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Development and Validation of UFLC Method for the Determination of Docetaxel in Rat Plasma and Its Application in Pharmacokinetic Interaction with Diclofenac Sodium

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Abstract

In cancer, the taxane of choice has historically been paclitaxel however, there is no substantial evidence that docetaxel may be the preferred taxane in this disease. Docetaxel has many preclinical advantages over paclitaxel and has been effective in both platinum and paclitaxel resistant disease. Even though, Docetaxel is the drug of choice for the treatment of cancer, its use is limited due to the limited or low bioavailability (8%). Hence, the present study is designed to develop a method on UFLC and validation of the developed method for the detection of Docetaxel in rat plasma. Chromatography was performed with an analytical Kromasil C18 column (250 x 4.6mm, 5µm), UFLC Chromatograph (Shimadzu SPD-M20A Photo Diode Array Detector and UFLC-20AD Pump (Japan), using acetonitrile and 20mmol Phosphate buffer pH-5 (57:43% v/v) as the mobile phase. The average extraction recovery of Docetaxel from rat plasma was greater than 92% with a good linearity of 0.997 in plasma over a concentration range of 60,600 and 6000ng/ml. Interday and intraday variability was < 10% in plasma. This newly developed UFLC method was applied to the pharmacokinetic study of Docetaxel in the presence of Diclofenac sodium after oral administration in rats.

Keywords: *Docetaxel, Diclofenac sodium, Pharmacokinetics, UFLC*

Contents

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1. Introduction

Cancer refers to a group of illnesses that result from cells in the body growing abnormally. These cells divide and produce new cells in an uncontrolled way that can spread throughout the body and cause damage to essential organs **[1]**. In cancer, the taxane of choice has historically been paclitaxel however, there is no substantial evidence that docetaxel also may be the preferred taxane in this disease.

Docetaxel has many preclinical advantages over paclitaxel and has been shown to be effective in both platinum and paclitaxel resistant disease. Docetaxel is (1S,2S,3R,4S,7R,9S,10S,12R,15S)-4-(acetyloxy)-15-{[(2R,3S)-3-{[(tert-

butoxy)carbonyl]amino}-2-hydroxy-3-phenylpropanoyl]oxy}1,9,12-trihydroxy-10,14,17,17-tetramethyl-11-oxo-6-oxatetracyclo[11.3.1.0{3,10} 0{4,7}] heptadec-13-en-2-yl benzoate, an anti-mitotic chemotherapy medication used mainly for the treatment of breast, ovarian and non-small cell lung cancer **[2,3]**. Docetaxel is the drug of choice for the treatment of cancer, its use is limited due to the limited or low bioavailability (8%) this is due to the hepatic first pass metabolism, limited absorption due to a poor water solubility and effect of the multidrug resistance transporter P-glycoprotein, which is responsible for an efflux mechanism resulting in a reduced crossing of the intestinal barrier **[4, 5]**.

Diclofenac sodium is a strong anti inflammatory drug that exerts its action by inhibiting cyclooxygenase–I. Diclofenac sodium was tested for its potential ability to inhibit CYP 3A4 metabolism and p-gp efflux **[6-8]**. Therefore, we hypothesized that the co-administration of Diclofenac sodium with Docetaxel can help in the improvement of bioavailability and show the significant effect on the pharmacokinetic parameters of Docetaxel. Hence, the present study is designed to develop a method on UFLC and validation of the developed method for the detection of Docetaxel in rat plasma and to evaluate the pharmacokinetics of Docetaxel in the presence of Diclofenac sodium in rats.

2. Experimental

2.1. UFLC method development

2.1.1. Chemicals and reagents

1. Docetaxel and Glimepiride were obtained from R&D dept. of Dr Reddy's laboratories limited, Hyderabad, India.
2. Diclofenac Sodium was obtained from Elan Life Sciences Private Limited, Hyd, India.
3. Acetonitrile and Potassium dihydrogen phosphate were of UFLC grade obtained from Finar chemicals (Ahmedabad), India.

2.1.2. Instruments and chromatographic conditions [9, 10]

The Lab India UV-3000+ model was used to determine the absorption maxima (λ_{max}) of Docetaxel. UFLC Chromatograph (Shimadzu SPD-M20A Photo Diode Array Detector and UFLC-20AD Pump (Japan). The column and UFLC instrument was maintained at room temperature. The reverse phase chromatography was performed with an analytical Kromasil C18 column. Acetonitrile and 20mmol Phosphate buffer P^H-5 (57:43% v/v), was used as the mobile phase. The flow rate was set at 1 ml/min and the injection volume was 20 µL. The UFLC detector was set at a wavelength of 232nm.

