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Enzymatic tumour tissue digestion coupled to SPE–UPLC–Tandem Mass Spectrometry as a tool to explore paclitaxel tumour penetration

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ABSTRACT

Paclitaxel is a good compound for regional (intraperitoneal) chemotherapy of peritoneal carcinomatosis. During IPEC, a cytotoxic solution is circulated in the peritoneal cavity, thereby promoting close contact between the cytotoxic agent and the exposed (residual) tumour tissue. To further explore the role of PTX in this type of treatment and study the impact of treatment modalities on tumour tissue penetration, in-vivo animal experiments were set-up.

In literature, PTX tumour uptake is frequently studied using autoradiography and/or fluorescence microscopy techniques. Owing to their semi-quantitative nature on one hand and the difficulty of incorporating imaging data within a pharmacokinetic–pharmacodynamic modelling framework on the other hand, we set out to develop a validated assay for the quantification of PTX in tumour tissue samples. Furthermore, in order to maximise spatial resolution, care was taken to minimise the sample weight necessary for the analysis.

Based on an enzymatic tumour tissue digestion protocol, an easy, less labour-intensive, when compared to mechanical tissue disruption techniques, method was developed. Through validation experiments we showed that our method reliably quantifies PTX in a working range of 30–8000 ng/g tumour tissue. Finally, using samples from the in-vivo experiments we demonstrated the suitability of the developed method.

1. Introduction

Paclitaxel (PTX), which binds and stabilizes the microtubule, causing cell death, has been used as an anticancer agent since 1984. Paclitaxel shows anticancer activity for a variety of human cancers, including breast, ovarian and prostate. Its favourable pharmacokinetic properties, e.g. slow peritoneal clearance, make it a good candidate compound for intraperitoneal perioperative chemotherapy (IPEC) [1] of peritoneal carcinomatosis (PC). During IPEC, a solution containing a cytotoxic agent, e.g. PTX, is instilled into the peritoneal cavity, thereby promoting close contact between the cytotoxic agent and the exposed (residual) tumour tissue.

Although IPEC as a tool for regional drug delivery has a strong pharmacokinetic rationale in the treatment of PC, controversy remains regarding the optimal treatment strategies that will optimise efficacy whilst minimising systemic exposure. Besides the ongoing debate on the duration of the perfusion or the optimal carrier solution, questions regarding the extent of tumour tissue penetration following IPEC treatment remain [2].

PTX penetration has been studied in-vitro as well as in-vivo, using, respectively, cell monolayers [3] or multicellular layer cultures [4,5] and human tumour xenografts [6–8]. Autoradiography [3,7,8], using ^{3}H-PTX, or fluorescence microscopy [5,6], using fluorescently labelled PTX, are used most often. Using these techniques, rather than an accurate determination of the PTX concentration in tumour tissue, a relative scale is used to describe penetration as a function of time and/or space. Because of the semi-quantitative nature of these imaging techniques, they are sometimes combined with a conventional whole tumour homogenisation and HPLC analysis [8,9] to be able to report accurate PTX concentrations in tissue. However, even in these instances questions remain with respect to the performance characteristics of the assay (imprecision/bias).

Besides the questions regarding the treatment modalities and the effective penetration of PTX in solid tumours, one of the reasons for its limited use is the toxicity (local and systemic) associated with IPEC administration of Taxol®. Although necessary for paclitaxel solubility, Cremophor EL, the main constituent of Taxol®, has been shown to cause severe adverse effects after IPEC

