

# Liquid Chromatography–Tandem Mass Spectrometry Method for Determination of Paclitaxel in Human Plasma

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## Abstract

A rapid and sensitive liquid chromatography – tandem mass spectrometry (LC-MS/MS) method for determination of the Paclitaxel in human plasma was developed and validated. Paclitaxel was extracted from plasma by a two-step extraction procedure with using of chloroform as Liquid–Liquid extractive organic solvent. LC-MS/MS analysis using electro-spray ionization (ESI+) was performed on Phenomenex C-18 Column (250x1.5 $\mu$ ) using as acetonitrile, water (80:20+0.1% acetic acid) as mobile phase. The method has a flow rate of 0.8ml/min. Retention of Paclitaxel was 4.60 minutes. An excellent linearity ( $r^2$  .099) between the peak ratios and Paclitaxel concentrations over the range of 10-100 ng/ml of plasma was studied. The lower limit of detection for Paclitaxel on mass was 10 pg/mL. There was about 100 percent of Paclitaxel was recovered in extracted samples. The study of Paclitaxel standard and extraction standard calibration curves were useful in pharmacokinetics analysis.

**Keywords:** Liquid chromatography-tandem mass spectrometry; Paclitaxel; Plasma; Chloroform extraction

## Introduction

Paclitaxel (Figure 1) is a natural product extracted from western yew tree, and takes its name from the Latin, *Taxus brevifolia*. It blocks mitosis by stabilizing the microtubules in cancer cells. During normal cell division, the microtubules are polymerized at the beginning of mitosis to be able to separate the daughter chromosomes. Then they depolymerise back to tubulin. Paclitaxel stops this depolymerisation so that the cells become filled with microtubules and cannot divide again. Paclitaxel is active against a number of cancers e.g. of the ovaries, breast, lung and stomach (1).

Paclitaxel is virtually insoluble in water and in most pharmaceutically acceptable solvents; such that it has poor oral bio availability 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 [2].

Various experiments have been performed to improve the bioavailability of this drug such as binding with polymers [3], co-administration with Cyclosporine A [4] or administration by liposome's [5]. To be able to compare the effectiveness of different formulations, 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 the side effects decrease. For example, Paclitaxel has demonstrated synergy with the pyrimidine analogue 5-fluorouracil (5FU) [6], gemcitabine [7], some anticonvulsants [8] and flavopirido [9]. Again, when Paclitaxel is given with other drugs, there is a need to evaluate pharmacokinetic parameters using a validated assay, with high specificity in order that co-administered drugs and/or metabolites do not interfere with the measurement of Paclitaxel. Various HPLC methods for validation of Paclitaxel was studied [14]. A review of early methods of determination of paclitaxel concentrations has been presented by Sparreboom et al. [2]. The aim of the present study was investigate the Paclitaxel extracted samples, and standard samples with high sensitivity, accuracy was studied with Liquid Chromatography- tandem mass spectrometry method which is use full for Pharmacokinetic analysis.

## Material and Methods

### Chemicals and reagents

Paclitaxel was provided by Dr.Reddys Research laboratory, Hyderabad-INDIA). HPLC grade acetonitrile & chloroform were procured from Merck. Ultra pure water was obtained from Mille – Q water (S G ultra pure waters system).

### Solutions

Stock solution of Paclitaxel was prepared by dissolving 1 mg of Paclitaxel in 1ml of acetonitrile. Standard solutions were obtained by diluting this solution with acetonitrile to give the final concentrations over the range of 10-100ng/ml for preparation of the standard calibration curve. Acetonitrile, water (80:20+0.1%acetic acid) was prepared for mobile phase and as well for reconstitution of Paclitaxel extracted sample from plasma.

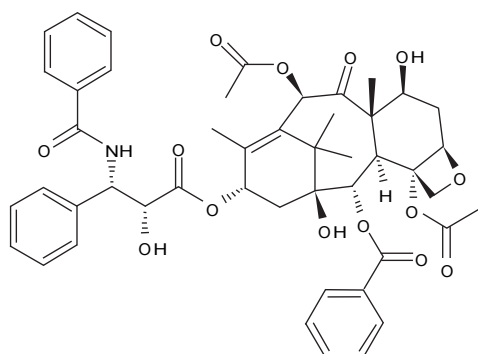


Figure 1: Chemical structure of Paclitaxel.

