Journal of Chromatography
Separation Techniques

Siddiqui et al., J Chromat Separation Techniq 2012, 3:4
DOI: 10.4172/2157-7064.1000135

Research Article Open Access

RP-HPLC Method for Estimation and Stress Degradation Study of Paclitaxel as per ICH Guidelines

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Abstract

A simple, accurate, selective & reproducible validated stability indicating assay method was developed for determination of Paclitaxel in presence of its degradation products. The best separation was achieved in the C18 analytical column at ambient temperature using a mobile phase composed of acetonitrile and phosphate buffer (60:40) in isocratic mode. The flow rate was set at 1.0 ml/min and detection wavelength was 226 nm. The drug gives peak at R_T 4.95 min and forced degradation studies gave two degradation products such as one peak of degradation product of alkaline hydrolysis were observed at RR_T 2.941(DP II) and at RR_T 0.382 (DP I) a peak for acidic hydrolysis product obtained along with the drug peak at R_T 4.95. The limit of detection (LOD) and limit of quantitation (LOQ) of developed method were found to be 2 µg/ml and 10 µg/ml respectively. The validation results obtained from the analysis also reveals that the developed method is specific and selective.

Keywords: Paclitaxel; Stability indicating assay; ICH; RP-HPLC

Introduction

Stress studies were carried out under the conditions mentioned in ICH Q1A (R2) viz dry heat, hydrolysis, oxidation and photolysis. Regulatory guidance in ICH Q2A, Q2B, Q3B and FDA 21 CFR section 211 all requires the development and validation of stability indicating assays [1-4].

Paclitaxel (PT) is chemically known as tax-11-en-9-one,5β,20-epoxy-1,2α,4,7β,10β,13α,hexahydroxy-4,10-diacetate-2-benzoate-13-α-phenylhippurate (Figure 1), a poly-oxygenated naturally occurring diterpene alkaloid, was first isolated by Wall and Wani from the bark of Taxus brevifolia. Paclitaxel is one of the broadest spectrum anticancer agent approved by the Food and Drug Administration FDA for the treatment of advanced ovarian cancer [5,6].

The literature shows that some analytical methods were developed for estimation of these drugs by individually or in combination with other drugs. Several analytical methods that have been reported for estimation of paclitaxel are HPLC [7,8] and LC-MS/MS [9,10].

The revised parent drug stability test guideline Q1A (R2) issued by International Conference on Harmonization (ICH) requires that stress testing on the drug substance should be carried out to establish its inherent stability characteristics and for supporting the suitability of the proposed analytical procedures. It is suggested that stress testing should include the effect of temperature, humidity, light, oxidizing agents as well as susceptibility across a wide range of pH values. It is also recommended that analysis of stability samples should be done through the use of validated stability-indicating testing methods [11,12].

A comprehensive high performance liquid chromatography (HPLC) study of the degradation behavior of PT under various ICH prescribed stress conditions has been lacking. So, the objective of the present work is to carry out forced decomposition studies according to the ICH requirements and develop a selective and validated stability-indicating HPLC method. An integral aim of the study is to separate the degradation products.

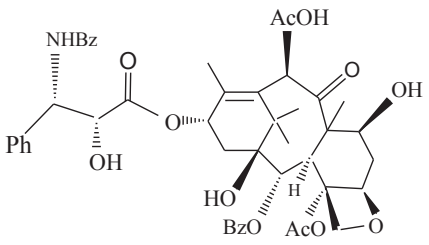

Figure 1: Chemical structure of Paclitaxel.

Experimental

Materials

Standard drug paclitaxel was obtained as working standard from Fresenius Kabi Oncology Ltd, Gurgoan, India. HPLC grade acetonitrile, glacial acetic acid and hydrochloric acid were procured from Merck Ltd., India. Analytical grade sodium hydroxide, hydrogen peroxide and other chemicals used in the study were procured from CDH chemicals Ltd, Mumbai, India.

Instrumentation

The HPLC system (Shimadzu, Japan) consist of a LC-10AT pump, a SPD-10AVP, PDA detector, phenomenex C18 (250 mmX4.6 mm, 5 µm) column, a Phenomenex, HPLC grade cartridge system and a class Nuchrom software. pH of the mobile phase was checked on microprocessor based water proof pH tester (pH tester 20, Eutech instruments, Oakton, USA). The overall illumination at the point of sample placement was tested using a calibrated lux meter (Lutron

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Received July 09, 2012; **Accepted** August 19, 2012; **Published** August 24, 2012

Citation: Siddiqui S, Paliwal S, Kohli K, Siddiqui AA, Sahu K (2012) RP-HPLC Method for Estimation and Stress Degradation Study of Paclitaxel as per ICH Guidelines. J Chromat Separation Techniq 3:135. doi: 10.4172/2157-7064.1000135

LX-102 digital light meter, Marcucci S.P.A, Vignate, Milan). Thermal stability study was performed in a hot air oven (oven universal with Thermotech thermostat TIC-4000N, S.M. Industries, New Delhi, India).