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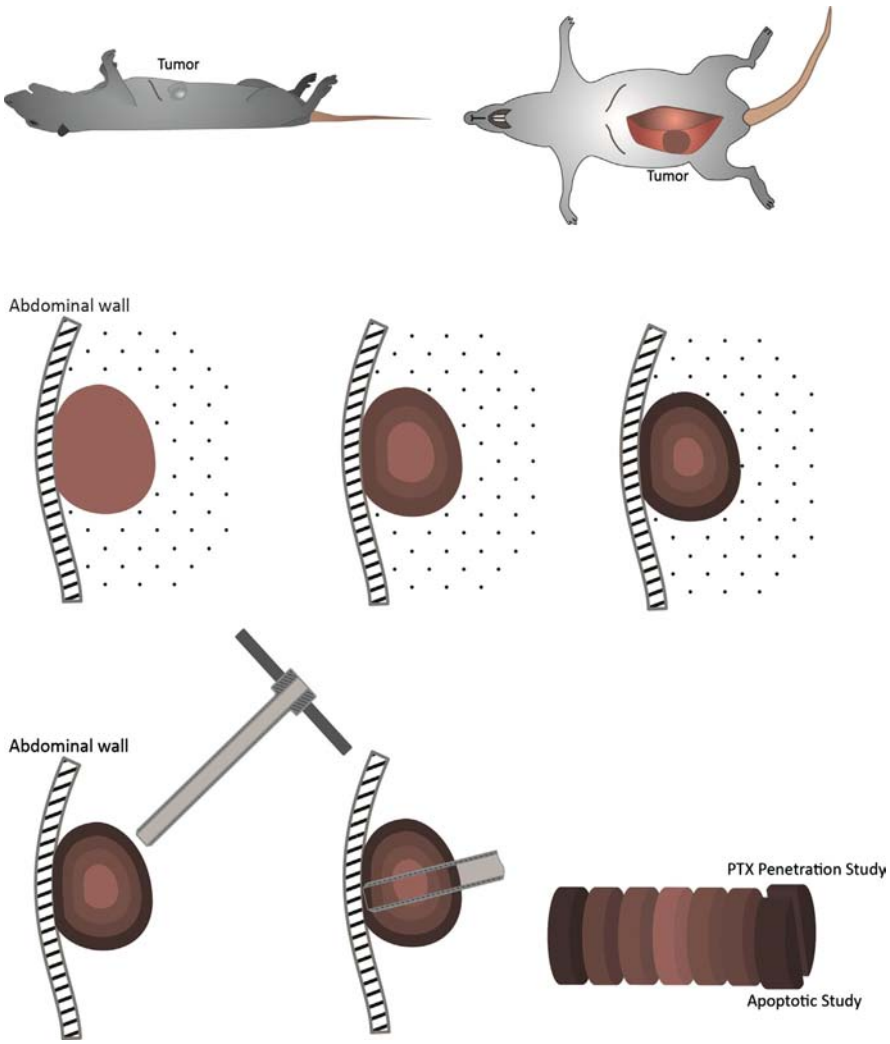

Fig. 1. Tumour harvesting protocol.

administration [10]. As such, despite its wide activity spectrum and its favourable pharmacokinetic properties, the use of PTX as a compound for IPEC remains limited.

Therefore, in support of the development of a new Cremophor EL-free paclitaxel formulation, a bioanalytical method to quantify paclitaxel in human tumour xenograft tissue was developed. Furthermore, in order to study the effect of carrier solution and treatment modalities on PTX tumour penetration, using the tumour harvesting protocol shown in Fig. 1, our sample pretreatment was optimised to handle low weight tumour tissue samples.

2. Material and methods

2.1. Reagents

Trizma®-base, Sodium dodecyl sulphate (SDS), Proteinase K (from *Tritirachium album*, 500 units/mL, buffered aqueous glycerol solution) and lipase (from porcine pancreas powder) were all from Sigma-Aldrich (Bornem, Belgium). EDTA, potassium dihydrogen phosphate and di-potassium hydrogen phosphate were from VWR® BDH Prolabo® (Leuven, Belgium). $^{13}C_6$-Paclitaxel (IS) and PTX were from Acros Organics (Geel, Belgium) and Chromadex (California, USA), respectively. ULC-grade water and methanol were from Biosolve (Valkenswaard, The Netherlands).

2.2. Buffers and enzyme solutions

A phosphate buffer (70 mM) in H_2O was made by accurately weighing and dissolving 2.0 g K_2HPO_4 and 0.8 g KH_2PO_4 in 250.0 mL of H_2O. Prior to dilution to 250.0 mL, the pH of the buffer was adjusted to pH 7.50 by titration with 1 M NaOH. Furthermore, a 50 mM Tris–HCl buffer was made by dissolving 0.607 g Trizma®-base into a final volume of 100.0 mL of H_2O after adjustment of the pH to 7.50 with a 1 M HCl solution.

The final proteinase K solution was made by diluting the buffered aqueous glycerol solution (500 units/mL) 100 fold in Tris–HCl buffer to a final concentration of 5 units/mL. Lipase solution (100 mg/L) was made by dissolving appropriate amounts of lipase in phosphate buffer.