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## Chromatographic condition

LC-MS/MS, Quattro micro API, triple quadrupole. Mass Lynx software, version 4.1. Consisted series of 2695 separation module and PDA (2996) detector all from Waters (Milford, MA, USA). Separation was achieved using phenomenax C-18 column (250x4.60 mm-5microns). The mobile phase contains 0.1% Acetic acid (80:20+0.1%Acetic acid) was prepared and degassed. Chromatographic separations were performed at 30°C. The flow rate was set to 0.8ml/min.

## LC-MS parameters

**Source (Es +):** Capillary (KV): 4.0; Cone (V): 40; Extractor (V): 1.0; RF lens (V): 0.1; Source Temperature (°C): 120; Desolvation Temperature (°C): 450; Cone gas flow (L/Hr): 50; Desolvation gas flow (L/Hr): 650

**Analyzer:** LM<sub>1</sub> Resolution: 12.0; HM<sub>1</sub> Resolution: 12.0; Ion energy<sub>1</sub>: 1.0; Entrance: 2.0; Collision: 30.0; Exit: 2.0; LM<sub>2</sub> Resolution: 12.0; HM<sub>2</sub> Resolutions: 12.0; Ion energy<sub>2</sub>: 1.0; Multiplier (V): 650; Syringe pump flow (lit/min): 10; Gas cell pirani pressure (mbar): < 1e-4 Bars

## Sample preparation

Plasma sample were obtained from healthy volunteers. For this samples 1 mL of Plasma was added into test tube and 50µl of standard (10, 20, 40, 60,80and 100ng) solution of Paclitaxel was added. These tubes are vortex for 1mint.After adding 1ml of chloroform to the test tubes, vortexed for 2 mints and centrifuged for 5min at 20°C and 1500g. That chloroform layer was completely removed and transferred to a clean test tube and evaporated to dryness at 37°C. These samples were reconstituted with mobile phase 80:20+0.1% Acetic acid.

## Sample extraction

Samples were extracted with liquid-liquid (L-L)method . Two of the common organic solvents used for the L-L extraction are methyl acetate [10] and methyl tertiary butyl ether (MTBE) [5]. The few articles mentioning SPE demonstrate the use of non-polar sorbents (C18 cartridges [11] or polar sorbents (Cn cartridges, [12, 13]. Here we used chloroform as extraction organic solvent. Once the drug has been extracted from the plasma to chloroform, then the drug sample was evaporated at 37°C. LC-MS/MS system and separation is performed generally by reversed phase HPLC. 50 µl of each sample

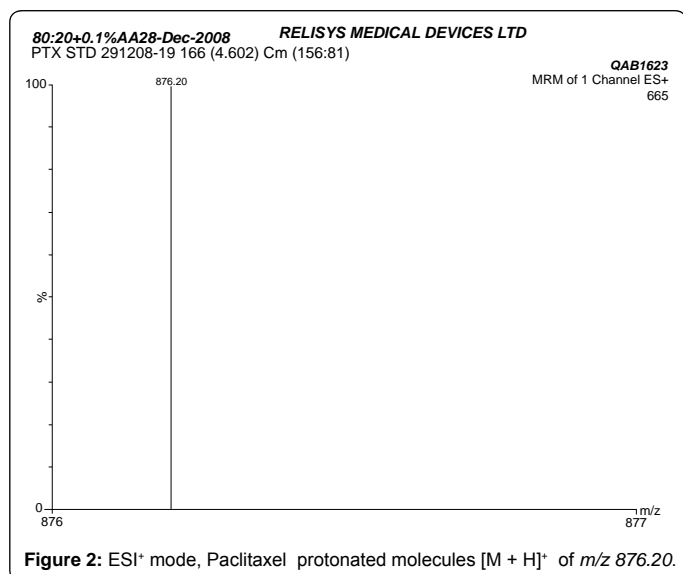


Figure 2: ESI+ mode, Paclitaxel protonated molecules [M + H]<sup>+</sup> of m/z 876.20.

