ARTICLE ORIGINAL

Mise au point et validation d'une technique de dosage du paclitaxel dans le plasma.

Development and validation method for estimation paclitaxel in plasma.

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RÉSUMÉ

Introduction : Le paclitaxel (PTX) est un anticancéreux cytotoxique, appartenant à la famille des taxoïdes, une famille d'agents antinéoplasique, dont l'originalité réside dans son mécanisme d'action. Il a en effet pour cible principale une protéine constitutive du fuseau mitotique, la tubuline. Il est largement utilisé dans de nombreux cancers, seul ou en association avec d'autres anti-tumoraux. Sa variabilité inter et intra individuelle, sa coadministration avec d'autres médicaments, son faible index thérapeutique et sa toxicité hématologique, justifient largement son suivi thérapeutique pharmacologique. Le paramètre le plus pertinent pour effectuer ce suivi semble être la durée pendant laquelle la concentration est supérieure à 0,05 µmol/L.

Objectif : Mettre au point et de valider une nouvelle méthode de dosage par chromatographie liquide haute performance avec détection UV (CLHP-UV) du PTX dans le plasma.

Méthodes : Dans ce travail nous avons mis au point une technique de dosage du PTX par CLHP utilisable en routine, en recourant à une détection UV. Nous avons optimisé également les conditions opératoires de cette technique de dosage, dont la température, le débit d'élution, la composition, le pH de la phase mobile et les modalités d'extraction. Finalement nous avons procédé à la validation de notre technique de dosage du PTX afin de pouvoir l'appliquer pour le suivi thérapeutique pharmacologique des patients

Résultats : Après déprotéinisation et extraction liquide/liquide la séparation chromatographique est faite sur une colonne C18 et détection UV-Visible (longueur d'onde :232 nm). La phase mobile est composée d'un mélange composé de tampon dihydrogenophosphate à 0,1 M et d'acétonitrile, 51/49 (v/v). Le clonazepam est utilisé comme étalon interne (EI). La linéarité de notre étude a été confirmée sur un intervalle de concentration allant de 50 à 1500 ng/mL avec un coefficient de corrélation r égale à 0,998. Elle est également spécifique et sensible avec une limite de détection égale à 10,3 ng/mL et une limite de quantification égale à 31,40 ng/mL. L'évaluation de la précision a montré que notre technique est répétable avec un coefficient de variation allant de 6,94 à 18,78 % et reproductible pour les trois concentrations étudiées Low, Medium et High avec un coefficient de variation respectivement de 14,92 ; 10,46 et 11,80 %. Dans ces conditions, le temps d'analyse du PTX est de 12,81 minutes.

Conclusions : Nous avons mis au point et valider une technique de dosage simple, spécifique, facile à mettre en œuvre et rapide. Elle permet donc des dosages en routine du PTX dans le plasma chez les patients traités par cet anticancéreux et nécessitant un suivi thérapeutique pour un éventuel ajustement de dose.

Mots clé : Paclitaxel – CLHP-validation – suivi thérapeutique.

SUMMARY

Background : Paclitaxel (PTX) is an anticancer drug used in the treatment of many cancer , alone or in combination with other anti-tumors. It has a narrow therapeutic range, a large inter and intra-individual pharmacokinetic variability and haematological toxicity. The most effective pharmacokinetic parameter seems to be the time during which the plasma concentration is over 0.05 µmol/L.

Aim: To develop and validate a new method for PTX quantitation in plasma using HPLC with UV/visible detection.

Methods: A rapid HPLC-UV method was developed for the determination of PTX level in plasma. All solvents used were HPLC grade.

Results: After liquid-liquid extraction, chromatographic separation was achieved using an RP 18 (250 mm) column. The mobile phase was composed of acetonitrile and 0.1 M potassium dihyrogenophosphate (49/51) (v/v). Clonazepam was used as internal standard. This technique was linear over the range 50 ng/mL to 1500 ng/mL (r= 0.998). The evaluation of precision showed that our method is repeatable with a within-day coefficient of variation (CV) ranging from 6.94 to 18.78 % and reproducible for three studied concentrations low, medium and high with day-to-day CV of 14.92, 10.46 and 11.8% respectively. Under these conditions, each analysis required no longer than 12.81 min.