2.3. Stock solutions, standards and quality control samples

A stock solution in acetonitrile was prepared for PTX at a concentration of 1.0 mg/mL. This stock solution was then further diluted with acetonitrile to obtain working solutions with PTX concentrations ranging from 0.016 μg/mL to 4.0 μg/mL. A separate stock solution as well as separate working solutions (PTX concentrations 0.04, 0.8 and 3.2 μg/mL, respectively) were prepared for the production of the quality control samples. The IS was dissolved in 1 mL acetonitrile and diluted to a concentration of 0.80 μg/mL using acetonitrile.

Blank bovine muscle tissue was spiked with the different working solutions to obtain calibrator/quality control samples. Full details of the preparation of the calibrators and quality control samples are given under Section 2.4.

2.4. Enzymatic tissue digestion protocol

Tumour samples and bovine muscle tissue were allowed to thaw at room temperature. Afterwards, excised tumour samples were rinsed using a physiological salt solution (9 g/L NaCl in H_2O) to remove most blood and to rinse off any left-over Taxol® that might have adsorbed to tumour's outer surface. After further sectioning and exactly weighing of the tumour samples to produce tissue specimens weighing 25 mg, tissue specimens were placed in an Eppendorf® vial.

To this Eppendorf® vial 500 μL lipase-solution, 250 μL proteinase-K solution, 30 μL SDS-solution (5 mg/100 μL in H_2O), 85 μL $CaCl_2$-solution (30 mM in H_2O), 50 μL EDTA-solution (100 mM in H_2O) and 85 μL of Tris–HCl buffer were added.

For the calibrator samples and the quality control samples, 50 μL of the corresponding working solution in acetonitrile was added to the Eppendorf® vial. Concurrently, for the tumour tissue samples, 50 μL of blank acetonitrile was added. Final concentrations of the calibrator samples ranged from 32 to 8000 ng/g. Quality control samples were prepared at PTX concentrations of 80, 1600 and 6400 ng/g, respectively.

Afterwards, the Eppendorf® vials were left to incubate overnight at 55 °C whilst continuously being shaken (IKA®-Werke GmbH & Co. KG, Staufen, Germany). After at least 16 h of incubation time, the Eppendorf® vials were vortex mixed and centrifuged for 10 min. at 3000 × *g* at 4 °C. Following centrifugation, samples were stored at 4 °C until further handling by solid phase extraction.

2.5. Solid phase extraction procedure

After centrifugation 50 μL of IS solutions was added to 950 μL of the supernatant. This mixture was then loaded onto the SPE cartridges (Oasis® HLC cartridges, Waters, USA) prior to washing of the SPE tubes with a sodium hydroxide (pH 10.0) solution and a 70% (v/v) MeOH-solution in H_2O. Following elution of PTX with MeOH, samples were concentrated by evaporation of the methanol under a mild nitrogen stream (30 min, 35 °C).

Finally, samples were reconstituted in 100 μL 50% (v/v) methanol in H_2O, transferred to the autosampler compartment and kept at 5 °C until injection onto the chromatographic system.

In case of samples above the ULOQ, re-analysis was performed using the supernatant. To this end, the supernatant was diluted with a physiological saline solution (20 and/or 400 fold) prior to spiking of the IS solution. Afterwards, this mixture was again subjected to SPE prior to injection onto the chromatographic system.

2.6. Instrumentation

Chromatographic separation was achieved on an Acquity UPLC system (Waters, Milford, MA) equipped with an Acquity BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm particle size) and an Acquity BEH C_{18} guard-column (5 mm × 2.1 mm, 1.7 μm particle size) all from Waters (Milford, MA). The column was kept at 50 °C. An aliquot of 1 μl was injected into the mobile phase stream using full loop injection. The mobile phases consisted of H_2O (MPA) and methanol (MPB). Components were eluted using gradient elution at a flow rate of 0.450 ml/min. From 0 to 0.5 min the mobile phase contained 55% of MPA. From 0.5 to 1.5 min the amount of MPB in the mobile phase was increased linearly to 100%. Between 1.5 and 2.5 min, the system was maintained at 100% MPB. Finally, at 2.5 min, the analytical column was re-equilibrated to initial gradient settings. Eluting compounds were detected using a Waters Quattro Ultima triple quadrupole system (Micromass Waters, Manchester, UK) equipped with an electrospray source (orthogonal Z-spray®) operated in positive ionisation (ESI+) mode. The MS/MS instrument was operated with a capillary voltage of 3.0 kV, source block temperature of 150 °C and cone voltage of 20 V. Nitrogen was used as desolvation gas and was heated to 400 °C and delivered to the source at 750 L/h. The system uses argon gas to induce fragmentation in the collision cell at a collision energy of 20 eV. The dwell time for each MRM transition was 100 ms and the interchannel and interscan delays were set to 20 ms. Details of the MRM-transitions for each compound are described in Table 1. Peak areas were integrated using MassLynx 4.1 software (Micromass Waters, Manchester, UK).

