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Title page

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A dried blood spot assay for paclitaxel and its metabolites

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Abstract

After being used for decades in clinical screening, dried blood spots (DBS) have recently received considerable attention for their application in pharmacokinetic and toxicokinetic studies in rodents. The goal of this study was to develop and apply a DBS-based assay for a pharmacokinetic study of paclitaxel (PTX) and its metabolites in SCID/Beige mice. A fast and sensitive UHPLC-MS/MS method has been developed for the simultaneous determination of PTX, its three metabolites (6*α*-hydroxy-paclitaxel, 3'-*p*-hydroxy-paclitaxel, and 6*α*,3'-*p*-dihydroxy-paclitaxel) and its stereoisomer 7-epi-paclitaxel. The 10 µL DBS sample was extracted with methanol for 20 min at 37°C. After dilution of the extracts with water in a ratio of 1:1, the analytes were separated on a reversed-phase 2.1 mm I.D. column using gradient elution. The total run time was 2.5 min. The analytes were detected by use of multiple reaction monitoring mass spectrometry. The extraction recoveries of the compounds were all greater than 60%, resulting in a quantification limit of 1 ng/ml. The calibration curves ranged from 1 to 1000 ng/ml. The intra-day and inter-day imprecision (%CV) across three validation runs over four quality control levels were less than or equal to 14.6%. The accuracy was within ±11.9% in terms of relative error. The described method is advantageous in terms of its ease-of-use and speed compared to other published PTX assays. The method's usefulness was demonstrated by applying it to a preclinical pharmacokinetic investigation of PTX and its metabolites in SCID/Beige mice with an intraperitoneal administration of 50 mg/kg Abraxane®.

Keywords

Paclitaxel, Metabolite, Dried blood spot, LC-MS/MS, Microsampling

1.Introduction

Paclitaxel (PTX, Fig.1) is a natural product discovered in 1968 by isolation from the bark of the pacific yew tree Taxus brevifola[1]. It is a widely used antineoplastic drug for the treatment of multiple types of tumors including ovarian, stomach, endometrial, colon, breast cancer and so on[2-4]. Despite its clinical efficacy, current PTX-based therapies are characterized by non-linear pharmacokinetics, a narrow therapeutic window, and severe dose-limiting toxicities (DLTs)[5, 6].

PTX is predominantly metabolized in the liver by cytochrome P450 (CYP) enzymes. The primary metabolite 6*α*-hydroxy-paclitaxel (6*α*-OH-PTX, Fig.1) is formed by CYP2C8, whereas the secondary metabolite 3'-*p*-hydroxy-paclitaxel (3'-*p*-OH-PTX, Fig.1) is generated by CYP3A4[7, 8]. These two metabolites can be further metabolized to a minor metabolite 6*α*,3'-*p*-dihydroxy-paclitaxel (6*α*,3'-*p*-di-OH-PTX, Fig.1). In addition, the C-7 chiral center in the taxane ring of PTX is notably labile and can readily undergo epimerization in an aqueous environment, resulting in 7-epi-paclitaxel (7-epi-PTX, Fig.1), a biologically active[9] and thermodynamically more stable stereoisomer under physiological conditions[6, 10].

Intraperitoneal chemotherapy (IPC) with PTX has a strong pharmacokinetic rationale in the treatment of peritoneal carcinomatosis. Hereto, PTX is directly administered into the abdominal cavity[11]. In this way, by lowering the systemic exposure, DLTs should be reduced [12]. However, controversy remains regarding the optimal treatment strategies that will optimize efficacy whilst minimizing systemic exposure. Quantification of PTX together with its metabolites is of major importance to gain insights into the absorption process and the extent of first-pass metabolism during IPC treatment. Current attempts to gain fundamental insights in the disposition of PTX following IPC treatment have focused on rodent models[13, 14]. In order to exploit the full potential of these animal models, an efficient sampling procedure coupled to a rapid and sensitive analytical method is highly desirable.

Dried blood spot (DBS) sampling is a form of microsampling where small volumes of blood samples are applied on absorbent paper[15]. The most notable benefits of DBS sampling are the low required volume thus resulting decrease in the number of animals to be included in pharmacokinetic (PK)/toxicokinetic (TK) studies[16-18], and the improved stability in the dried blood spot matrix as compared to whole blood/plasma samples[19]. However, some challenges need to be overcome for the implementation of DBS analysis. The most common disadvantages are hematocrit, complex matrix (whole blood plus paper), required high sensitivity, and the comparability of DBS and plasma derived concentrations[20].

Different bioanalytical techniques have been described for the determination of PTX, including immunoassays[21], capillary electrophoresis[22], HPLC with UV detection[23] and liquid chromatography-tandem mass spectrometry (LC-MS/MS)[24-26]. Of these, the LC-MS/MS technique has been the most frequently applied method due to its sensitivity and selectivity[27]. There have been several LC-MS/MS assays published for the quantification of PTX and its metabolites[3, 28-30] in various biological matrices such as plasma, serum, urine and tissue. As far as we know, there is only one publication reported for the determination of PTX in human DBS[31]. This method has a relatively narrow calibration range (0.2-20 ng/ml) and its clinical utility has not been demonstrated. Our report here is describing the quantification of PTX including its metabolites in dried blood spots obtained from animal experiments.

The goal of the present study was to develop a rapid and sensitive LC-MS/MS method for the simultaneous quantification of PTX and its metabolites (3'-*p*-OH-PTX, 6*α*-OH-PTX, and 6*α*,3'-*p*-di-OH-PTX) as well as the stereoisomer 7-epi-PTX, in mice DBS samples based on a simple extraction procedure. The use of DBS, a short gradient elution, and satisfactory sensitivity demonstrate that this LC-MS/MS method is superior to previous methods. This method to quantify PTX and its metabolites was successfully applied to a pharmacokinetic study where 10 SCID/Beige mice received an intraperitoneal dose of 50 mg/kg Abraxane®.

