INDO AMERICAN JOURNAL OF PHARMACEUTICAL RESEARCH

DETERMINATION AND QUANTIFICATION OF PACLITAXEL IN HUMAN PLASMA BY LC-MS/MS: APPLIED METHOD TO THERAPEUTIC DRUG MONITORING

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ARTICLE INFO

Article history
Received 03/08/2017
Available online
31/08/2017

Keywords
Paclitaxel,
LC-MS/MS,
Human Plasma,
Liquid-Liquid Extraction.

ABSTRACT

A high throughput liquid chromatography-tandem mass spectrometric (LC-MS/MS) method for the determination and quantification of anti-cancer drug Paclitaxel in human plasma is described for the application to therapeutic drug monitoring. It is rapid and sensitive binary phase reversed phase LC-MS/MS method equipped with electro spray ionization (ESI) source and C_{18} column (100 mm x 4.6mm, 5µm), operating in the positive ion and multiple reaction monitoring (MRM) mode. The extraction of Paclitaxel and Carbamazepine (Internal standard) from the human plasma was carried out by two phase liquid-liquid extraction (LLE) method using methyl tert butyl ether (MTBE) as an extractive solvent giving extracts free from endogenous interferences. The retention time of Paclitaxel is 1.44 minutes with the flow rate of 0.5 mL/minutes. Sample preparation by this method yielded very good and consistent mean recoveries of Paclitaxel and IS. The method was linear over the dynamic range 5.00 to 3000.00 ng/mL (r^2 0.997). The lower limit of detection and quantification for Paclitaxel on mass was found to be 5 ng/mL. This method was fully validated as per USFDA and EMEA guidelines. Conclusion: The proposed LCMS/MS method has better performance in terms of simplicity, sensitivity, stability and specificity than the previously reported methods. Moreover, there is rapid sample preparation, adequate retention and better extraction efficiency with less matrix interferences. Therefore, it can be considered as a suited bio-analytical tool for therapeutic drug monitoring and pharmacokinetic analysis during chemotherapy.

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*Please cite this article in press as **Pallavi H. Tank** et al. Determination and Quantification of Paclitaxel in Human Plasma by Lc-Ms/Ms: Applied Method to Therapeutic Drug Monitoring. Indo American Journal of Pharmaceutical Research.2017:7(08).*

INTRODUCTION

Paclitaxel is a natural product containing anticancer agent and it is extracted from western yew tree. It was discovered in 1968 by isolation from the bark of Taxus brevifolia, a species of yew tree originally from Pacific Northwest in North America. Paclitaxel has limited usage due to less content availability from yew trees. Therefore, the leaves of yew tree are used to produce semi synthetic precursor (10-decaacetylbaccatin) of paclitaxel. Microtubules are the key target for natural anticancer drugs. Paclitaxel binds to β-subunit of tubulin. It blocks mitosis by stabilizing the microtubules in cancer cells. Normal cell division includes polymerization of the microtubules at the beginning of mitosis and releases the daughter chromosomes. Paclitaxel blocks this de-polymerization so that the cells become filled with microtubules and cannot able to mitosis. It induces apoptosis of malignant tumor cells during breast, ovaries, lung and stomach cancer. Chemotherapy treatment of cancer patient includes intravenous or oral administration of drugs. The treatment plan for paclitaxel depends on which cancer is diagnosed. Paclitaxel metabolism occurs in liver with cytochrome p450 (CYP) enzymes. Paclitaxel metabolizes to 6a-hydroxypaclitaxel (6a-OHP) and p-3'-hydroxypaclitaxel (C3'-OHP) by CYP2C8 and CYP3A4, respectively. Paclitaxel involves multiple side effects therefore, quantification of it and its metabolites have important role in pharmacokinetics measurement of paclitaxel. Therefore, the dose and the exposure time of the drug can be measured to optimize the therapy. Paclitaxel is virtually insoluble in water and in most pharmaceutically acceptable solvents; such that it has poor oral bioavailability therefore it is mainly administered by the intravenous (IV) route. Currently the vehicle to administer paclitaxel by IV is a mixture of Ctenophore EL (polyethoxylated castor oil) and ethanol. This vehicle provokes adverse effects, such as hypersensitivity [1,2].