2.1.3. Standard solutions

Primary stock solutions of Docetaxel and Glimepiride (Internal standard) were prepared in methanol at a concentration range of 1 mg/ml and stored at -80^0 C until use. Primary stock solution of Glimepiride was diluted in methanol to obtain the working solution of 20 µg/ml concentration. Primary stock solution of Docetaxel was firstly diluted with methanol to give working solutions with concentrations of 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5 and 7.5 µg/ml. Each of these samples (100 µL) were added with 100 µL of internal standard from 20 µg/ml working solution and the resultant solution was evaporated to dryness and the resultant residue was dissolved in 0.1 ml of mobile phase and 20 µL of this solution is used for the determination of LLOD and LLOQ.

2.1.4. Extraction procedure for Plasma samples [11]

To 100 µL of plasma, 100 µL of internal standard from 20 µg/ml of working solution was added and the proteins were precipitated by adding 200 µL of methanol, the resultant solution was mixed for 2 minutes on vortex shaker at room temperature and centrifuged at 4000 rpm for 30 min and the supernatant was evaporated to dryness, the residue was dissolved in 100 µL of mobile phase and after filtration through 0.2 µ syringe filter, 20 µL of the solution was used for the UFLC analysis.

2.2 UFLC method validation [12-14].

2.2.1. Specificity and selectivity

The chromatographic interference from endogenous compounds was assessed by comparing the chromatograms of blank plasma with that of the samples spiked with docetaxel and IS (glimepiride).

2.2.2. Sensitivity

The lowest limit of quantification (LLOQ) was determined as the minimum concentration that could be accurately and precisely quantified with the relative standard deviation of $< \pm 10\%$.

2.2.3. Linearity

Calibration curve was plotted by taking nine concentrations of docetaxel ranging from 0.025 to 7.5 µg/ml. Blank samples were analyzed to confirm the absence of interferences. Calibration curves were plotted by taking Peak area

ratio of docetaxel to glimepiride on Y-axis and Concentration of corresponding values on X-axis. The minimally acceptable correlation coefficient (r^2) for the calibration curve was 0.99 or greater.

2.2.4. Precision and accuracy

In order to assess the intra- and inter-day precision and accuracy for the method, docetaxel samples at low, medium and high concentrations were prepared as described above. The intra-day precision of the method was assessed by calculating the coefficient of variation (CV) for the analysis of samples in three replicates and inter-day precision was determined by the analysis of samples on three consecutive days. Accuracy was calculated by comparing the measured values to the true values and was expressed in percent. The precision was accepted when the coefficient of variation for each concentration doesn't exceed ± 10, and accuracy was accepted when the average values were > 95% of true concentration except for the LLOQ where the limit was > 92%.

2.2.5. Range

The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing the amount of analyte within or at the extremes of the specified range of the analytical procedure.

2.2.6. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness study was carried out by changing the flow rate, ratio of mobile phase acetonitrile and phosphate buffer (57:43) & (50:50) ratio and P^H of buffer solution.

2.2.7. Ruggedness

In this a standard solution of the drug substance with in a matrix should be analyzed while systematically varying operating condition. The condition examined should include different operator in the same lab, different instrument in same lab, different laboratory, changing source of reagent and solvent, changing a new column. Ruggedness studies were carried out by changing the operator and instrument in same lab with same condition containing same concentration (1µg/mL) of docetaxel in rat plasma.

2.2.8. Solution stability study

The stability of docetaxel in plasma was tested at the concentrations of the low and high QC samples. Short-term storage stability of sample up to 24 hours (6, 12, and 24 hrs) at room temperature in plasma, was analysed by the extraction from plasma and then reconstituted with 50 % acetonitrile and was assessed in triplicate. Results were expressed as the percent recovery relative to the initial (nominal) concentration at time zero. Stability was defined as less than 10% loss of the initial concentration.

2.3 Pharmacokinetics of docetaxel in the rats (15)

Female wistar rats weighing 200-250 g were purchased from Mahaveer enterprises (Hyderabad). Rats were housed at Animal Care Facility of Sri Shivani College of pharmacy. upon arrival rats were given diet free from antioxidant and acclimatized for a period of 1 week. The animals were maintained on a 12 hr light–dark cycle (light on from 8:00 to 20:00 h) at ambient temperature (22–24 °C) and 60% relative humidity.