2.Material and Methods

2.1 Reagents and chemicals

Paclitaxel, $^{13}C_6$-Paclitaxel (internal standard (IS)), 3'-*p*-hydroxy-paclitaxel, 6*α*-hydroxy-paclitaxel, 6*α*-hydroxy-paclitaxel-d5 (IS), 6*α*,3'-*p*-dihydroxy-paclitaxel, and 7-epi-paclitaxel were all purchased from Toronto Research Chemicals (Toronto, Canada). ULC-MS grade water and methanol were obtained from Biosolve (Valkenswaard, The Netherlands). The PKI Bioanalysis Card (GR2261004) was supplied from PerkinElmer Health Sciences (Greenville, South Carolina, USA). All other solvents or chemicals were analytical grade or better.

2.2 Instrumentation and set-up

2.2.1 UHPLC-MS/MS

An Acquity BEH C18 column (50 × 2.1 mm, 1.7 µm) with an Acquity BEH C18 VanGuard pre-column (5 × 2.1 mm, 1.7 µm) installed in an Acquity UPLC H-class system (Waters, Milford, MA, USA) was used for chromatographic separation. Mobile phases, delivered at 0.45 ml/min, consisted of 0.2% formic acid either in 90/10 (*v/v*) water/methanol (eluent A) or in methanol (eluent B). The total run time for each separation was 2.5 min, and the linear gradient elution program was as follows: 0-1.25 min,

50% B to 80% B; 1.25-1.5 min, 80% B to 100% B; 1.5-2.0 min, 100% B to 50% B; 2.0-2.5 min, 50% B. A divert valve program switching alternatively between detector and waste was incorporated as follows: 0-0.75 min: to waste; 0.75-2.0 min: to detector; 2.0-2.5 min: to waste. The column temperature was kept at 50 °C, and the injection volume was 10 µL.

The LC eluate was led directly into a Waters Xevo TQS tandem mass spectrometer (Waters, Milford, MA, USA) with electrospray ionization (ESI) in the positive-ion, multiple reaction monitoring (MRM) mode. The MS/MS instrument was operated with a capillary voltage of 3.0 kV and source temperature of 150 °C. The desolvation gas (nitrogen) flow rate was set to 1000 L/h at a temperature of 600 °C, and collision gas (argon) flow was maintained at 0.15 ml/min. Details of the MRM-transitions, dwell time, cone voltage, and collision energy for each compound are described in Table 1.

2.2.2 Microflow UHPLC-MS/MS

A microflow UHPLC (µUHPLC)-MS/MS, i.e. the Waters ionKey/MS system, was evaluated for increasing sensitivity during method development. The system was comprised of the ACQUITY UPLC M-Class, the Xevo TQ-S, and the ionKey/MS™ separation device (Waters, MA, USA). Mobile phase A was 10% methanol in water containing 0.2% formic acid and mobile phase B was 0.2% formic acid in methanol. In a full loop injection mode, 5 µL was injected on to a trap column (M-Class Trap Symmetry C18, 100 Å, 5 µm, 300 µm × 25 mm) using a trapping flow of 30 µl/min with 20% methanol for 3.0 min. After trapping, the flow was reversed and directed to a Waters BEH C18 iKey™ analytical column (150 µm × 100 mm, 130 Å, 1.7 µm).

The flow over the analytical column was 2 µl/min and the column was operated at 50°C. PTX and its metabolites were eluted from the analytical column using the following gradient: 0-5 min: 90-0.5% A and 10-99.5% B, 5-6 min: 0.5-90% A and 99.5-10% B, 6-6.5 min 90% A and 10% B, 6.5-7 min 90-0.5% A and 10-99.5% B, 7-12 min 0.5% A and 99.5% B, 12-12.5 min: 0.5-90% A and 99.5-10% B. In the µUHPLC eluate, the analytes were detected using the same MS/MS parameters as described above (Table 1).

2.3 Preparation of standards and quality controls

Primary stock standards were prepared in ethanol at concentrations of 0.96, 0.943, 0.0345, 1.104, and 0.257 mg/ml for PTX, 7-epi-PTX, 3'-*p*-OH-PTX, 6*α*-OH-PTX, and 6*α*,3'-*p*-di-OH-PTX, respectively. Stock standards were mixed in the appropriate proportions and were further diluted with acetonitrile to obtain working solutions. Blank human whole blood from healthy volunteers (Ghent University

Hospital, Belgium) was spiked with the different working solutions to obtain calibrator samples at 7 concentration levels. In order to preserve the integrity of the blood matrix, the blood standards contained at most 5% (*v/v*) of working solution. The final concentrations for the calibrators ranged from 1 to 1000 ng/ml for all analytes. The blood quality control (QC) samples were also prepared as a mixture from the different working solutions at concentrations of 1 (lower limit of quantitation QC, LLQC), 2.5 (low QC, LQC), 25 (medium QC, MQC) and 1000 (high QC, HQC) ng/ml. Internal standard stock solutions of $^{13}C_6$-PTX and 6α-OH-PTX-d5 were prepared in ethanol at concentrations of 0.1 and 0.5 mg/ml, respectively. After mixing and further dilution of these stock solutions with methanol, an internal standard working solution at a concentration of 2 ng/ml was obtained. All stock and working solutions were stored at -20°C.

2.4 DBS sample preparation and extraction

Aliquots (10 µL each) of whole blood samples were spotted onto PKI Bioanalysis Cards (PerkinElmer Health Sciences, USA) with a calibrated pipette and left to dry for at least 3 h at room temperature prior to analysis. The entire DBS was punched out and collected into a 1.5-ml Eppendorf tube. Extraction was performed by adding 200 µL of IS working solution. The sample tubes were closed and thermostatted at 37 °C for 20 min whilst continuously being shaken by a Biosan TS-100 Thermo shaker (Biosan, Riga, Latvia) at 500 rpm. Then, 100 µL of the extract was added to 100 µL water and vortex-mixed for 30s. After transferring the resulting solution to an autosampler vial, a volume of 10 µL was injected into the UHPLC-MS/MS system.