Preparation of standard solution

Accurately weighed 10 mg of PT was transferred to a 10 ml volumetric flask, sufficient amount of acetonitrile was added to dissolve it and volume was made up to 10 ml (stock A; 1000 µg/ml). Aliquots of stock A were further diluted with mobile phase up to 10 ml to get concentration of 20, 40, 60, 80,100 µg/ml for the linearity study.

Mobile phase selection

In order to select a suitable mobile phase for the analysis of PT, various combinatorial ratios of various solvents were tried on the basis of trial and error. Considering the system suitability parameters viz retention time, tailing factor, number of theoretical plates and HETP, and the mobile phase found to be most suitable for analysis was acetonitrile: phosphate buffer pH 7.4 in the ratio of 60:40. The mobile phase was filtered through 0.22 µ filter paper to remove particulate matter and then degassed by sonication. Flow rate employed for analysis was 1.0 ml/min and the concentrations were detected at 226 nm.

Linearity and calibration curve

To establish the linearity of analytical method, a series of dilutions ranging from 20-100 µg/ml were prepared in the same manner as described in earlier section. All the solutions were filtered through 0.22 µ membrane filter. The solutions were injected in triplicate into the HPLC column, keeping the injection volume constant (20 µl). Chromatograms were recorded at 226 nm (Figure 2) and calibration curve was plotted between the mean peak area *vs.* respective concentration (Table 1 and Figure 3).

Stress (forced decomposition) studies

Conditions employed for performing stress studies were as follows:

- **Hydrolytic decomposition:** Acidic and alkaline hydrolysis of PT was conducted in 0.1M HCl and 0.1M NaOH respectively. Neutral hydrolysis was performed in water. Acidic and neutral hydrolytic studies were carried out at 80°C and the alkaline hydrolysis was carried out at 50°C.

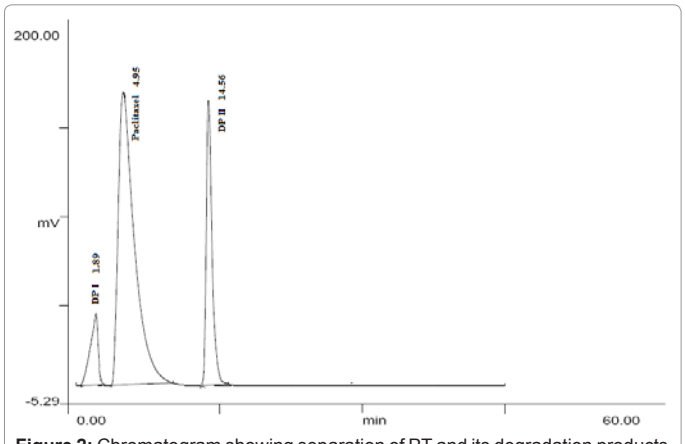

Figure 2: Chromatogram showing separation of PT and its degradation products in a mixture of stressed samples.

System suitability parameters	RT	AUC	No. of theoretical plates	Tailing factor	HETP
Rep-1	4.95	3240977	3499	1.18	27.88
Rep-2	4.96	3241054	3649	1.18	27.91
Rep-3	4.95	3240002	3679	1.21	28.71
Rep-4	4.94	3240998	3644	1.18	28.71
Rep-5	4.95	3241956	3687	1.21	27.92
Rep-6	4.97	3240945	3655	1.21	27.69
Mean	4.953	3240988.667	3635.500	1.195	28.137
S.D.	0.010	618.974	69.009	0.016	0.452
R.S.D. (%)	0.209	0.019	1.898	1.375	1.606

Table 1: Result of system suitability parameters for PT.

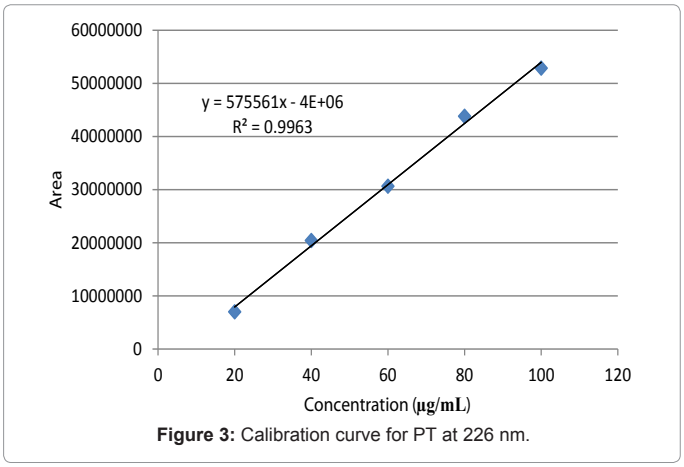

Figure 3: Calibration curve for PT at 226 nm.