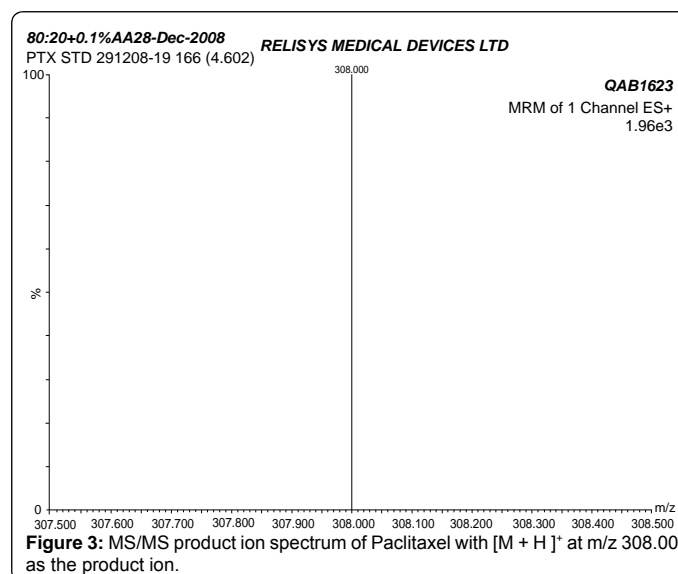


Figure 3: MS/MS product ion spectrum of Paclitaxel with [M + H]<sup>+</sup> at m/z 308.00 as the product ion.

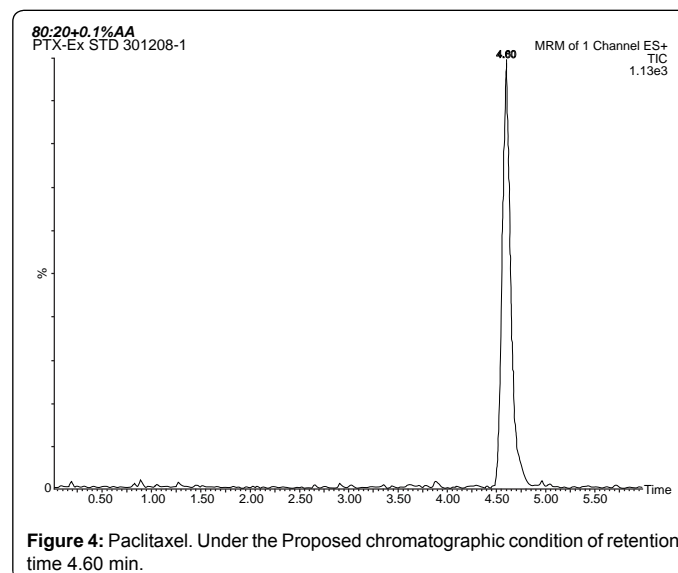


Figure 4: Paclitaxel. Under the Proposed chromatographic condition of retention time 4.60 min.

was injected to LC-MS/MS. The experiment was repeated on three consecutive days

## Quantification

Calibration standards of Paclitaxel were prepared by spiking 1µg Paclitaxel standard to 1ml of blank human plasma to give final concentrations over the range of 1-100ng /mL. Calibration curves were prepared by plotting the measured peak area ratios of Paclitaxel vs. concentration of standard samples. The intra day (with in run) and interday (between run) accuracy and precision of the method was determined by measuring sample of Paclitaxel standards solution (10, 20, 40, 60, 80,100ng/ml) on three separate days.

## Results

In the present study simple liquid-liquid extraction procedure was performed. The extraction efficiency was increased when liquid-liquid extraction (LLE) using dichloro-methane, Petroleum ether, methanol, chloroform extraction procedure was used. The extraction recovery was increased when chloroform as extractive solvent. It was found that all solvents gave high extraction efficiency for Paclitaxel.