The study was approved by the Institutional animal ethical committee. Rats (n=3) were fasted overnight and administered docetaxel at a dose of 40 mg/kg in a 5.0 % suspension of gum acacia through oral route. Blood samples (300 µL) were collected at 0, 1, 2, 4, 6, 8, 12, and 24h following docetaxel administration through retro-orbital puncture under mild ether anesthesia. Plasma was separated immediately by centrifugation and stored at -80^0C until analysis. At the time of analysis the plasma samples were subjected to extraction procedure as stated above and the concentration of the drug in it was determined by using the calibration curve of plasma. The obtained plasma concentration data of docetaxel was analyzed to obtain the appropriate pharmacokinetic parameters by applying Non-compartmental open model using Kinetica 5.0 software (Kent scientific).

3. Results and Discussion

3.1 Method development

The UV-Vis absorbance of Docetaxel was scanned from wavelength of 200-400 nm on a Lab India UV-Vis spectrophotometer (Uv3000+) and maximum absorbance was observed at a wavelength of 232 nm in methanol (Fig-1) therefore, wavelength of 232nm was chosen for UFLC-UV detection in this method. The mobile phase used for

the method was very simple and achieved optimal separation of Docetaxel and I.S. (Glimepiride) without interference from the other components in rat plasma (fig-3).The flow rate was selected as 1 ml/min.

Figure-1: Showing UV-Visible spectrum of docetaxel within a range of 200-400 nm

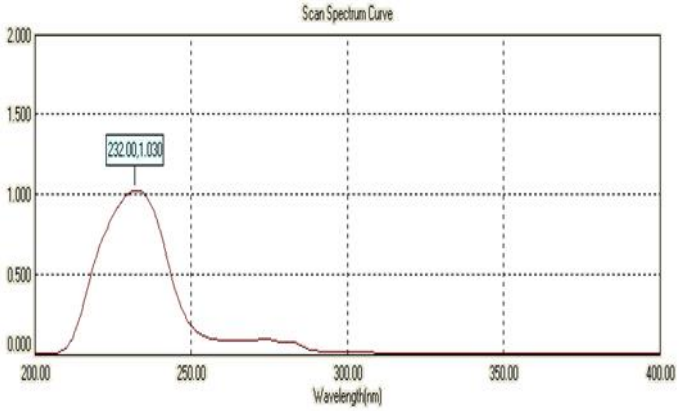

Figure 1

3.2. UFLC method validation

3.2.1. Specificity and selectivity

Chromatograms of Docetaxel and Glimepiride (Internal Standard) obtained after extracting the drugs from rat plasma were represented in Fig-3. No interference of endogenous compound peaks was detected with Docetaxel or Glimepiride at their respective retention times (Docetaxel R_t = 10.5 min, Glimepiride R_t = 8.5 min) in blank rat plasma (fig-2).

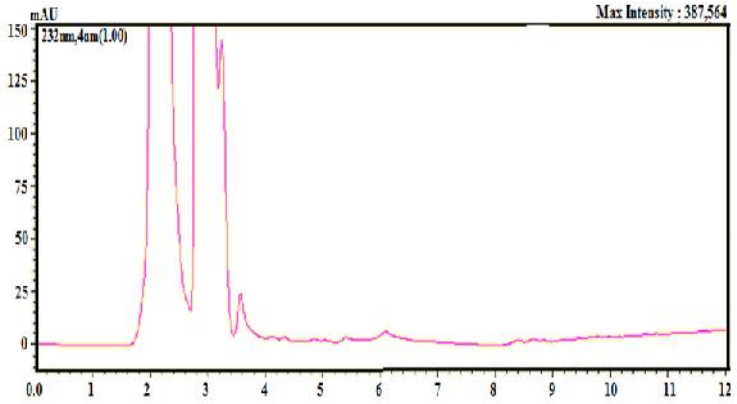

Figure 2: Showing blank rat plasma

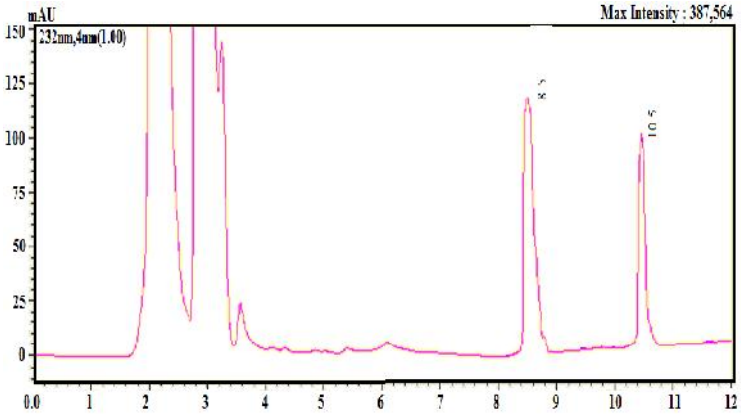

Figure 3: Showing Chromatogram of Docetaxel and glimepiride

3.2.2. Sensitivity

The LOQ of Docetaxel was found to be 0.154µg/mL. The mean percent accuracy value for plasma samples was 99.225% and coefficient of variation was below 4.4% at the LOQ. Hence it is a sensitive method.