- **Oxidative decomposition:** The oxidative stress studies, PT was dissolved at a concentration of 4 mg/ml in 3% H_2O_2 and kept at room temperature for 24 hr Subsequently, the drug was exposed to 30% H_2O_2 at room temperature for a period of 5 days.
- **Photolytic decomposition:** Photodegradation studies were carried out by exposing the drug to sunlight for 2 days.
- **Thermal decomposition:** Thermal studies were also conducted on solid drug, which was heated at 50°C for 2 months in hot air oven. Samples were withdrawn at appropriate time period for analysis.

Method validation

Validation of optimized HPLC method was done with respect to following parameters.

Linearity

The linearity of the developed method was performed with a concentration range of 20 to 100 µg/ml at R_T 4.95.

Accuracy

Accuracy was evaluated by fortifying a mixture of degraded solution with three known concentrations of the drug. The recovery of added drug was determined by calculating the pre-analyzed drug concentration and correlating with the concentration of spike drug. In Table 2, shows that excellent recoveries were made at each added

concentration, despite the fact that the drug was fortified to a mixture that contained drug as well as the degradation products, formed under various stress conditions.

Precision

Intra-day precision: The procedure precision (intra-day repeatability) was established by analyzing three replicates over three concentrations of PT shown in Table 3.

Inter-day precision: As shown in Table 3. day to day precision (inter-day) was carried out by three concentrations with three replicates.

Robustness

The robustness study was done by making small changes in composition of mobile phase in the optimized method. There was no significant impact on the retention time and tailing factor. The data of robustness is given in Table 4**.**

Specificity and selectivity

Specificity is the ability of a method to discriminate between the intended analyte and other components in the sample. Specificity of the HPLC method is demonstrated by the separation of the analytes from other potential components such as impurities, degradants, or excipients. The purity data of developed method is given in Table 5.

Limit of detection (LOD) and limit of quantitation (LOQ) for developed method

The limit of detection and limit of quantitation was calculated experimentally. The limit of detection was defined as the concentration that yields a signal to noise ratio of 3. The limit of quantitation was calculated to be the lowest concentration that could be measure with a signal to noise ratio of 10.

Results and Discussions

Stress studies were performed under conditions of dry heat (thermal studies), hydrolysis (acidic, alkaline and neutral), oxidation, and photolysis, as mentioned in ICH Q1A (R2). A minimum of four samples were generated for every stress condition, viz., blank solution stored under normal conditions, the blank subjected to stress in the same manner as the drug (Paclitaxel), a zero time sample containing the drug (which was stored under normal conditions), and the drug solution subjected to stress treatment. The stressed samples were initially analysed by HPLC using a RP C18 column and a mobile phase composed of water and acetonitrile (50:50). As the separation and

Spiked concentration (µg/mL)	Measured concentration	± S.D.,	R.S.D. (%)	Recovery (%)
20	19.963	0.803	4.023	**99.817**
40	40.060	0.252	0.628	**100.150**
60	59.934	0.219	0.365	**99.889**

S.D.= Standard deviation.
R.S.D.=Relative standard deviation.
n=no. of replicates

Table 2: Recovery data for Paclitaxel spiked into a mixture of stressed samples (n=3).

Actual concentration (µg/mL)	Intra-day			Inter-day		
	Measured concentration	± S.D.,	R.S.D. (%)	Measured concentration	± S.D.,	R.S.D. (%)
20	20.157	0.201	0.999	20.080	0.130	0.647
40	40.150	0.339	0.844	39.873	0.202	0.508
60	59.997	0.274	0.457	59.886	0.188	0.314

S.D.= Standard deviation.
R.S.D.=Relative standard deviation
n = no. of replicates

Table 3: Reproducibility and precision data evaluated through intra-day and inter-day studies (n=3).

Spiked concentration (µg/mL)	ACN: Phosphate buffer(58:42)			ACN: Phosphate buffer (62:38)		
	measured concentration	± S.D.,	R.S.D. (%)	measured concentration	± S.D.,	R.S.D. (%)
20	20.160	0.078	0.387	20.117	0.155	0.771
40	40.086	0.161	0.402	40.119	0.392	0.977
60	60.034	0.311	0.518	59.917	0.276	0.462

ACN=Acetonitrile
n=Number of determination.