Paclitaxel concentrations need to be measured with a fully validated method with sufficient sensitivity to measure plasma levels from the "poor" formulation as well as the improved formulation. Paclitaxel is rarely used as monotherapy but is administered with other anticancer drugs to create a synergy of action allowing paclitaxel doses to be lowered, consequently its pharmacokinetic response is necessary to measure. When paclitaxel is given with other drugs, there is a need to evaluate pharmacokinetic parameters using a validated assay, with high specificity and high sensitivity in order that co-administered drugs and/or metabolites do not interfere with the measurement of paclitaxel [3,4]. Various bio-analytical methods has been reviewed like HPLC method for determination paclitaxel in human serum [5], method for the quantification of paclitaxel in rat plasma and brain tissue by LCMS [6], HPLC method for determination of paclitaxel in human plasma [7,8], LCMS method for determination of paclitaxel in rat tissues [9], LCMS/MS quantitative analysis of paclitaxel and its major metabolites in serum, plasma and tissue [10], LCMS method for determination of paclitaxel in human plasma [11].

Although the previously published bio-analytical methods have various successful approaches, the proposed LCMS/MS method for quantification of paclitaxel in human plasma has better performance and application for clinical study of pharmacokinetic parameters during therapeutic drug monitoring in cancer patients. Therefore, the objectives of the present study includes-

- To develop a highly sensitive, high throughput and simple bio-analytical method with ease of sampling procedure.
- To develop rapid method for the quantification of Paclitaxel with adequate retention time and uniform sharp peak shape.
- To develop easy and fast extraction process with better extraction efficiency and less ionic suppression in terms of good recovery.
- To increase the selectivity and specificity by eliminating matrix influences due to usage of human plasma.
- To evaluate the practicability of the developed validated bio-analytical method in therapeutic drug monitoring (TDM) by quantification of Paclitaxel at lower concentration level (up to < 10ng level).
- To compare the proposed method with the previously reported and reviewed methods in terms of effective validation parameters.

MATERIALS AND METHODS

Chemicals and reagents

Working standard of Paclitaxel was provided by Khandelwal Laboratories Pvt. Ltd., Mumbai, India and Internal Standard (IS) Carbamazepine was obtained from Cadila Pharmaceuticals Pvt. Ltd. Purity of both the standards were more than 95%. HPLC grade methanol was purchased from Spectrochem Pvt. Ltd., Gujarat. MS grade formic acid and methyl tert-butyl ether (MTBE) were purchased from Merck. Banglore. Ultra pure water was obtained from Mille – Q water (S G ultra pure waters system). Control Human Plasma (heparin anticoagulant) for preparation of Quality Control (QC) samples was obtained from Blood bank, Rajkot, Gujarat.

Preparation of standard and quality control samples

Working solutions of Calibration Standards (CC) and Quality Control samples (QC) of Paclitaxel were prepared from Stock solution (1mg/ml) of Paclitaxel which was prepared by dissolving 10 mg of Paclitaxel in 10ml. Standard solutions were obtained by diluting this solution with methanol to give the final concentrations over the range of 10-100ng/ml for preparation of the standard calibration curve. Methanol: Water (90:10 + 0.1%formic acid) was prepared for mobile phase and as well for reconstitution of Paclitaxel extracted sample from plasma. Stock solution of Internal Standard Carbamazepine (1mg/ml) was prepared in methanol. CC and QC stock solutions were diluted with methanol: water (90:10 + 0.1 % formic acid) to produce working solutions of 5, 10, 25, 75, 200, 600, 1500, 3000 (ng/mL) for CC samples and 100, 300, 2500, 18000, 54000 (ng/mL) for QC samples. Calibration standards in plasma were prepared by freshly spiking 190µL of control human plasma with the appropriate working solution of the analyte (10µL). All the prepared solutions were vortexed for complete mixing. Stock and working solutions of Analyte and Internal Standard were stored at 2-10°C. Samples for the determination of stabilities and effects were prepared by spiking control human plasma in bulk with analyte in appropriate concentrations [5(LLOQ), 15 (LQC), 125 (MQC) and 2700 (HQC) ng/mL)] and 300 µL aliquots were distributed in different tubes.

