water bath at 60 ºC for 1 h at the end of which the solution was neutralized by adding 10 mL of 0.5 N NaOH. An appropriate volume (1 mL) of internal standard working solution (50 µg/mL) was added before adjusting the volume to 25 mL with water. 30 µL of sample was injected to HPLC apparatus.

Oxidative exposure: An appropriate volume (0.25 mL) of the paclitaxel stock solution (1 mg/mL) was taken and exposed to oxidative degradation by adding 10 mL of 30 % hydrogen peroxide (H_2O_2). This mixture was incubated in a water bath at 60 ºC for 1 h. An appropriate volume (1 mL) of internal standard working solution (50 µg/mL) was added before adjusting the volume to 25 mL with water. 30 µL of sample was injected to HPLC apparatus.

Heat exposure: An appropriate volume (0.25 mL) of the paclitaxel stock solution (1 mg/mL) was taken and exposed to hydrolysis by adding 10 mL water. This mixture was incubated in a water bath at 60 ºC for 1 h. An appropriate volume (1 mL) of internal standard working solution (50 µg/mL) was added before adjusting the volume to 25 mL with water. 30 µL of sample was injected to HPLC apparatus. Concentration of paclitaxel and internal standard in the stress condition were 10 and 2 µg/mL, respectively.

RESULTS AND DISCUSSION

Assay specificity: Representative chromatograms of low and high concentrations of paclitaxel in acetonitrile are shown in Fig. 2. Paclitaxel and the internal standard (hydrocortisone acetate) were eluted at 4.2 min and 7.5 min, respectively, with a total run time being 10 min. No endogenous or extraneous peaks were observed interfering with the assay.

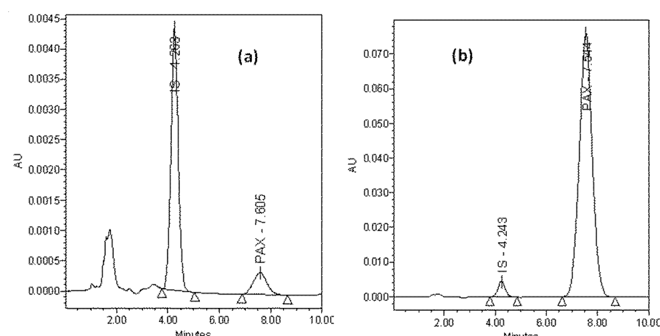

Fig. 2. Representative chromatograms showing 0.1 µg/mL (a) and 40 µg/mL (b) of paclitaxel. PAX = paclitaxel and IS = internal standard

Validation characteristics

Linearity and range: A standard curve was constructed for paclitaxel by plotting the peak area ratios of the drug *vs.* internal standard. There is an excellent linearity over the concentration range from 0.1 µg/mL to 40 µg/mL (Fig. 3). A typical equation obtained by the weighted regression was y = 0.7576x + 0.1189, with a correlation coefficient of 0.9996 (R^2).

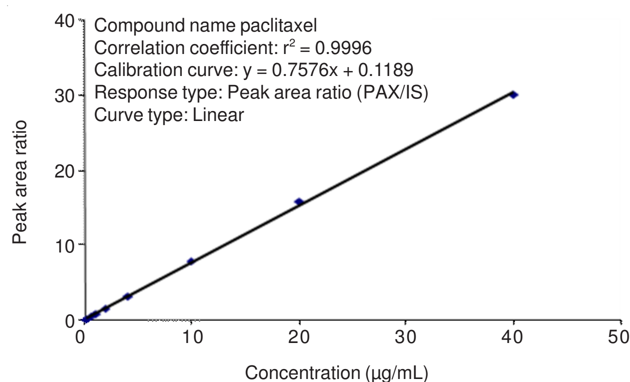

Fig. 3. Calibration curve of paclitaxel in acetonitrile by HPLC method

Accuracy: The accuracy was expressed as the closeness to the true value and is calculated as the per cent recovery related to the nominal values. Tables 1 and 2 present the percentage of drug recovered relative to the nominal values. The recovered values were close to the true value suggesting the accuracy of the assay.

TABLE-1
HPLC DATA OF VALIDATION PARAMETERS FOR INTER-DAY ACCURACY AND PRECISION OF PACLITAXEL (n=3)

Nominal value (µg/mL)	Recovered conc. (µg/mL)	SD (µg/mL)	RSD (%)	Recovery (%)
0.1	0.0989	0.003	3.02	98.91933
0.5	0.495	0.008	1.6	98.93044
1.0	1.002	0.028	2.7	100.1551
2.0	2.064	0.074	3.6	103.2074
4.0	4.068	0.097	2.4	101.7065
10.0	10.39	0.301	2.9	103.9472
20.0	20.64	0.441	2.1	103.2087
40.0	39.66	0.093	0.23	99.15517

TABLE-2
HPLC DATA OF VALIDATION PARAMETERS FOR INTRADAY ACCURACY AND PRECISION OF PACLITAXEL (n=3)

Nominal value (µg/mL)	Recovered conc. (µg/mL)	SD (µg/mL)	RSD (%)	Recovery (%)
0.1	0.10	0.0022	2.1	100.3
0.5	0.472	0.013	2.7	94.4
1.0	0.95	0.029	3.1	94.7
2.0	1.98	0.067	3.5	98.8
4.0	4.14	0.019	0.47	103.4
10.0	10.14	0.38	3.72	101.4
20.0	20.22	0.23	1.14	101.1
40.0	40.04	0.27	0.67	100.1

Precision: The precision was measured as the relative standard deviation (RSD) expressed as percentage over the concentration range of paclitaxel during the course of validation. This is presented in Tables 1 and 2 for the interday and intraday precision. The results indicated an acceptable precision for all concentrations assayed for both intraday and interday samples.