3.2.3. Linearity of calibration curve

The calibration curves of docetaxel were linear over the different concentration range in plasma. The correlation coefficient was found to 0.997 (fig-4).

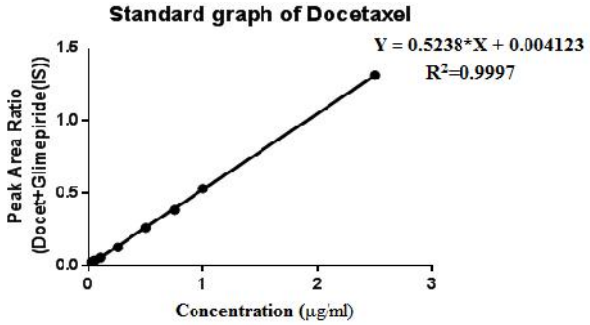

Figure 4: Standard graph of Docetaxel

3.2.4. Precision and accuracy

Table-1 shows a summary of intra- and inter-day precision and accuracy. Intra- day accuracy of 60, 600 and 6000 ng/ml was found to be 98.15, 99.73 and 101.35 respectively and inter- day accuracy was found to be 99.32, 99.26 and 97.59 respectively. Therefore, the intra- and inter- day accuracies (% deviation) were within < ±10% for the LLOQ. The intra- and inter-day assay precision (CV) ranged from 1.19 to 0.9 and 1.49 to 1.28 % respectively. These results indicated that the present method has very good accuracy and precision.

Table 1: Showing inter- and intra-day variation of spiked plasma samples.

	Trial-1	Trial-2	Trial-3	Mean	SD	Accuracy%		CV%
						Mean	SD	
Intraday								
60	59.92	58.54	58.95	59.13	0.70	98.15	1.18	1.198
600	598.9	594.00	605.00	599.30	5.51	99.73	1.10	0.91
6000	5974.0	6079.1	6191.12	6081.4	108.6	101.3	1.81	1.7
Interday								
60	58.78	100.2	55.98	59.65	0.76	99.32	1.18	1.28
600	585.9	599.1	545.9	595.9	8.92	99.26	1.14	1.49
6000	6004.1	5912.3	5651	5855.8	153.2	97.59	3.05	1.3

3.2.5. Robustness

Robustness study was carried out by changing the ratio of mobile phase acetonitrile and phosphate buffer (57:43) & (50:50) and flow rate (0.9, 1 and 1.1mL/min) of the mobile phase. The retention time for both mobile phases (1&2) were found to be 10.5 and 6.2 with %CV 0.225 and 1.95 and for three different flow rates (0.9, 1 and 1.1 mL/min) the R_t values were found to be 9.3, 10.5 and 11.6 with %CV 0.21, 0.40 and 1.7 respectively (Table-2 and 3).

Table 2: Showing robustness in mobile phase 1 and 2 of spiked plasma samples

S.No	**Mobile Phase-1**		**Mobile phase-2**	
	Peak area	**Retention time**	**Peak area**	**Retention time**
1	213254	10.5	217111	6.2
2	214321	10.5	213415	6.2
3	214381	10.4	214409	6.2
Average	214044.4		216651.2	
S.D.	481.64		4235.13	
%CV	0.225		1.95	

Table 3: Showing robustness in different Flow rates

S.No	**Flow rate**	**Peak area**	**Average**	**S.D.**	**%CV**	**R_t**
1	0.9 mL/min	215198	214877.5	453.25	0.21	9.3
		214557				
		214587				
2	1 mL/min	217751	218372	878.2	0.40	10.5
		218993				
		218340				
3	1.1 mL/min	213325	215923	3674.1	1.7	11.6
		218521				
		215899				

3.2.6. Ruggedness

Changing the analyst and system we checked the ruggedness of the method (Table- 4). The retention time obtained by system 1 is 10.5, for system 2 is 10.9 respectively and the number of theoretical plates for system 1 is 2756 for system 2 is 2345 (Table-5).