2.5 Method validation

The method was validated for human DBS and cross-validated for mice DBS following the “Guideline on bioanalytical method validation” released by European Medicines Agency (EMA), 2011 and EBF recommendation on the validation of bioanalytical methods for dried blood spots[32, 33].

Selectivity was evaluated by the analysis of blank DBS from two different human whole blood sources and three mice whole blood sources. The LLOQ was defined as the lowest point of the calibration curve for which the bias (%) was within ± 20% and imprecision (coefficient of variation, CV%) < 20%. Calibration models were evaluated by comparing recovery values and residual distributions. Bias and imprecision were determined from six replicates at four QC concentration levels over three days. Bias was determined as % relative error of nominal concentration and imprecision was expressed as % CV. Carry-over was assessed by injecting blank samples directly after an injection of the highest concentration level of the calibrators.

Matrix effect was evaluated following Viswanathan et al., through the calculation of the internal standard-normalized (IS-normalized) matrix factor (MF)[34]. Extraction recoveries were determined at low and high concentrations in six replicates by comparing the peak area for extracted QC samples with the directly injected solution containing the same nominal concentrations.

Dilution integrity (10-fold) was assessed for PTX, 7-epi-PTX, and 6*α*-OH-PTX by processing six human DBS samples at 10-fold above the HQC sample. The dilution integrity of these three compounds were chosen due to their higher expected concentrations in vivo on a standard treatment regimen. In this procedure, the methanol-based extract of a DBS sample was first diluted with water in a ratio of 1:1 and further diluted with a blank matrix, followed by an addition of IS. In our study, three blank matrices were evaluated: i) a 50/50 (*v/v*) water/extract of blank DBS samples, ii) a 50/50 (*v/v*) water/extract of PKI bioanalysis cards, and iii) a 50/50 (*v/v*) water/methanol mixture. Dilution integrity was acceptable if the mean concentrations of analytes were within ± 15% of target.

Analyte stability was evaluated with human DBS QC samples at low and high concentrations in six replicates. The stability of the processed QC samples in an autosampler (10 °C) was determined for 24 h. Storage stability was tested using QC samples stored at room temperature (± 22°C) for 20 days. Samples were dried overnight and stored in a sealed plastic bag. QC samples were analyzed after 10 and 20 days of storage against a freshly prepared calibration curve, the spots of which were dried at least 3 h before the analysis.

A cross-validation was performed to ensure that the method is applicable to mice DBS samples. The DBS calibrators were made in human whole blood, and DBS QC samples at four concentration levels in six replicates were prepared in mice whole blood. Analytes' concentrations in mice DBS samples were quantified according to the human DBS calibration curve. For QC samples, the obtained mean accuracy and precision should be within 15% except the LLQC samples that should be within 20%.

3. Results and discussion

3.1 Method development

3.1.1 Mass spectrometric detection and chromatography

The individual parameters for the mass spectrometric detection were optimized by infusion of the analytes and internal standards in 50/50 (*v/v*) methanol/water containing 0.1% formic acid. Detection was evaluated in the positive electrospray ionization (ESI^+) mode alone due to the different

basic functional groups present in all compounds. The MS parameters such as cone voltage and collision energy were optimized in order to achieve the highest MS response for each analyte. The most abundant adduct ion for all of them was $[M+Na]^+$, and their major product ions come from the rupture of an ester bond (depicted in Fig. 1). The optimization parameters and selected MRM transitions are listed in Table 1.

Considering the structural diversity of the compounds, gradient elution was applied, using methanol containing 0.2% formic acid as the organic eluent. Different gradient conditions were evaluated to achieve a baseline separation of PTX and its stereoisomer 7-epi-PTX as well as to maximize chromatographic resolution of other compounds whilst minimizing overall run time. After optimization of gradient elution parameters, we achieved full chromatographic separation for all target analytes in only 2.5 min. This 2.5 min includes the equilibration time required for the next run. A chromatogram of a calibrator sample is presented in Fig. 2. As shown in the chromatogram, PTX is eluted earlier than 7-epi-PTX, demonstrating 7-epi-PTX is less polar than PTX. This is possibly due to intramolecular hydrogen bonding between the 7α-hydroxyl group and the carbonyl oxygen of the 4α-acetoxy group[35].

3.1.2 Extraction procedure

Different challenges should be overcome when developing a sample extraction protocol for DBS samples.

Firstly, as stated in the European Bioanalysis Forum (EBF) recommendation on DBS, “hematocrit is currently identified as the single most important parameter influencing the spread of blood on DBS cards, which could impact the validity of the results generated by DBS methods, affecting the spot formation, spot size, drying time, homogeneity and, ultimately, the robustness and reproducibility of the assays”[32]. In order to remove the variability resulting from the spread of and non-homogeneity in the blood, we have chosen to analyze the entire blood spot in our study. This approach would produce more consistent DBS concentrations regardless of HCT levels. Additionally, the correlation between DBS and plasma concentrations is to be established, and is even mandatory for the DBS assay to be implemented clinically[20]. Usually bridging studies, where both plasma and blood (DBS) are collected from each time point, need to be conducted to evaluate the relationship or agreement of the DBS and plasma concentrations[36]. In our situation working with mice, collecting both the plasma and DBS samples at each time point is extremely challenging due to the very limited amount of blood that can be taken. In this case, it is more advantageous to predict the plasma concentrations

from DBS data using ex vivo blood to plasma ratio data from an experimental aspect[36]. Therefore, the correlation between the DBS and plasma concentrations was not investigated in our study.