Limit of detection (LOD) and lower limit of quantification (LLOQ): The LLOQ of the assay was found to be 100

ng/mL. This is estimated to be the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision for the paclitaxel analyte.

Stability indicating assay: The per cent amounts of drug remaining after exposure to different stress conditions are given in Table-3. Fig. 4 shows representative chromatograms for the drug after being exposed to different stress conditions. The data in Table-3 indicate the susceptibility of the drug for heat, oxidation and alkaline and acidic conditions with the alkaline hydrolysis being the most detrimental for the drug. The assay method was shown to be suitable for monitoring the drug stability as indicated from Fig. 4, which showed no interference from the degradation product. This further indicates the selectivity of the assay.

TABLE-3
PERCENT REMAINING OF PACLITAXEL AFTER EXPOSURE TO DIFFERENT STRESS CONDITIONS DATA EXPRESSED AS MEAN ± SD (n = 3)

Stress conditions	Remaining (%)
Oxidative stress	57.4 ± 1.7
Alkaline hydrolysis	2.53 ± 0.1
Acid exposure	49.4 ± 5.8
Heat exposure	75.6 ± 14.7

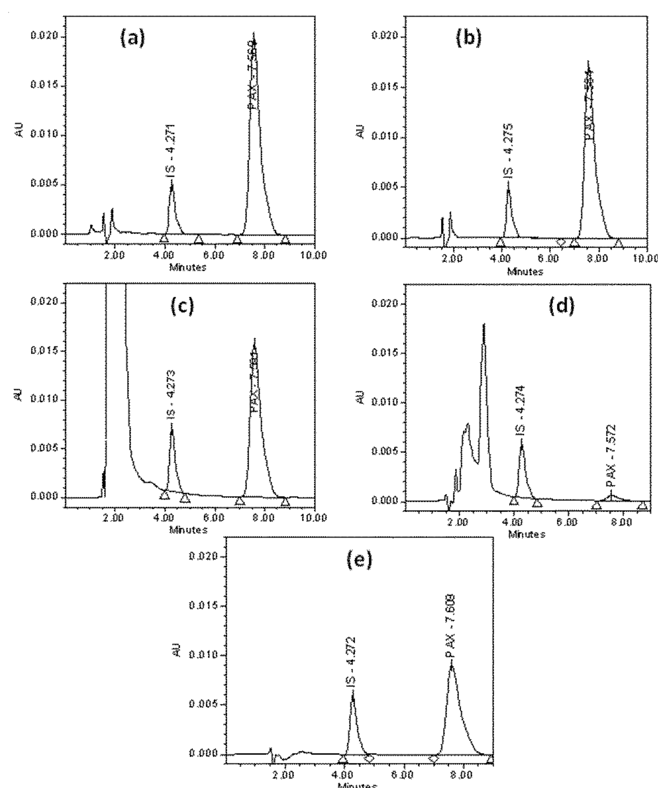

Fig. 4. Representative chromatograms showing paclitaxel after exposure to different stress conditions as (a) intact drug, (b) water, (c) hydrogen peroxide, (d) alkaline hydrolysis and (e) acid hydrolysis

The selectivity of the assay was further investigated by analysis of the drug in plasma samples. Fig. 5 shows representative chromatogram of blank plasma as well as the plasma spiked with the drug and internal standard.

The figure clearly shows the absence of any interference of any endogenous materials in the plasma with the drug or its internal standard. This indicates the suitability of the protein precipitation method used in extraction of the drug and the internal standard from the plasma samples. The method was linear over a range of 0.2 to 20 μg/mL. It should be noted that the concentration of 0.1 μg/mL was detected with this assay but at low precision.

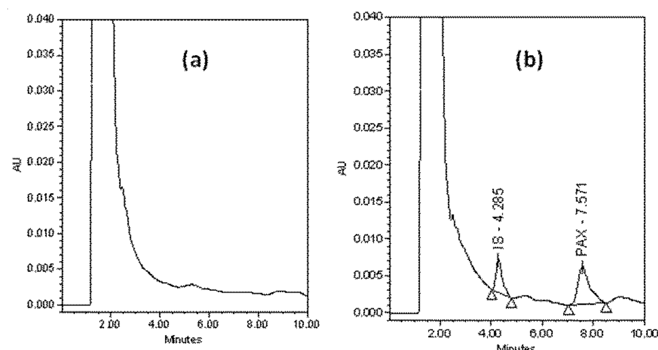

Fig. 5. Chromatograms represent (a) blank plasma and (b) the plasma spiked with 1 μg/mL of paclitaxel and its internal standard

Conclusion

A simple rapid accurate and precise stability-indicating HPLC analytical has been developed and validated in the current studies for analysis of paclitaxel in both solution form and spiked in rabbit plasma. The results of stress condition, undertaken according to the ICH guidelines, reveal that the method is selective and stability-indicating. The developed method can thus be considered suitable for determination of the drug throughout the future work.

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