Conclusion: We have developed and validate a new assay for PTX monitoring using HPLC with UV detection which is sensible, specific, reliable and easy to carry out in clinical use for its therapeutic drug monitoring

Key Words: HPLC - Validation – paclitaxel-therapeutic drug monitoring

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INTRODUCTION

Paclitaxel (PTX) isolated from Taxus brevifolia, the Western Yew tree, is a hydrophobic drug with a molecular weight of 853.92 g/mol. Paclitaxel is drug used for the treatment of breast and ovarian cancers as well as lungs and AIDS -related Kaposi's sarcoma [1]. The most effective pharmacokinetic parameter seems to be the time during which the plasma concentration is over 0.05 µmol/L [2]. Different methods have been developed for the determination of PTX, such us capillary electrophoresis [1], LC-MS [3-4] and high performance liquid chromatography (HPLC) [5-6-7-8- 9].

The aim of this study was to establish and validate simple, accurate and precise method for quantitatively determination plasma concentration of PTX using HPLC-UV technique.

METHODS

Preparation of standard solutions

Standard solution of PTX (1 mg/mL) and Clonazepam (125 µg/mL) was prepared in methanol (MeOH) and stored at -20°C. Appropriate dilutions of the standard solution were made in MeOH to produce solutions in the range of 0 to 1500 ng/mL. Samples for the determination of recovery, precision and accuracy were also prepared by spiking control in appropriate concentrations (60, 400 and 1200 ng/mL) and stored at −20°C.

Apparatus and chromatographic conditions

The HPLC system manufactured by Varian® 31 (Australia) was used for the present study. For the data acquisition and integration, Galaxy Chromatography data system version1.9.3.2 software operated by Pentium 4HT was used. The analytical colomn was C18 (4.6 × 250 mm) having 5 µm particle size (Merck, France). The mobile phase consisted of a mixture of phosphate buffer and acetonitril at a ratio of 51:49. The mobile phase pH was adjusted at 5 with orthophosphoric acid, filtered through a 0.45 µm membrane filter and degassed using ultrasonic bath sonicator. All experiments conducted on the HPLC were carried out in isocratic mode. 50 µL of aliquot was injected on HPLC at a flow rate of 1 mL/min. The column temperature was maintained at 30°C and elution was monitored at 232 nm using an ultraviolet detector.

Sample extraction.

Aliquots of 200 µL plasma samples (blank, standard or patient sample) and 15 µL of IS solution were mixed for 1 min. Sodium carbonate (1000 µL) were added for deproteinisation and 8 mL diethyl ether for extraction. Each sample were shaken for 15 min and centrifuged at 4°C (4000 g x 10 min), then the supernatant was dried under nitrogen flow at room temperature. Finally, we added 100 µL of mobile phase for reconstitution and 50 µL was injected into HPLC column for quantification.

Validation of the analytical method

The developed method was validated as per the US Food Drug Administration (FDA) FDA guidelines for linearity, accuracy, precision and specificity. Limit of detection (LOD) and limit of quantification (LOQ) were determined using US Pharmacopoeia (USP).

Linearity

The calibration curves were prepared (n= 6) with six increasing concentrations of PTX. The final concentration of PTX in plasma samples were 0, 50, 100, 500, 1000 and 1500 ng/mL. The linearity of the analytical procedure was evaluated by plotting detector response (the peak area) against analyte concentration. Linear regression analysis was carried out to calculate the slope, intercept and linear correlation coefficient (r).

Accuracy and precision

Accuracy and precision of the analytical method was determined by analyzing quality control (QC) samples at three different concentrations in six replicate analysis (n= 6). QC standards were prepared in the same media and were independent of those used for the preparation of calibration curves.

The precision (% RSD) of the analytical procedure was evaluated by determining the intra- and inter-day coefficients of variation and reported as % RSD for a statistically significant number of replicate measurements. The intra-day precision of the selected method was estimated by the analysis of three different concentrations of the drug in six times on the same day. The inter-day precision was assessed by analyzing samples in the same

way as for the intra-day precision assay and was repeated for 6 consecutive days.

Specificity

Specificity is the ability of the analytical method to measure accurately and specifically the analyte of interest in the presence of other components that might be expected to be present in the sample.