Secondly, the ideal option for introducing an IS in DBS method development is to add IS into the blood before spotting it onto the sample card. The benefit of this option is that the IS can interact with the liquid blood matrix and the analytes[37]. However, it is very cumbersome to implement this in our rodent PK/TK study due to the limited amount of blood that can be sampled during venipuncture. Therefore, like in most DBS assays, in our assay the IS were introduced via an extraction solvent. A potential drawback of this choice is that the IS may not be fully incorporated into the matrix components and sample card, thus unable to compensate for fluctuations in extraction from the DBS samples[33]. It is, therefore, important to evaluate the efficiency of the extraction process during method development. Under 37°C with a rotate speed of 500 rpm for 20min, the extraction efficiencies of methanol and acetonitrile were evaluated for the DBS samples. As shown in Table 3, extremely low recoveries (3.0-5.2%) were found when using acetonitrile (low concentration even resulted in no detectable signal). The recoveries were approximately 13-23 fold lower in comparison to methanol. This is most likely due to the insolubility of the dried blood matrix in pure acetonitrile[37], resulting in poor extraction of the compounds and low elution efficiency. Based on these results, methanol was selected as the extraction solvent for the further validation.

Thirdly, compared with analysis of liquid samples, the sensitivity of the DBS method is expected to be lower because of the small spotting volume (i.e. 10-20 µL). An evaporation and reconstitution procedure is commonly implemented to counteract the loss of sensitivity. In contrast, a simple extraction procedure with a further dilution with water to match the initial phase of the gradient elution was applied in our study. This procedure resulted in a simple and time-saving sample extraction process. The loss of sensitivity is partly compensated through the use of an advanced mass spectrometer (i.e. Waters TQS system).

3.1.3 Microflow-UHPLC as a means to increase sensitivity

In an attempt to increase the sensitivity of the assay, we evaluated a novel micro-fluidics platform, i.e. the Waters ionKey/MS system. Theoretically, compared to analytical scale LC (e.g. 2.1 mm I.D. chromatography), micro LC benefits from signal enhancements for small molecule analysis through enhanced ionization efficiency and reduced matrix interference[38]. To test this hypothesis, we evaluated the sensitivity between the UHPLC-MS/MS and ionKey/MS system by injecting 1 ng/ml of PTX on both systems. As shown in supplementary Fig. A.1, for the same injection volume, a similar S/N ratio was obtained on both systems. Therefore, a trap-and-elute strategy was explored. This set-

up can handle relatively large injection volumes (e.g. 20 µL) and facilitate the loading of the samples containing a relatively high proportion of organic solvent compared to direct injection[39].

The configuration of the optimized dual pump trapping and subsequent sample elution is presented in Fig.A.2. The schedule of events in the optimized dual-pump trapping workflow is shown in supplementary Table A.1. The representative chromatograms of PTX and its metabolites, eluted under above conditions are shown in Fig. A.3. In order to compare the sensitivity difference between the dual pump trapping strategy and the analytical scale LC (i.e. 2.1 mm I.D. chromatography), the signal to noise of PTX obtained from these two approaches was normalized with the amount of PTX on column, giving a ratio of about 0.8 (dual pump trapping/analytical scale LC). This is in line the finding with the direct injection mode.

The main hurdle we encountered when trying to improve the sensitivity for PTX and its metabolites using the dual pump trap-and-elute strategy was the carry-over. Initially, a very high carry-over (~ 5.0 %) was found for a 20 µL full loop injection of PTX with only an iKey flushing procedure. By adding the trap column washing procedure, the carry over reduced to ~0.6 %, indicating contamination in the trap column or sample loop. In order to identify the sources of carry over, the trap column was disconnected and a tubing (I.D. 40 µm × length 6") was connected instead. The no injection (i.e. run gradient without injection) and blank injection were performed after an injection of PTX with only an iKey flushing procedure. Blank signals were found for both no injection and blank injection, demonstrating that the carry over originates from the trap column. Various attempts for cleaning the trap column and also the injection path were further investigated, including using acetonitrile (stronger organic) and ethanol (higher solubility of PTX) as the wash solvents for the trap column, increasing the flushing flow rate (e.g. 50 µL/min) and/or flushing time (e.g. 7 min) of the wash solvents, adding 0.1% plasma in the sample, running multiple rinses through the trap column by cycling between auxiliary pump A (Aux A, 20% methanol) and B (Aux B, 100% methanol) multiple times. However, none of these solutions helped to reduce the carry over. A scale down to 5 µL full loop injection further reduced the carry over to ~0.12%, albeit at the expense of sensitivity. Reducing the operational a range of PTX in DBS samples to 1 - 100 ng/ml, the carry-over of the 100 ng/ml PTX DBS sample injections became less than 20% of the LLOQ, using the optimized trapping workflow shown in Table A.1.

The sensitivity we were able to achieve with the ionKey/MS system configured as such was 1 ng/ml in the DBS sample. This is comparable with that of the UHPLC-MS/MS method using a 10 µL injection. Considering the rapid analytical run time, wide calibration range, and lack of carry over that

UHPLC-MS/MS provides for PTX, we choose to abandon µUHPLC-MS/MS in favor to UHPLC-MS/MS for the further method optimization.

3.2 Validation

3.2.1 Selectivity, carry over, calibration model, and LLOQ

None of the blank samples gave any interference at the retention times of PTX and its metabolites. No detectable carry-over for the analytes was found after injection of the highest calibrator sample. The optimal calibration curves were fitted using a quadratic function for describing the relationship between responses and concentrations for all compounds. Due to the wide calibration concentration range (i.e. 1 to 1000 ng/ml), a log-transformation was required to correct for heteroscedasticity. The limit of quantification for the DBS sample was 1 ng/ml for all compounds. A typical chromatogram of a LLOQ calibrator sample is presented in Fig. 2.

3.2.2 Bias, imprecision, and cross-validation

Bias and imprecision results from human and mice DBS QC samples are presented in Table 2. In human DBS samples, within- and between-run bias were 99.0-111.9 % and 98.4-111.0 % of nominal concentrations, and within- and between-run imprecision were 5.6-14.6 % CV and 8.8-13.6 % CV.