Table 1
Detection parameters for the compounds under study. For the different compounds, a quantifier and qualifier trace were monitored. Qualifier traces were used to calculate ion ratios. These ion-ratios were monitored during the analysis to confirm the identity of the measured compound.

Compound	Detection	MRM-transitions		CE (eV)
	Ionisation mode	Quantifier	Qualifier	
PTX	ESI+	876.5 > 307.5	876.5 > 531.0	20
$^{13}C_6$-PTX	ESI+	882.5 > 313.7	882.5 > 531.0	20

2.7. Validation

The validation experiments were based on the "Guidance for Industry-Bioanalytical Method Validation" recommended by the Food and Drug Administration (FDA) of the United States. Batches of quality control samples at three different concentration levels were prepared and analysed on separate occasions.

2.7.1. Accuracy and imprecision

Accuracy was evaluated by analysing results from QC samples measured under repeatability conditions on different days (QC samples were analysed in sextuplicate, triplicate or duplicate on 5 different days). It was calculated as the mean relative error of QC samples analysed on five different days. Within-run imprecision was calculated as the average relative standard deviation (RSD) of the concentration of the QC samples. Between-run imprecision was evaluated by replicate measurements of QC samples on five different days. Between-run imprecision was calculated as the RSD of the average QC concentrations measured on five different days.

2.7.2. Selectivity

Selectivity was assessed by examining peak interference (signal-to-noise ratio < 9) from four independent sources of blank bovine muscle tissue.

2.7.3. Stability

The stability of PTX in tissue, stored at −80 °C was assessed using bovine muscle controls. On day 0, blank bovine muscle tissue was homogenised and PTX working solutions were spiked to achieve PTX concentrations at the respective concentrations of the QC samples. Using fresh calibrator samples PTX concentrations in bovine muscle tissue were quantified on day 0 and after 1 month of storage at −80 °C. The measured concentrations (measured in sextuplicate on three different concentration levels) were then used in a two-way ANOVA analysis in R®(R foundation for statistical computing, Vienna, Austria) to test whether significant degradation had occurred during this 1 month period.

2.7.4. Robustness evaluation

To further explore the performance characteristics of the developed method with tumour tissue samples rather than bovine muscle tissue quality control samples, tumour tissue samples for which a duplicate sample was available ($n=20$) were analysed 1 month after the original analysis.

3. Results and discussion

3.1. Method development

3.1.1. Mechanical tissue homogenisation

Based on the work by Eckert et al. [11] we explored, as an initial sample preparation method, mechanical tissue homogenisation using an Ultra-Turrax® (IKA®-Werke GmbH & Co. KG, Staufen, Germany) homogeniser in combination with a Potter–Elvehjem system (VWR International, Leuven, Belgium). The combination of both techniques, rather than only using an Ultra-Turrax®, is believed to produce a more complete cell disruption, making PTX bound to cell organelles freely available for extraction using SPE. Before homogenisation a 6% phosphoric acid solution was added in a 1:1 (v/v) ratio to disrupt PTX protein binding. After centrifugation at $10{,}000 \times g$ for 20 min the supernatant was subjected to SPE.

Based on the method described by Matuszewski et al. [12], we assessed the performance of the sample preparation technique, in terms of recovery and matrix effect, using bovine muscle quality control samples at three different PTX concentration levels (0.2, 0.4 and 2.0 ng/mg). Measurements of pre-/post- and pure standards were made in triplicate. As shown in Table 2, the average recovery was low (69.1%). Furthermore, significant ion suppression was observed (average matrix effect 30.8%).