Quantification limits

LOD and LOQ decide about the sensitivity of the method. LOD is the lowest detectable concentration of the analyte, whereas LOQ is the lowest amount of the analyte in a sample, which could be quantitatively determined with suitable precision and accuracy. LOQ was assessed by the standard deviation of the response and the slope method. Slope S was calculated from the calibration curve of the analyte and the standard deviation was estimated by running six blank samples while LOD was taken as the one - third of LOQ for their simultaneous analysis, LOQ and LOD were estimated by the serial dilution method.

RESULTS

Method development

An isocratic method was used. Different ratios, flow rates, pHs of mobile phase and column temperatures were studied in order to shorten the retention time of PTX and to improve peak symmetry. However, optimal assay conditions were found when using ration of (51/49 Buffer/ACN). The pH was adjusted to 5 with othophosphoric acid, the column temperature set at 60°C and the flow rate at 1 mL/min. Retention time of PTX in these conditions was 13.96 + 0.05 min.

The choice of the internal standard (IS) is important in the method development. The IS must be added at the beginning of extraction and should follow the drug in all stages of the extraction. It must be well separated from the peak of PTX, having a retention time close to the molecule and a total analysis time as short as possible. Several molecules were tried in our work such as mitotane, teïcoplanine, Barbital sodique and clonazepam. Mitotane was no detected, barbital sodique and clonazepam were detected at retention time of 2.23 and 7.7 min, respectively. Finally clonazepam was kept as internal standard.

A precipitation of plasmatic proteins was carried out in this study using sodium carbonate and extraction was done with diethyl ether.

Validation of the method

The specificity of the method was examined by comparing both chromatograms of blank human plasma and plasma spiked with PTX. Representative chromatogram of blank plasma extracted of PTX's standard plasma and clonazepam are shown in fig.(1A-B) there were no interfering pics in blank plasma at the retention time of PTX. Mean regression coefficient (r^2) of all PTX calibration curves was 0.99 +0.0007 (n= 6). So the method was linear in concentration range 50 to 1500 ng/mL.

The precision and accuracy of data obtained from controls have been presented in Table 1. Intra-day within day CV% of the assay varied from 6.94% at 1200 ng/mL to 18.77% at 60 ng/mL. Intra-day accuracy of estimation in different standards samples was found to be 17.25% for 60 ng/mL, 16.46% for 400 ng/mL and 16.12% for 1200 ng/mL. Inter assay between day coefficient of variation was 11.80% at 1200 ng/mL to 14.92% for 60 ng/mL. The inter-day accuracy of estimation in the seeded samples varied from -8.66% to -4.47% for 60 ng/mL and 1200 ng/mL, respectively. These results suggest a good precision and accuracy of the method.

The recovery was assessed by comparing the response of five replicates of extracted sample (60, 400 and 1200 ng/mL) to the response of pure standard at the same concentration level. The standard specimens were diluted in bidistilled water and directly injected into the HPLC system. The recovery of PTX was greater than 90 % (data not shown) for all tested concentrations.

The LOD and LOQ calculated were 10.6 ng/mL and 32.14 ng/mL, respectively. The calculated LOD and LOQ concentrations confirmed that the methods were sufficiently sensitive.

Table 1. Precision and accuracy of PTX in human plasma

	Observed	Accuracy	Precision
Added PTX cocentration (ng/mL)	PTX concentration (Mean + SD; ng/mL)	(%) Bias	(%) CV %
Intra-day*			
60	49.65 + 9.32	- 17.25	18.77
400	334.13 +35.46	-16.46	10.61
1200	1006.49 + 64.86	-16.12	6.94
Inter-day*			
60	54.8 + 7.95	-8.66	14.92
400	382.08 + 40	-4.47	10.46
1200	11146.01 +140.44	-4.49	11.80

*n= 6 assays in the same day for each concentration; **n=6 assays per day during 3 days for each concentration. Daily (intra-day and day to day (inter-day) precisions are represented as mean values + SD of six different assays for each concentration.

DISCUSSIONS

We describe a simple and rapid method for quantification PTX using HPLC with UV detector, suitable for the clinical implementation of therapeutic drug monitoring of this chemotherapeutic drug.