For the cross validation of mice DBS sample, the within-run bias and imprecision were 94.3-118.3 % of target concentration and 1.8-16.0% CV. For 6*α*-OH-PTX and 6*α*,3'-*p*-di-OH-PTX, the measured bias at the LLOQ level are respectively 118.3% and 117.7%, which fell within the regulated bioanalytical guidelines. For 3'-*p*-OH-PTX at the high QC level, the imprecision was considered acceptable albeit it slightly deviated (16.0% CV) from the pre-set limit of 15%. The outcome of the cross validation demonstrated the feasibility of using human DBS calibrators to quantify mice DBS samples. This removes the need for fresh mouse blood for the production of the calibrator samples.

3.2.3 Extraction recoveries and matrix effects

Extraction recoveries and IS-normalized matrix effects in DBS samples are summarized in Table 3. The extraction recoveries for all compounds processed using methanol were 60.5-87.6%, demonstrating a satisfactory extraction efficiency. The IS-normalized matrix factors for the compounds except 6*α*,3'-*p*-dihydroxy-paclitaxel were within 85%-115% with a low variation indicating that the IS compensate well for the matrix effects. Moderate signal suppression (67.5-79.1%) was observed for the metabolite 6*α*,3'-*p*-dihydroxy-paclitaxel. This could be related to its chemical structure. 6*α*,3'-*p*-dihydroxy-paclitaxel has an extra hydroxyl group with respect to the IS 6*α*-hydroxy-paclitaxel-d5. This makes 6*α*,3'-*p*-dihydroxy-paclitaxel more polar, thus early eluting, and therefore probably more

prone to ion suppression in contrast to the IS. Although this limited matrix effect was observed during validation, we considered this acceptable given the good reproducibility of the observed absolute matrix effect and in view of the scope of the assay.

3.2.4 Stability

The mean of the calculated concentrations of all compounds were within -12.1% to 7.8% of target when fortified at low and high QC concentrations and kept for 20 days at room temperature, or 24 h post-extraction in the autosampler at 10 °C.

3.2.5 Dilution integrity

Evaluation of dilution integrity during method validation can ascertain the method is applicable to the samples with analyte concentrations higher than the upper limit of quantification. The principles of dilution used for traditional liquid samples also apply for DBS. However, a DBS sample cannot be simply diluted as is typically done for bio-fluids, due to the dried matrix. The common approach is to dilute the processed high concentration samples with the extracts of blank DBS samples. It works well for a few samples with a relatively small dilution factor, but can be costly or impractical when the samples require large dilution factors[37]. This implies a lot of blank whole blood for each run, while the blank matrix (especially the blood) from mice is not freely available and very expensive to obtain.

In our study, two alternative blank matrices, a 50/50 (*v/v*) water/extract of PKI bioanalysis cards and a 50/50 (*v/v*) water/methanol mixture, were compared to the above dilution procedure using a 50/50 (*v/v*) water/extract of blank DBS samples, and subsequently validated. As graphically shown in Fig. 3, the dilution integrity with these three matrices was acceptable with the mean bias within ±15% of expected diluted concentrations. There was no significant difference in accuracy and precision obtained from the two alternative procedures compared with the standard procedure. The procedure with a 50/50 (*v/v*) water/methanol mixture can easily be configured to DBS sample analysis without processing additional (blank DBS) cards in each analytical run. It was thus selected as the blank solvent for the dilution.

3.3 Application of the method

As a proof-of-concept, the method was applied to DBS samples from a mice PK study. In this study, a 50 mg/kg Abraxane® dose was administered intraperitoneally. To obtain the DBS samples, after venipuncture (taking approximately 12 - 15 µL of blood), 10 µL of blood was accurately transferred to a DBS card. The concentration time curves for PTX and its metabolites from ten SCID/Beige mice are

shown in Fig. 4. 6*α*-OH-PTX is shown to be the major metabolite of PTX in mice. 3'-*p*-OH-PTX is a secondarily abundant metabolite, which is measurable from 1 to 4 hours post dosing. 6*α*,3'-*p*-di-OH-PTX was detected in some samples but was below the LLOQ and is therefore not shown. 7-epi-PTX is abundant in vivo and it is detectable up to 8 hours after dosing. To our knowledge, it is the first time that the PK profiles of the metabolites and 7-epi-PTX in mice are presented. Further interpretation of the pharmacokinetic results, however, is beyond the scope of this publication.

It is worth noting that two partially overlapping peaks were eluted at 1.54 and 1.57 min, respectively (shown as an insert in Fig.1) in the mice sample. These chromatographic peaks were observed in the MRM-transition optimized for 6*α*-OH-PTX. The peak at 1.57 min is thought to represent 6*α*-OH-PTX due to the same retention time with that of the calibrators, whereas the peak at 1.54 min is assumed to be the stereoisomer of 6*α*-OH-PTX at the C-6 chiral center in the taxane ring. This phenomenon is possible because there are two potential directions for a hydroxyl group to be added onto the C-6 center during metabolism. This previously unreported stereoisomer of 6*α*-OH-PTX was observed in our study most likely due to the improved resolution of the UHPLC system. Calculated 6*α*-OH-PTX concentrations were therefore taken as the sum of these two species, thereby assuming an identical MS/MS response factor.

4. Conclusion

The proposed method of analysis provides a rapid and sensitive LC-MS/MS assay for the simultaneous quantification of PTX, its three metabolites and the stereoisomer 7-epi-PTX in DBS samples over the concentration range of 1-1000 ng/ml. The validated method with a low sample volume needed was demonstrated to be selective, accurate and precise. The simple extraction procedure and short LC-MS/MS run time imply that a large amount of samples can be analyzed in a short time frame. The feasibility of using human DBS calibrators to quantify mice DBS samples was shown. This facilitates the analysis of mice samples as the source of blank mice blood are limited in comparison to human blood. The utility of this assay was demonstrated through its successful application for the analysis of DBS samples from the pharmacokinetic studies in mice.

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Table 1 Detection parameters for the compounds under study.