**Quantify Compound Summary Report MassLynx 4.1**

Dataset: C:\MassLynx\PTXSTD151107.PRO\PTX-100-10ng-10109.qld

Last Altered: Wednesday, December 31, 2008 11:09:36 India Standard Time

Printed: Thursday, January 08, 2009 12:29:18 India Standard Time

A

Method: C:\MassLynx\PTXSTD151107.PRO\MethDB\PTX60208-2.mdb 04 May 2008 22:26:13

Calibration: 31 Dec 2008 11:09:36

**Compound name: PTX STD**

Correlation coefficient:  $r = 0.995033$ ,  $r^2 = 0.990090$

Calibration curve:  $6.4653 * x + 6.31373$

Response type: External Std, Area

Curve type: Linear, Origin: Include, Weighting: Null, Axis trans: None

	# Name	Type	Std. Conc	RT	Area	IS Area	Response Detecti...	ng	%Dev Mod.U...	Mod.C...
1	1 PTX STD 29120...	Standard	100.000	4.63	659.553		659.553 bb	101.0	1.0	
2	2 PTX STD 29120...	Standard	80.000	4.63	483.926		483.926 bb	73.9	-7.7	
3	3 PTX STD 29120...	Standard	60.000	4.63	429.126		429.128 bb	65.4	9.0	
4	4 PTX STD 29120...	Standard	40.000	4.63	282.728		282.728 bb	42.8	6.9	
5	5 PTX STD 29120...	Standard	20.000	4.63	118.322		118.322 bb	17.3	-13.4	
6	6 PTX STD 29120...	Standard	10.000	4.63	74.783		74.783 bb	10.6	5.9	

**Quantify Calibration Report MassLynx 4.1**

Dataset: C:\MassLynx\PTXSTD151107.PRO\PTX-100-10ng-10109.qld

Last Altered: Wednesday, December 31, 2008 11:09:36 India Standard Time

Printed: Thursday, January 08, 2009 12:29:18 India Standard Time

B

Method: C:\MassLynx\PTXSTD151107.PRO\MethDB\PTX60208-2.mdb 04 May 2008 22:26:13

Calibration: 31 Dec 2008 11:09:36

**Compound name: PTX STD**

Correlation coefficient:  $r = 0.995033$ ,  $r^2 = 0.990090$

Calibration curve:  $6.4653 * x + 6.31373$

Response type: External Std, Area

Curve type: Linear, Origin: Include, Weighting: Null, Axis trans: None

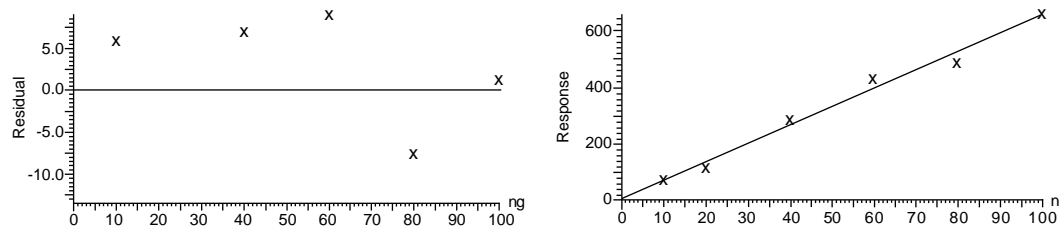


Figure 5: Calibration curves (A) List of samples in nano grams for calibration (B) The linearity of Paclitaxel calibration curve.

In this study, ESI<sup>+</sup> was chosen as the ionization source. Total Ion Current (TIC) was increased when adding if Acetic acid 0.1% to the mobile phase. It was found that the signal intensity of Paclitaxel in human plasma was high using ESI<sup>+</sup> source. The ESI<sup>+</sup> source provided satisfactory data on method validation and subsequent quantitation for plasma samples from healthy volunteers. ESI<sup>+</sup> mode, Paclitaxel formed predominantly protonated molecules  $[M + H]^+$  of  $m/z$  876.20 (Figure 2). The paclitaxel parent molecule was fragmented while applying collision energy as organ, and formed product ions (Figure 3). Product ion is necessary for quantification of paclitaxel.

This method showed on interfering peaks of any other contamination other than Paclitaxel. Under the Proposed chromatographic condition of paclitaxel retention time 4.60 min

(Figure 4). The extracted samples chromatograms were co-related to the standard. The recovery of paclitaxel in different concentration, determined by comparing peak areas from extracted standard samples. There was about more than 99% of Paclitaxel was recovered from plasma.