Table 4: Data for robustness (n=3).

Peak	R_T	RR_T	Resolution	Peak purity index
DP I	1.89	0.382	-	0.9989
PACLITAXEL	4.95	1.000	2.20	1.0000
DP II	14.56	2.941	4.91	0.9891

Table 5: Peak purity data of the degradation products.

peak shape were not good, therefore, organic modifier concentration was changed from 80 to 95%, but no improvement was observed. Subsequent attempts were made by lowering of pH of the mobile phase (using acetic acid) and replacement of acetonitrile by methanol. In both cases, marked improvement was observed. Further trials were carried out by varying the composition of mobile phase using ACN: Phosphate buffer. Eventually, a mobile phase composition of phosphate buffer: acetonitrile (40:60, pH 7.4) gave the best results. During these studies, injection volume was 20 µl and the mobile phase flow rate was constant at 1 ml/min. The analytical wavelength was 226 nm.

A simple, precise and significant analytical method has been developed and validated for PT. The stability of the PT was evaluated using different conditions as per the guidelines. The drug is linear at 20 to 100 µg/ml concentration and the regression equation obtained from the data (Table 1).

Y= mX +C,

Where Y=AUC, m=slope (575561), X=conc. (µg/ml), C=intercept ($-4E^{06}$ in this equation)

The chromatogram of mixture of stress samples (Figure 2) obtained from the study shows that the method was sufficiently specific to the drug. The resolution factor for the drug peak was greater than 2 from the nearest resolving peak. The method was also selective to degradation products as all the peaks were pure, which was proved through purity curve studies. System suitability was determined by calculating the percent relative standard deviation (RSD) for area and retention time for six replicates injections of 50 µg/ml PT standard. The area %RSD was calculated to be 0.019 and the retention time %RSD was calculated to be 0.209.

In this study, a selective and validated stability-indicating HPLC assay method for PT on a HPLC C-18 column was developed, which could separate the drug and its degradation products formed under a variety of stress conditions. The recovery results obtained from the developed method shows that the method recover 99.90% drug. The accuracy result shows that the method gives %RSD of <1. Robustness is the measure of the performance of a method when small, deliberate changes are made to the specified method parameters. The intention of robustness is to identify critical parameters for the successful implementation of the method. Robustness is partially evaluated during method development when conditions are optimized to improve resolution and other method performance criteria (e.g. peak shape, sensitivity). The %RSD values for precision (intra-day and inter-day) is less than 1%, indicating that the method is sufficiently precise. The limit of detection (LOD) and limit of quantitation (LOQ) of developed method were found to be 2 µg/ml and 10 µg/ml respectively.

The method demonstrated linearity over a large range of concentration of 20-100 µg /ml. The calibration curve was used to establish the concentration behavior of PT when subjected to the various stress conditions (Table 3). The degradation studies show that no degradation product was observed even on exposure of the drug to 30% H_2O_2, sunlight (2 days at 60,000-70,000 lux) & hot air oven for 1 month at 50°C, revealing that it was stable against oxidation, photolytic studies & thermal studies respectively. The drug showed susceptible behavior towards alkaline condition and it was observed that around 60% of the drug degraded on heating at 80°C it in 0.1N NaOH for 2 hr. In alkaline hydrolysis, one peak of degradation product were observed at R_T 14.56 along with the drug peak at R_T 4.95, in mixture of stressed samples the degradation products appeared at RR_T 2.491 to DP II.

Acidic hydrolysis was carried out by exposing the drug in 0.1M HCl at 80°C for 12 hr. One peak of degradation product was observed at R_T 1.89 along with the usual drug peak at R_T 4.96. In mixture of stressed samples the degradation product appeared at RR_T 0.382, pertaining to DP I, it was observed that around 20% of the drug degraded in acid hydrolysis.

Neutral hydrolysis was carried out by charging the drug in water at 80°C for 24 hr. No extra peak was found which shows that the drug was stable in neutral hydrolysis. The forced-degradation study with PT shows that the drug degraded 55% in alkaline and 20% in acidic stress conditions. The specificity and selectivity of the method with the samples under these stresses was demonstrated through the evaluation of R_T, RR_T, resolution, and purity data for all peaks in the chromatograms.

Conclusion

This study concluded that developed validated method is statistically significant. The degradation study results shows that the drug is stable at alkaline and oxidative conditions. This method proved to be simple, accurate, precise, specific and selective. Hence the method thus developed is recommended for the routine studies in the industries.

Acknowledgments

The authors gratefully acknowledge the Vice Chancellor, Banasthali Vidyapeeth, Rajasthan for providing the experimental facilities for this research work.

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