Based on the strong protein binding affinity of PTX [13] and in an attempt to improve recovery and reduce the ion suppression, we decided to introduce an additional protein precipitation step prior to SPE. To this end, the 6% phosphoric acid solution was replaced by a 1:1 (v/v) mixture of 0.06 N $Ba(OH)_2$ and 0.06 N $ZnSO_4$ (both Sigma-Aldrich, Bornem, Belgium). The average recovery and matrix effect of this improved sample preparation method were 96.8% and 160.3%, respectively. Although the average recovery was significantly better than the earlier method we were not satisfied with the results from this sample preparation method.

On one hand the average matrix effect being significantly larger than 100% (i.e. significant ion enhancement) and on the other hand the laborious procedure of mechanically processing the tissue with two separate techniques and the difficulties we faced, in terms of instrument maintenance/cleaning, when trying to mechanically homogenise low weight (± 25 mg tumour tissue) samples, in a high-throughput fashion, made us reconsider our approach. Although mechanical tissue homogenisation has proven to be reliable in e.g. the accurate quantification of brain PTX concentrations [9], in our application, given the vast heterogeneity in tumour tissue composition and tumour tissue density it was regarded impracticable. For example, several of the tumours we encountered contained a high degree of dense fibrous-tissue, making it impossible, using mechanical techniques, to homogenise the tumour to a satisfactory degree (i.e. appearing like a homogeneous suspension).

Therefore, based on a publication by Yu et al. [14], we explored enzymatic tissue digestion as an alternative sample preparation technique.

Table 2
Recovery and matrix effect for the different sample preparation protocols under study.

	Recovery		Matrix effect	
	Average (%)	SE (%)	Average (%)	SE (%)
Mechanical homog.	69.1*	12.3	30.8*	1.6
Mechanical + PP	96.8	5.8	160.3*	9.1
Enzymatic digestion	118.4	9.8	110.0	9.0

Measurements were made in triplicate on 3 different PTX concentration levels. The reported recoveries and matrix effects are the average over the replicates and different concentration levels.

* indicates an average recovery or matrix effect being statistically significantly different from 100% at the 5% level of significance.

3.1.2. Enzymatic tissue digestion

The enzymatic digestion described by Yu et al. [14] was based on two different enzymes (collagenase or proteinase K) that are known to degrade connective tissue. Apart from these two enzymes, other enzymes, e.g. subtilisin A [15], capable of digesting tissue samples have been described in literature. However, proteinase K, which is known to break up the cell nucleus (hence its widespread use in DNA extraction [16] protocols), in our opinion, since PTX resides, bound to the microtubules, inside cell's nucleus, would provide a more complete release of tissue bound PTX.

Furthermore, in order to get rid of fatty residues from the tumour tissue which could disturb the SPE clean-up, we found it feasible to add lipase to the enzymatic digestion protocol. In literature, lipase has been used as an add-on enzyme in digestive protocols for the analysis of trace elements [17] as well as small-molecules [18] from a.o. human tissue samples.

Based on these reports in literature, in a first step, we studied the effect of different concentrations of proteinase K (0.5, 1.0 and 5.0 units/mL) and lipase (100, 200 and 1000 mg/L) on the degree of digestion of tissue samples after an overnight incubation (approximately 16 h) at 55 °C.

After centrifugation of the Eppendorf® vials, containing the incubated tissue samples, to precipitate any leftover solid particles, no residual solid pellet was observed for the highest proteinase K concentration (5.0 units/mL). Furthermore, by weighing of the residual pellet, obtained at the lower proteinase K concentrations, we found that the different lipase concentrations did not affect the degree of tissue digestion. Furthermore, using post column infusion of a PTX solution whilst injecting SPE extracts of bovine muscle tissue treated according to the different protocols, a qualitative assessment was made of the matrix effect. These results (data not shown) revealed that the most stable baseline following injection of the tissue sample extracts was obtained from the protocol with the highest proteinase K solution. However, no effect was observed for the different lipase concentrations tested. Therefore, it was decided to include lipase in the lowest concentration tested (i.e. 100 mg/L).

In an attempt to compare this enzymatic tissue digestion protocol with the earlier evaluated mechanical tissue homogenisation techniques, the Matuszewski [12] experiments were repeated using the final digestive protocol described under Section 2.4. From Table 2 it is observed that neither the average recovery, nor the average matrix effect differs significantly from the theoretical optimal 100% value, making the enzymatic tissue digestion the best sample preparation amongst the ones compared in our study. Moreover, albeit the fact that it takes overnight to perform the digestion, this protocol requires very limited operator involvement, whereas the mechanical disruption based approaches are, indeed, faster but highly labour intensive.