Compound	Cone voltage (V)	Collision energy (CE)	MRM-transitions	Dwell (secs)
PTX	36	28	876.3 > 308.1	0.017
$^{13}C_6$-PTX	74	28	882.3 > 314.2	0.017
7-epi-PTX	10	30	876.6 > 308.2	0.017
3'-*p*-OH-PTX	60	30	892.5 > 324.1	0.030
6*α*-OH-PTX	36	30	892.5 > 308.1	0.017
6*α*-OH-PTX-d5	28	32	897.5 > 313.2	0.017
6*α*,3'-*p*-di-OH-PTX	18	30	908.5 > 324.1	0.047

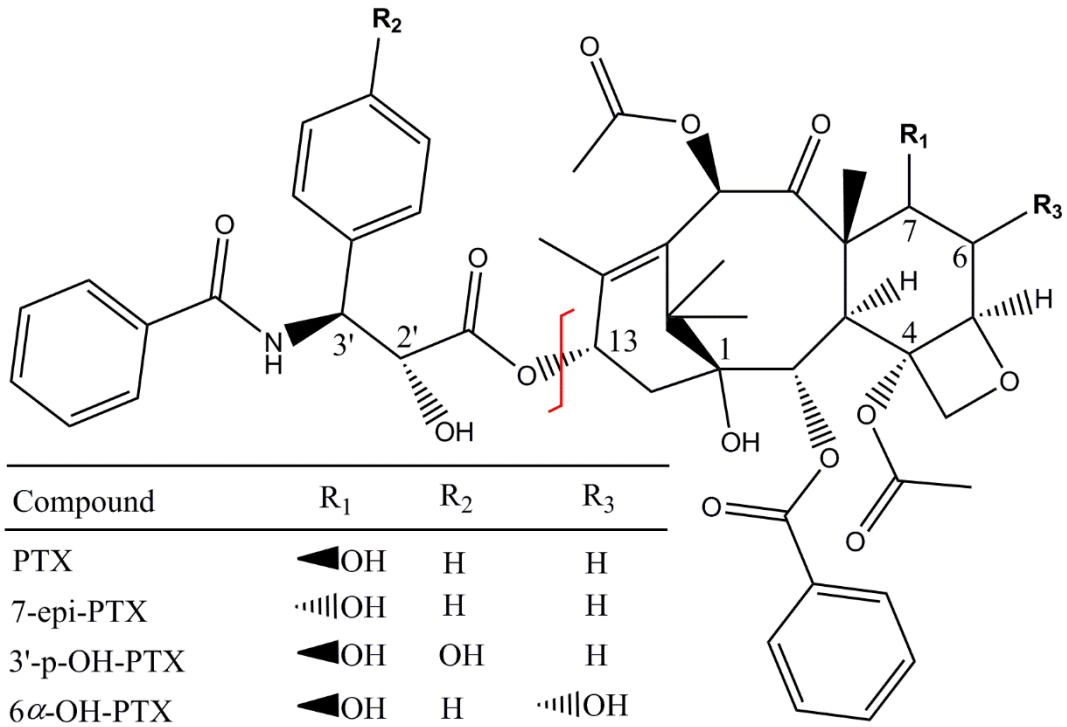
Table 2 Bias and imprecision data for human and mice DBS QC samples of the method.

Compound	Nominal concentration (ng/ml)	Human DBS sample				Mice DBS sample (cross-validation)	
		Within-run (n=6)		Between-run (n=18)		Within-run (n=6)	
		Bias (%)	Imprecision (RSD%)	Bias (%)	Imprecision (RSD%)	Bias (%)	Imprecision (RSD%)
PTX	1-2.5-25-1000	109.2-103.1-107.8-101.0	5.9-12.4-12.8-13.6	108.4-101.1-107.0-99.6	11.3-10.9-13.0-12.4	101.5-95.6-103.0-100.7	12.5-6.7-1.8-13.9
7-epi-PTX	1-2.5-25-1000	103.7-99.7-101.2-101.5	11.2-11.8-11.2-14.6	105.1-99.3-104.9-99.0	12.9-9.8-12.2-13.6	109.3-94.3-96.1-99.5	8.1-3.6-3.5-15.0
3'-*p*-OH-PTX	1-2.5-25-1000	103.6-101.9-109.4-99.3	5.6-11.4-13.2-13.6	105.8-99.3-108.5-99.3	8.8-8.9-10.6-11.2	112.9-103.8-102.8-106.1	8.2-6.3-11.7-16.0
6*α*-OH-PTX	1-2.5-25-1000	103.4-101.1-105.9-99.7	9.3-11.1-11.7-14.6	105.5-99.6-106.6-98.8	11.7-9.9-11.1-12.5	118.3-105.3-102.7-109.0	4.6-4.7-3.9-15.0
6*α*,3'-*p*-di-OH-PTX	1-2.5-25-1000	104.8-110.1-111.9-99.0	6.8-14.6-12.6-12.5	104.2-101.1-111.0-98.4	10.0-12.1-12.1-11.6	117.7-112.7-100.3-101.8	11.6-8.0-5.8-13.3

Table 3 Extraction recoveries and IS-normalized matrix effects of the method.

Compound	Matrix effect (%, n=6)		Extraction (via methanol) recovery (n=6)		Extraction (via acetonitrile) recovery (n=6)
	Low (RSD%)	High (RSD%)	Low (RSD%)	High (RSD%)	High (RSD%)
PTX	99.3 (1.3)	100.5 (0.9)	87.6 (8.4)	70.5 (10.1)	4.6 (3.0)
7-epi-PTX	104.4 (3.4)	97.1 (5.5)	85.4 (8.2)	70.5 (13.8)	5.2 (2.3)
3'-*p*-OH-PTX	85.7 (2.5)	91.7 (4.6)	70.6 (6.6)	69.0 (6.3)	3.0 (3.2)
6*α*-OH-PTX	101.5 (1.5)	101.0 (2.4)	71.0 (5.4)	64.5 (9.1)	3.7 (3.7)
6*α*,3'-*p*-di-OH-PTX	67.5 (6.1)	79.1 (2.5)	64.7 (4.2)	60.5 (7.7)	3.0 (3.5)

Figure captions



Compound	R_1	R_2	R_3
PTX	◀OH	H	H
7-epi-PTX	····OH	H	H
3'-p-OH-PTX	◀OH	OH	H
6α-OH-PTX	◀OH	H	····OH
6α,3'-p-di-OH-PTX	◀OH	OH	····OH

Fig. 1 Chemical structures of paclitaxel, 7-epi-paclitaxel, 3'-*p*-hydroxypaclitaxel, 6α-hydroxy paclitaxel, and 6α,3'-*p*-dihydroxypaclitaxel, and their fragmentation pattern.