EXPERIMENTAL

Chromatographic Operating Conditions:

Shimadzu (Shimadzu Scientific Instruments) Prominence LC system equipped with degasser (DGU-20A_5R), pumps (LC-20ADvp) along with auto-sampler (SIL HTc) was used. The binary mobile phase, a mixture of methanol and 0.1% formic acid in Milli Q Water (90:10 v/v) was entered into Gemini C_{18} (100 mm x 4.6mm, 5µm) column and delivered at a flow rate of 0.5ml/min into electro spray ionization chamber with total run time of 3.25 minutes. Column oven temperature and auto sampler temperature were set 40 ± 0.3°C and 10 ± 3°C, respectively.

Mass Spectrometric operating conditions:

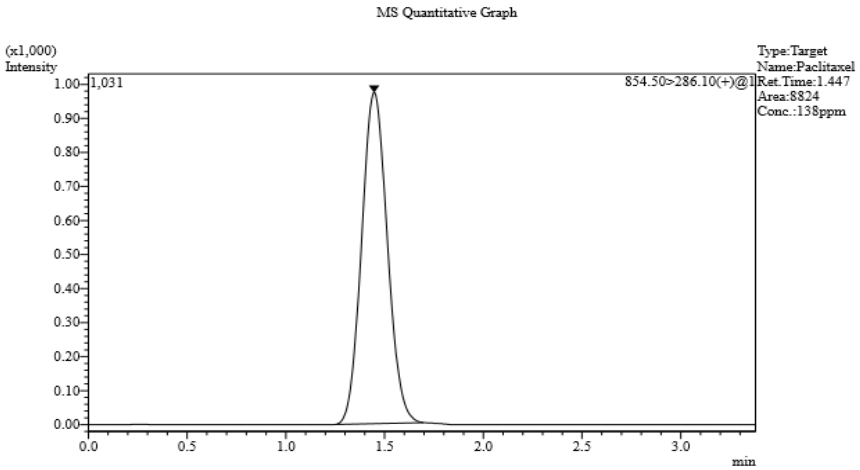
Quantification of paclitaxel was achieved by MS/MS detection using carbamazepine as iternal standard (IS) by AB Sciex mass spectrometer, equipped with a turbo ion spray interface at 450°C. Shimadzu Lab solution 5.53 SP3C was used as an operating system. Mass spectral data of analyte and IS were obtained in positive ion mode (ESI+) in MRM (Multiple Reaction Monitoring) mode shown in Figure 1 and Figure 2. Two ionization agents (acetic acid and formic acid), and two volatile salts (ammonium acetate and ammonium format) at different concentration levels were tested to compare the influence of pH on ionization. Formic acid led to highest ionization efficiency by comparing signal-to-noise (S/N) ratios and therefore, its concentration was set 0.1% v/v. Paclitaxel shows best results without sodium salt form with higher intensity of peak area results. The optimized mass spectrometric conditions were mentioned in Table I and ESI (+) conditions were shown in Table II.

Table I: Optimized Mass Spectrometric Conditions for Paclitaxel Quantification.

Parameters	Paclitaxel (Analyte)	Carbamazepine (IS)
Ion mode	ESI (positive ion mode)	ESI (positive ion mode)
Parent Ion m/z (Da)	854.5	237.0
Daughter Ion m/z (Da)	286.1	194.1
Dwell (milliseconds)	100	100
Collision energy (CE)	20	25
Retention time (minutes)	1.44	1.40

Table II: Electrostatic Ionization Conditions (ESI +).