Table 4: Showing Chromatographic conditions for system-1 & 2

Parameter	System-1	System-2
Company	Shimadzu	Shimadzu
Detector	SPD-M20A PDA	SPD-10A UV-Visible
Pump	UFLC-20AD	LC-10AD

Table 5: Values of RT & No. of Theoretical plates in system-1 & 2

S.No	Parameter	Values found	
		System-1	System-2
1	Retention time	10.5	10.9
2	No. of theoretical plates	2756	2345

3.2.7. Stability

Solution stability study was performed by analyzing the prepared samples of 100 ng/ml and 1000 ng/ml in plasma at different time intervals of 6h, 12h and 24h at room temperature. The samples were stable up to 12hours (93.31% and 929.54%) and the results were shown in table-6.

Table 6: Values of solution stability study

Time	**Conc. Of Docetaxel (mean of 3 replicates)**	
	100ng/ml	**1000ng/ml**
6 h	97.14	965.2
12h	93.31	929.54
24h	84.4	829.21

3.3. Application of the developed UFLC method to pharmacokinetics study

Plasma concentrations of docetaxel in pharmacokinetic studies were successfully quantified by this method up to 24 h. The mean plasma concentration-time profiles after oral (40 mg/kg) administration of docetaxel in rats were shown in Fig-**5.** The basic pharmacokinetic parameters of docetaxel in rats were determined using Non-compartmental analysis of Kinetica program and were listed in Table-7**.**

Table 7: Pharmacokinetic parameters of Docetaxel (40 mg/kg) and Docetaxel + Diclofenac sodium (Docet + Diclo) treated groups using determined by using Kinetic 5.0

Group		**C max**	**T max**	**AUC (ng/ml*hr)**	**AUMC (ng/ml*hr^2)**	**$t_{1/2}$ (hr)**	**MRT (hr)**	**Cl (L/hr)**	**Vd (L)**
Doce	Mean	1536.1	2	14.25333	135.746	5.788	9.523	564.29	4709
	SD	22.83	0	0.014	0.722	0.044	0.057	0.5694	37.03
Doce+Diclo	Mean	1818.4	2	17.604	176.957	6.215	10.14	456.91	4097
	SD	11.326	0	0.084	4.198	0.112	0.295	2.209	57.02

The significance was analyzed by unpaired t-test using Graph pad Prism 5.0, where *p<0.05.

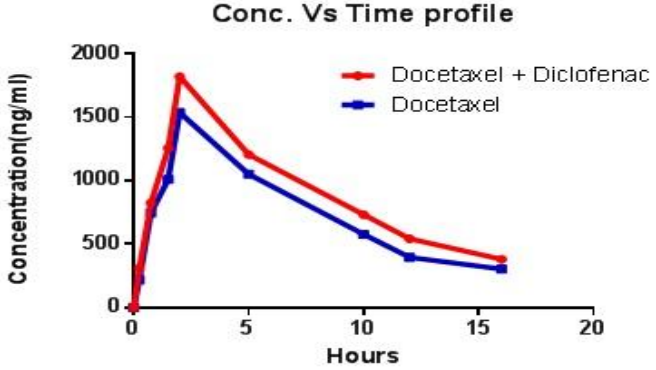

Figure 5: Showing the docetaxel concentration Vs Time profile of Docetaxel (40mg/kg) with and without continuous administration of Diclofenac sodium (25 mg/kg) for 8 days in healthy rats. (Using Graph padprism 5.0)

4. Conclusion

A simple, sensitive, accurate and precise UFLC method was developed and validated to quantify docetaxel in rat plasma. Systematic pharmacokinetic study was conducted to study the effect of Diclofenac sodium on the pharmacokinetics of Docetaxel. Pharmacokinetic studies were conducted in wistar rats and the results demonstrated the significant changes in pharmacokinetics of Docetaxel after administering the drug with Diclofenac sodium. Mean plasma concentrations of Docetaxel were also increased with prior administration of Diclofenac sodium. Diclofenac sodium also significantly improved the bioavailability of Docetaxel by increasing the area under the curve of mean plasma concentration and time profile and decreasing the clearance. This may be due to the competitive inhibition of CYP 3A4 enzyme metabolism of Docetaxel. Based on these findings it was concluded that the mean plasma concentration and bioavailability of Docetaxel can be enhanced by co-administration of Docetaxel with Diclofenac sodium. However, further studies in large group of animals at different doses of Diclofenac sodium were needed to confirm the enhancement of Docetaxel bioavailability with co-administration of Diclofenac sodium.

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