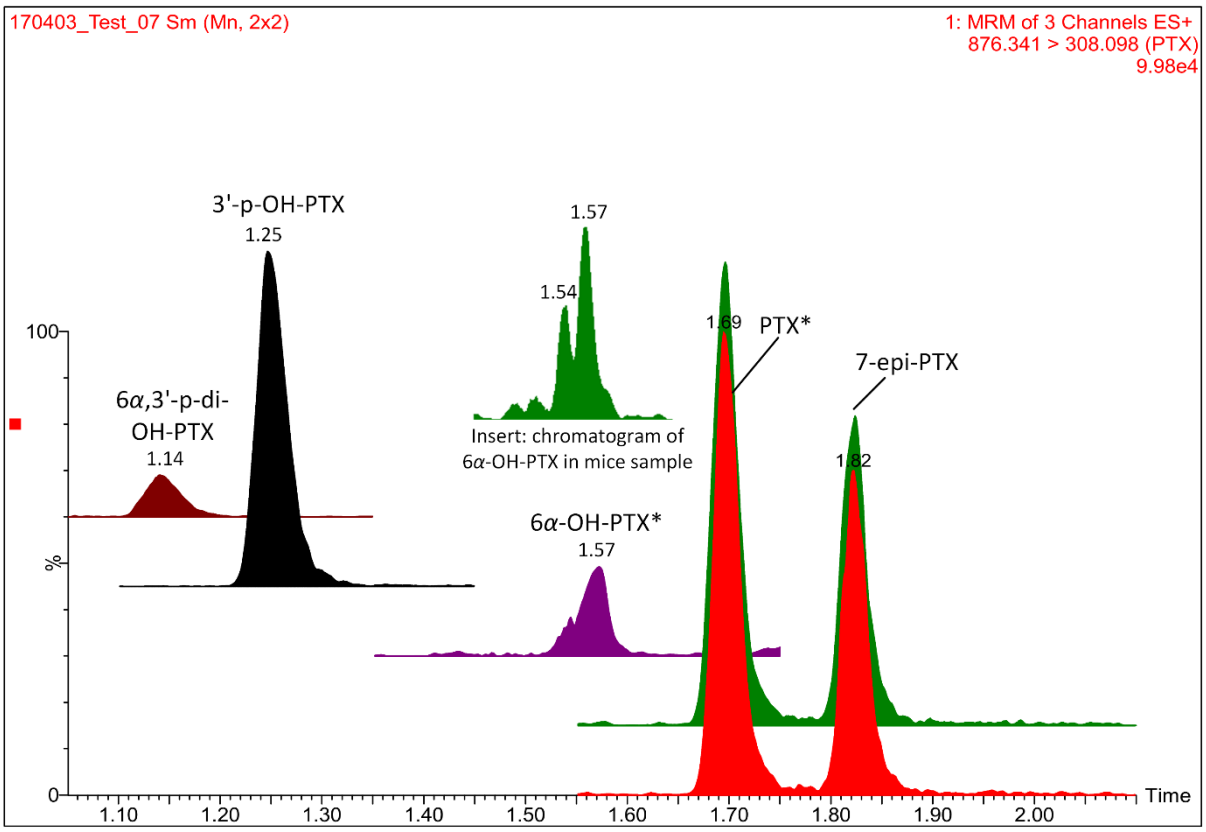


Fig. 2 Chromatogram of a LLOQ calibrator sample. An asterisk denotes compounds for which a stable isotope labelled internal standard was included. A chromatogram of 6α-OH-PTX in a real mouse DBS sample post intraperitoneal administration of 50 mg/kg PTX was also inserted.