Variable	Experimental tested range	Optimized value
DL temperature (˚C)	200-350	250
Nebulising gas flow (L/min)	2-10	3
Heat block temperature (˚C)	200-450	400
Drying gas flow (L/min)	10-20	15
Capillary voltage (v)	2500-4000	4000



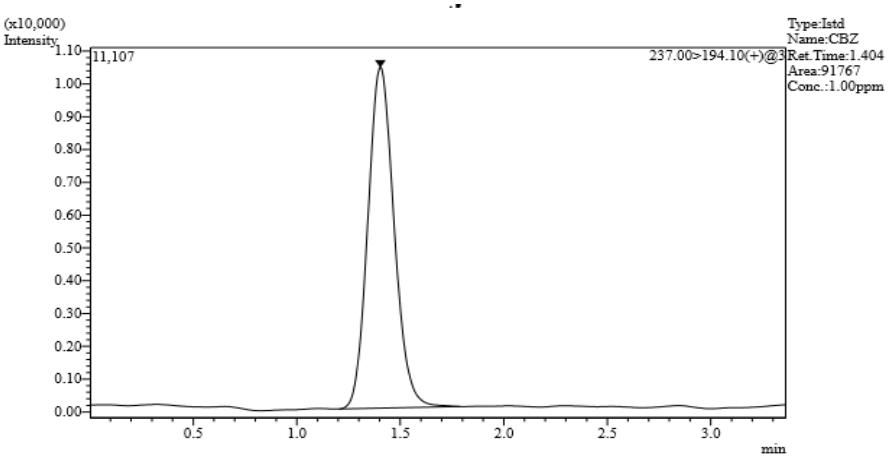


Figure 1: Comparative Chromatograms (Intensity vs time) of paclitaxel and internal standard carbamazepine shows retention time 1.44 and 1.40 minutes, respectively

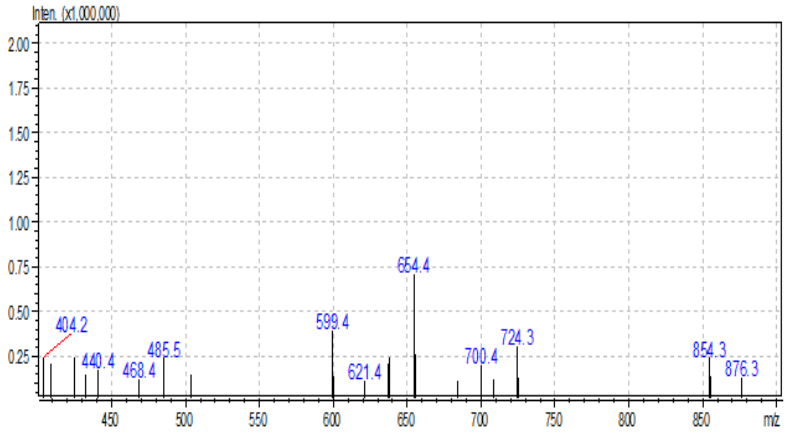


Figure 2.1: Mass spectra of Paclitaxel Parent Ion.