**Calibration**

Standard Calibration curve for Paclitaxel in different range of concentrations (10, 20, 40, 60, 80, 100ng/ml) in plasma were prepared. The linearity of calibration curve ( $r^2 > 0.99$ ) (Figure 5) concentration ranges are investigated. The calibration curve obtained in plasma sample Calibration curve:  $4.4653 * X + 6.31373$ . The accuracy and precision was determined by preparing with 5 samples of Paclitaxel at concentrations of 1, 10, 20, 40, 60, 80, 100ng/ml in plasma on three



separate days. According to the intraday (within- run) and interday (between- run) a very good data of accuracy and precision were observed over the entire concentration range.

### Method validation

**Selectivity:** This method was specific for Paclitaxel with no interference of peaks at the retention time of Paclitaxel.

**Linearity:** A linear range equation was judged to produced the best fit the concentration / response relationship. The regression type was 1/ concentration and peak area for a 6-point calibration curves were found to linear from 10 nano grams to 100 nano grams. The goodness to fit for concentrations were consistently greater than 0.99 during the course of validation and study period.

**Recovery:** The percentage of recovery of Paclitaxel from PBS is observed as more than 99%.

**Accuracy:** The accuracy of the assay was defined as the absolute value of the ratio of the back calculated mean values of the quality control samples to their normal values, expressed as percentage. Within the batch, accuracy range from 90.54% to 112.93%. Between the batch, accuracy range from 97.70%-105.37%.

**Precision:** The precision of the assay was measured by the percent coefficient of variation over the concentration range of quality control samples, respectively of Paclitaxel during the course of the study. Within batch Precision ranged from 0.04% to 15.19%. Between batch Precision ranged from 3.84% to 10.49%.

**Stability:** Short-term stability of Paclitaxel was determined by comparing the mean of the area responses obtained from 3 replicate analysis of aqueous standard (40 ng /ml) at 0.0 hours and after 6.0 hours. Ratio of means of area was 102.5%. Which is within the acceptance range of 90-110%.

Long- term stability of Paclitaxel was determined by comparing the mean of the area responses obtained from 3 replicate analysis of aqueous standard (80 ng/ml) after 12 days and freshly prepared aqueous standard. Ratio of means of area was 101.7%. Which is within the acceptance range of 90-110%.

### Discussion

In the present study the importance of LC-MS/MS techniques for determination of paclitaxel in biological fluids were observed. Paclitaxel was extracted from aqueous medium into an organic solvent. The LC-MS/MS method was validated and subsequently used to analyze samples from a comparison study.

The LC-MS/MS method demonstrated high specificity because only ions derived from the analytes of interest were monitored. The selectivity towards endogenous plasma matrix was tested in different batches of human plasma by analysis blanks and samples at LLOQ levels. Paclitaxel chromatograms indicate no significant visible interference at the expected retention time of the analyte since Paclitaxel was modified to elute in a region where visible interference is not observed. The retention time of Paclitaxel was 4.6 min. This is the shortest total run time (6.0 min) for determination of Paclitaxel in plasma.

In the present study, a two step extraction procedure was

described using chloroform as extracting solvent. The accuracy and precision for this sample and rapid procedure support the reliability of the assay for the determination of paclitaxel in biological fluids and for pharmacokinetics studies.

### Conclusion

A two step extraction procedure for paclitaxel from human plasma and an improved method for determination of paclitaxel were reported. The LCMS/MS method need a cleaner extract at it less specific and any endogenous compounds with the same retention time and similar maximum of absorption would interfere with the assay, UV detection can be used and an adequate PK profile can be obtained with a method validated between 10 and 100 nano gram/mL [13].

In this study, we reported a newly developed LC-MS/MS method for the determination of Paclitaxel in human plasma. The LC-MS/MS technique is suitable for determination of small amount of Paclitaxel with good accuracy and reproducibility. The sample pretreatment was easy and fast using chloroform extraction. ESI<sup>+</sup> technique with satisfactory mass spectral response generated for paclitaxel parent molecule. This method was successfully applied to determined Paclitaxel concentration in human plasma, for processing of multiple samples in a limited amount of time for pharmacokinetics studies.

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