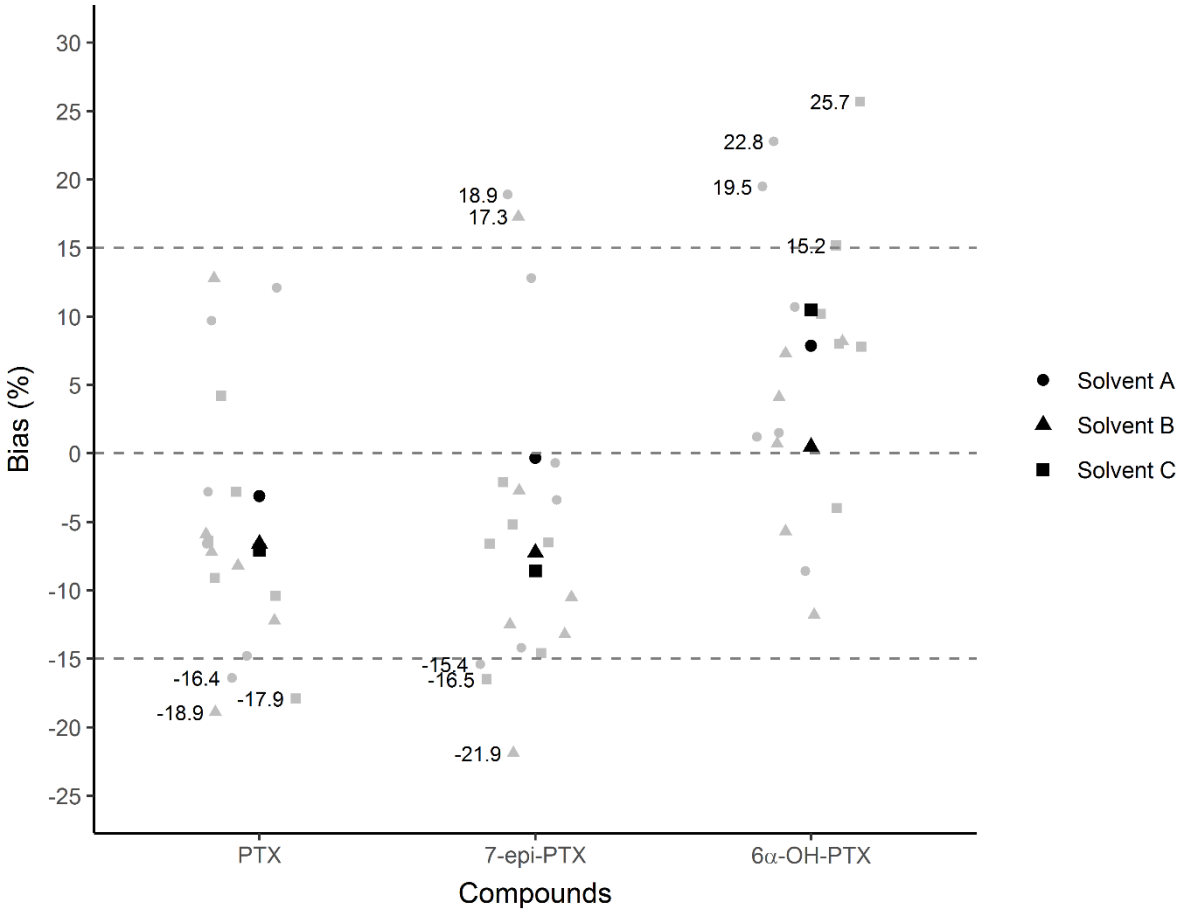


Fig. 3 Dilution integrity (10-fold) of PTX, 7-epi-PTX, and 6α-OH-PTX by processing six human DBS samples at 10-fold above the HQC sample. Three blank matrices were evaluated for the dilution: Solvent A (●): 50/50 (*v/v*) water/methanol; Solvent B (▲): the 50/50 (*v/v*) water/extract of PKI bioanalysis cards; Solvent C (■): the 50/50 (*v/v*) water/extract of blank DBS samples. The black signs stand for the mean bias from six replicates of the DBS samples diluted with different blank matrices. The individual points (grey signs) beyond ±15% were labeled with the found bias.

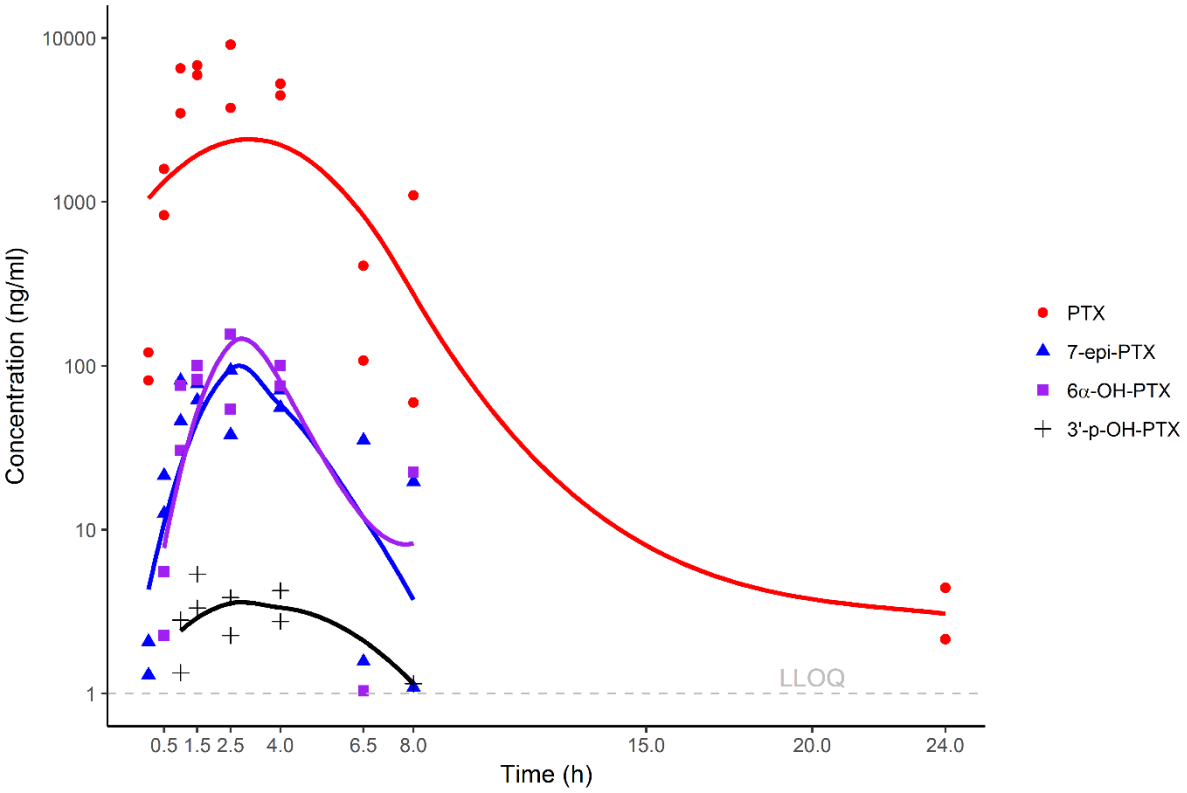


Fig. 4 The concentration-time curves of PTX and its metabolites in mice post intraperitoneal administration of a 50 mg/kg dose of PTX. A Loess smoothing line was added to the plot for each analyte.