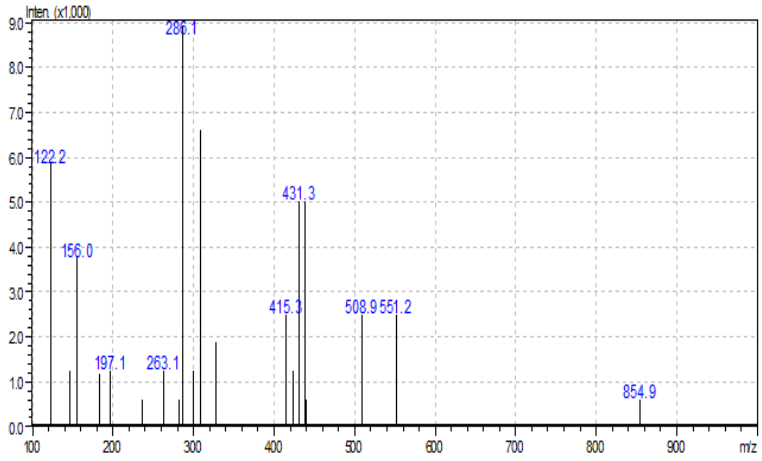


Figure 2.2: Mass spectra of Paclitaxel Daughter Ion.

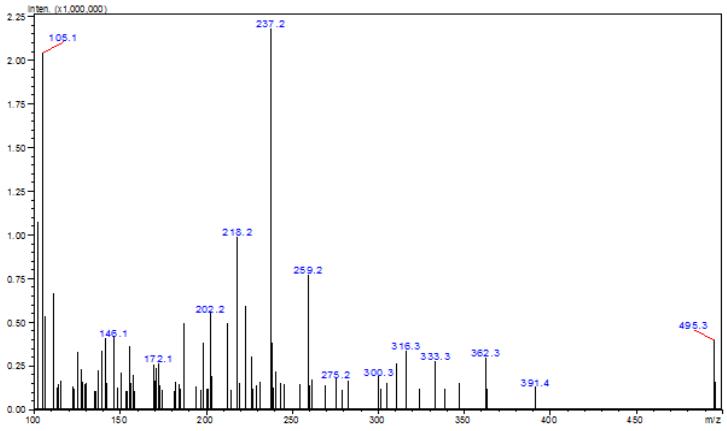


Figure 2.3: Mass spectra of internal standard carbamazepine parent ion.

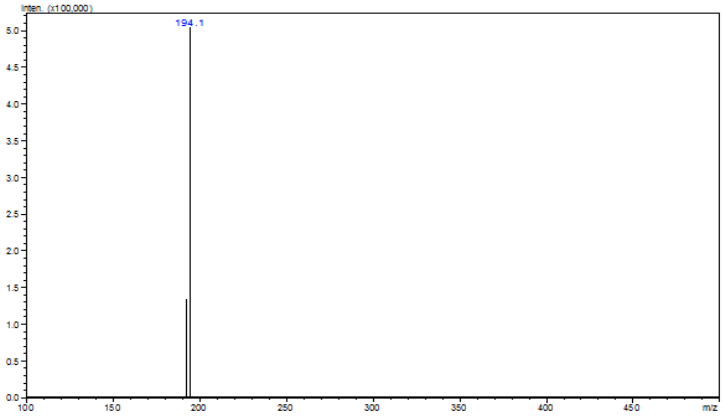


Figure 2.4: Mass spectra of Internal standard carbamazepine daughter ion.

Figure 2: Mass spectra with parent ion peak and daughter ion peak of paclitaxel and carbamazepine internal standard

Quantification:

Calibration standards of paclitaxel were prepared in blank human plasma from stock solution (1mg/ml) to give final concentrations over the range of 5-3000ng/ml. The chromatograms were acquired by using Lab Solution Software 5.60 SP2D supplied by Shimadzu. The calibration curves were plotted as the peak area ratio (Drug/ISTD) on Y-axis Vs the nominal concentration of paclitaxel on the X-axis. The intraday (within run) and inter day (between run) accuracy and precision of the developed method was determined by measuring standard samples of paclitaxel over entire concentration range on three separate days.

Method validation:

System suitability

System suitability experiment was performed by injecting six consecutive injections using aqueous MQC with internal standard at the start of the method validation and on each day.

Auto-Sampler Carryover

For checking auto-sampler carryover two blank, ULOQ and LLOQ samples were processed; as per extracted sample preparation these samples were acquired by auto-sampler in order to check auto-sampler carryover.