Different mobile phase solvents have been reported in literature at various compositions for the separation of PTX. Here, PTX and clonazepam were separated on the ODS column using the mobile phase consisting of buffer and acetonitrile in the ratio of 51:49. The isocratic mode was employed for the elution of these drugs. Several internal standards are described in the literature such as 2-méthyl PTX [7], ^{3}H-PTX [10], doxétal [11], and carbamazepine [12]. In our method we used clonazepam. Two extraction methods have been described for PTX in literature, a solid-liquid [7-11] and a liquid-liquid extraction [6-9-11-13]. Several extraction solvents have been described; such as a mix of acetonitrile, methanol and n-butylchloride [13]; ethyl acetate [9]. In our method, plasma samples were pretreated with very simple liquid-liquid extraction procedure before analysis requiring only about 14 min.

The method developed for simultaneous analysis of clonazepam and PTX was validated for analytical performance parameters such as linearity, accuracy, precision, specificity and quantification limits as per the ICH guidelines. Linear regression analysis confirmed that the r^2 values for both drugs were found to be 0.9995, confirming the linear relationship between the concentration of the drug and the area under the curve. The calculated LOD and LOQ concentrations confirmed that the methods were sufficiently sensitive.

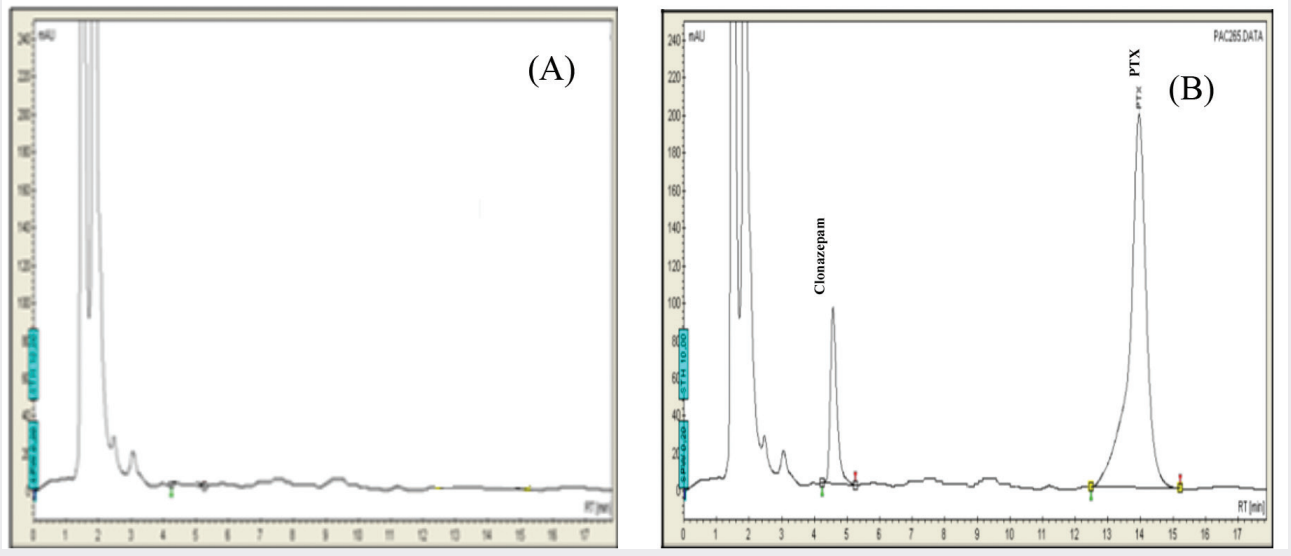

Figure 1 (A-B). Representative chromatograms of blank and PTX spiked plasma (A) representative chromatogram of blank plasma extracted of PTX (B) representative chromatogram of PTX standard plasma extracted

CONCLUSION

The HPLC method was developed for simultaneous estimation of PTX and Clonazepam in human plasma sample. The developed method is simple, rapid and reliable enough employed for analysis of the two drugs simultaneously using the mobile phase, ACN and Bufffer. Run time for Clonazepam (6 min) ensures its rapid estimation without any interference from PTX (12 min). Validation report confirms that the method has good linearity, accuracy, precision, adequate specificity, and it can be employed to find out the concentration of PTX in human samples.

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