As mentioned by Yu et al. [14], analyte instability should be considered and assessed when using an enzyme based digestive protocol. Therefore, we performed a stability study by comparing the PTX response in samples where PTX and IS were added prior to and after enzymatic tissue digestion ($n=6$ in all groups).

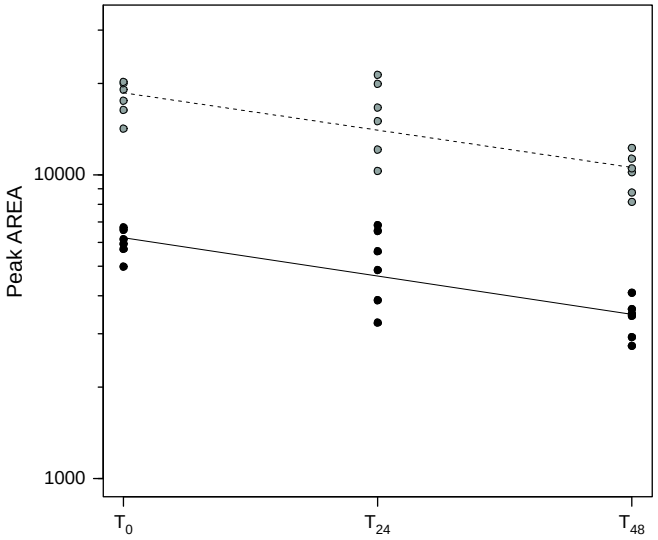

Fig. 2. PTX and IS stability during enzymatic tissue digestion. The black symbols represent the measured peak areas for PTX, whereas the grey symbols denote the peak areas as a function of time for the IS. All 18 samples were analysed in a single run. The solid and dashed line are the predicted peak areas, according to a linear model treating time as a continuous covariate, for PTX and the IS, respectively. From the model estimated slopes it was estimated that the half-live for degradation for PTX and the IS were 57.4 h and 58.7 h, respectively.

In short, PTX and IS were added to (i) blank bovine muscle tissue prior to digestion or (ii) digested blank bovine muscle tissue, at a final concentration of 2000 ng/g. As shown in Fig. 2, PTX as well as the IS undergo mild degradation during enzymatic digestion. The half-life of degradation, calculated from the estimated slopes (as the reciprocal of $\log_{10}(2)$ and the estimated slope, on a $\log_{10}$-scale) of the solid and dashed lines, are 57.4 h and 58.7 h, respectively. From these estimated half-lives we calculated that during our 16 h incubation period approximately 17.6% and 17.2% of PTX and IS will be degraded. From a sensitivity perspective, this is definitely acceptable, moreover, given that PTX and the IS are degraded to the same extent, this degradation will have no influence on the quantification, since the latter is based on the ratio of peak areas from PTX and IS.

Although, in general, in order to correct for variability during enzymatic digestion, one would choose to add the IS prior to the digestion, we choose to add it post digestion and prior to the SPE procedure. This way, samples above ULOQ could easily be diluted post digestion with a physiological saline solution, without having to use an IS supplemented dilution medium.

3.2. Validation

3.2.1. Accuracy and (intermediate) imprecision

As seen from Table 3, bias and imprecision were within specifications dictated by the EMA guidance on bioanalytical method validation [19]. Since bias and imprecision for the lowest QC level were $<20\%$, this level was accepted as LLOQ. Based on these results, our method proved to be precise and accurate.

3.2.2. Selectivity

No interferences were observed at the retention time of PTX and the IS when analysing blank bovine muscle tissue from 4 independent sources (data not shown).

3.2.3. Stability

Fig. 3 depicts the measured QC concentrations (in sextuplicate) at day 0 and 1 month after the start of the stability experiment.

Table 3
Results for the validation experiments based on bovine muscle tissue quality control samples.

	Concentration (ng/g)	Bias (%)	Within-run imprecision (%)	Between-run imprecision (%)
QC low	80	10.7	17.2	7.9
QC medium	1600	7.3	5.0	13.6
QC high	6400	7.9	6.3	11.8

QCs were analysed on 5 different days. Analysis on day 1, day 2 and days 3–5 were carried out in sextuplicate, triplicate and duplicate, respectively. For QC low and QC medium 1 and 2 outliers were excluded, respectively.