Linearity

The linearity of the method was determined by using a $1/x^2$ weighted least square regression analysis of standard plots associated with eight-point standard curve. A straight-line fit is made through the data points by least square regression analysis and a constant proportionality is observed.

Accuracy and precision

Accuracy was evaluated by measuring percentage mean accuracy at each concentration level of QC and precision was calculated by measuring percentage co-efficient of variation at each concentration level of QC. The within batch Precision and Accuracy was established by using 6 replicate samples at HQC, MQC, LQC and LLOQ QC samples. The between batch precision will be established by using all the replicate samples at HQC, MQC, LQC and LLOQ QC level for the three precision and accuracy batches.

Selectivity

Selectivity was proved by determining two different parameters matrix factor and specificity.

1. Matrix factor

The matrix factor is given as the ratio of analysis of the analytical response obtained from analysis of extracted blank matrix samples spiked after extraction with the analyte, at 3 concentrations (low, middle and high) and ISTD (at the working concentrations) relative to the analytical response obtained from reference solutions.

2. Specificity

The specificity of the intended method will be established by screening the standard blank plasma (without spiking with drug or internal standard). The specificity will be evaluated by comparing the responses of interfering peak at the retention time of Drug and ISTD in the standard blank against the response of the respective extracted LLOQ and AQ LLOQ.

Recovery

Recovery for the drug, metabolite and internal standard of method were determined by using six replicates of HQC, MQC and LQC were analyzed by following the procedure for Aqueous sample preparation and compared with same concentration level of QC samples.

Stability

Short term and stock solution stability was determined by six replicates of stock dilution stability standards (internal standard and analyte which prepared and stored between 2-8℃) and freshly prepared stock dilution of comparison standard (analyte and internal standard) after 4hr. The response of stability sample was corrected by multiplying with correction factor.

Dilution integrity

Six replicates of dilution integrity samples (approximately 1.7 times of highest standard concentration) were examined by diluting them twice and another six replicates by diluting them 4-5 times using drug free human plasma. Processed and analyzed DQC samples Along with freshly spiked CC standards in duplicates and QC samples (at Higher, Middle, and Lower) in duplicate as per extracted sample preparation.

RESULTS AND DISCUSSION

Linearity, Accuracy and Precision of calibration curve standards

The calibration curve of paclitaxel was linear over the range of 5-3000 ng/mL and the representative of it was shown in Figure 3. The correlation co-efficient was 0.997 for multiple analytical runs. The best straight line fit of the calibration curve data points was obtained by using least square regression analysis and weighting factor of $1/x^2$. The mean accuracy observed for the CC standards of Paclitaxel were ranged from 94.18-112.14% which is within the acceptance limits of 85.00 to 115.00% for all CC standards except LLOQ standard and 80.00 to 120.00% for LLOQ standard. The precision observed for the CC standards of paclitaxel were ranged from 0.37-14.22 which is within the acceptance limit of 15.00% for all CC standards except of LLOQ standard and 20.00% for LLOQ standard (Table III and Table IV).

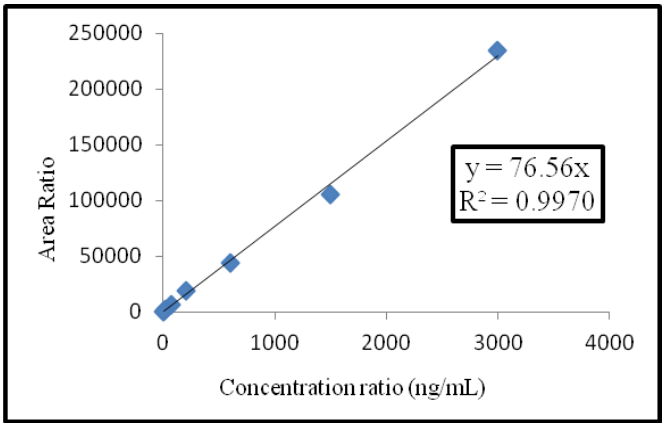


Figure 3: Calibration curve (Peak area vs. Concentration) of paclitaxel with regression analysis equation.

Table III: Inter-batch accuracy and precision data.

Parameters	HQC	MQC	LQC	LLOQ QC
Actual conc. (ng/ml)	2700	125	15	5
Measured Concentration mean (ng/ml) (n=24)	2764.17	132.39	14.83	4.89
S.D.	46.23	9.87	1.64	0.64
% CV	1.67	7.363	11.053	13.07
% Mean Accuracy	101.71	102.27	101.13	99.72

Table IV: Intraday accuracy and precision data.

Parameters	HQC	MQC	LQC	LLOQ QC
Actual conc. (ng/ml)	2700	125	15	5
Measured Concentration mean (ng/ml) (n=6)	2793	132.66	14.5	4.83
S.D.	35.25	12.24	1.5	0.68
% CV	1.26	9.23	10.34	14.22
% Mean Accuracy	98.78	102.6	100.13	99.29

Recovery study

The average recoveries of paclitaxel at three different concentration levels were found to be 94.11%, 99.01% and 90.23% at high, medium and low quality control (HQC, MQC and LQC) levels, respectively (Table V).

Table V: Recovery study data.

Parameters	HQC	MQC	LQC	ISTD
Mean peak area of extracted sample	247681.83	14881.50	1279.66	324830.5
Mean peak area of un-extracted sample	263186.67	15030.5	1418.17	325308.3
% Mean recovery	94.11	99.01	90.23	99.85
% Mean recovery with Correction factor (1.2)	112.93	118.81	108.28	119.82

Matrix Effect

The recovery yield of this method shows that recovery rate was consistent over the calibration range. No effect of matrix (six different lots of EDTA plasma including one hemolysed and lipemic sample) was observed on analyte quantification. The overall precision of the matrix factor is expressed as co-efficient of variation (% CV). Normalized matrix factor for paclitaxel and internal standard were determined 4.22 and 4.76%, respectively.

Stability studies

Short term and stock solution stability study for PTX and ISTD at concentration 3000 ng/mL for PTX and 100 ng/mL for ISTD was carried out after the storage for 9 hours at ambient temperature. Stability was assessed by comparing against the freshly prepared stock solution which was having the same concentration. The % mean concentration after 9 hours at ambient temperature was found as 95.61% for PTX and 94.88% for ISTD, which were within the acceptance limits of 90.00 to 110.00%. As this bio-analytical method can be applied to cancer patients who are taking chemotherapy in duration of 21 days, so it is advisable to do the real time TDM and no need to require measurement of all stability studies parameters [12,13].

CONCLUSION

A highly sensitive and simple method was developed and validated in human plasma for the quantification of paclitaxel using LCMS/MS as per USFDA and EMEA guidelines. It can be successfully applied to perform sample analysis of a pharmacokinetic and bioavailability studies during chemotherapy of paclitaxel in cancer patients. This method offers various advantages over the other published methods, such as less sample volume, high throughput, rapid, greater sensitivity and simplicity, adequate retention without matrix interferences. The present LC–MS/MS method provides a simple, robust, quick and sensitive analytical tool for quantification of paclitaxel in human plasma and can be successfully applicable to clinical studies and therapeutic drug monitoring as well. There is the recommended future research requirement with the protocol permission of Human Ethics Committee for practicability of the proposed method in toxicology and pharmacokinetic field by therapeutic drug monitoring of cancer patients during chemotherapy for individual dosage adjustment for effectiveness of chemotherapy schedules.

ACKNOWLEDGEMENT

The authors would like to thank, Khandelwal Laboratories Pvt. Ltd., Cadila Pharmaceuticals Pvt. Ltd. for providing necessary drug samples and Department of Pharmaceutical Sciences, Saurashtra University, Rajkot for experimental work facilities. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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