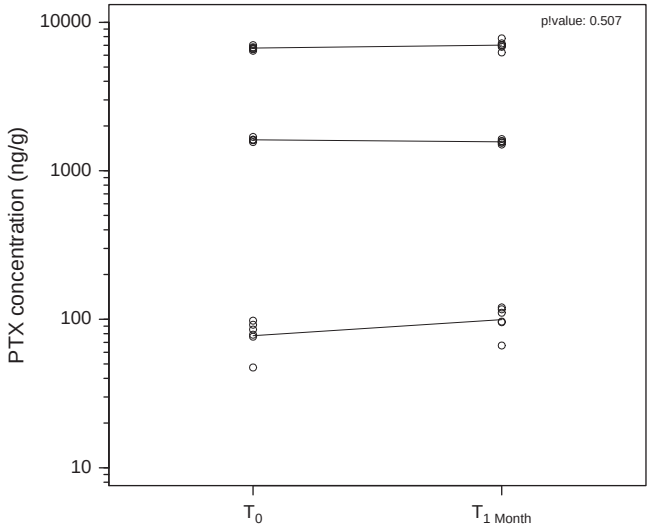

Fig. 3. The measured PTX concentrations at day 0 and 1 month after the start of the stability experiment. Spiked bovine muscle QC samples were stored for 1 month at -80 °C prior to analysis. The *p*-value indicates that using a two-factor ANOVA analysis, at the 5% level of significance, no evidence was present in the data in favour of significant degradation.

From the graph it is seen that no significant degradation has occurred during storage at -80 °C for 1 month. Moreover, a formal statistical significance test, using a two-factor ANOVA analysis to test the null hypothesis of no degradation, revealed a *p*-value (0.507) well in excess of the 0.05 level of significance cut-off, indicating that in our data, no evidence is present in favour of significant degradation.

3.2.4. Robustness evaluation

The results from the original analysis and the reanalysis (1 month after the original analysis) of a subset of tumour tissue samples are shown in Fig. 4. Results for samples that were below the lower limit of quantification (BLOQ; $n=8$ in original and reanalysis) are not depicted on this figure. When comparing the results from both analyses, it stands out that for some samples the results were similar, whereas for other samples results differed significantly between both analyses (This is observed from the high degree of variability in the slopes of the solid lines in Fig. 4). We calculated that only in 6/12 cases (without taking into account the measurements that were BLOQ), results were within -46.9% and 49.9% of their average value, indicating a high degree of variability between the results of both analyses.

Although our method proves to be accurate and precise, based on the validation data in bovine muscle quality control samples, the robustness experiment indicates that some aspect of the

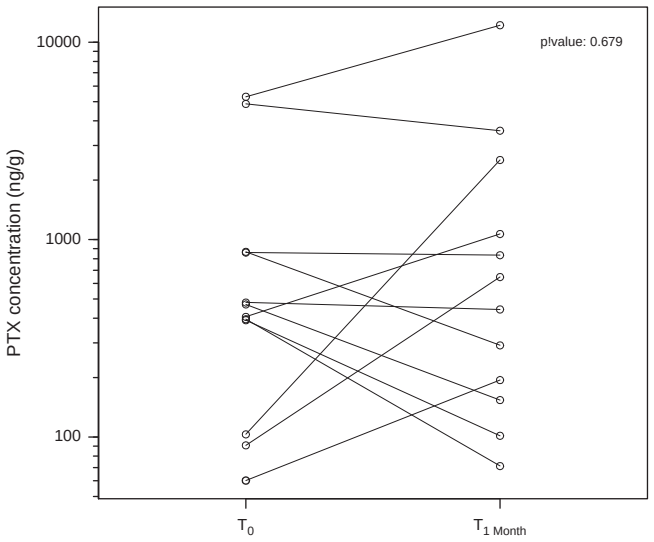

Fig. 4. Results from the robustness evaluation. The individually measured concentrations from "duplicate" samples are depicted by open circles and are joined together by a solid line. The *p*-value indicates that, using a paired sample *t*-test, no evidence is found in favour of significant degradation when tumour tissue is stored at −80 °C.

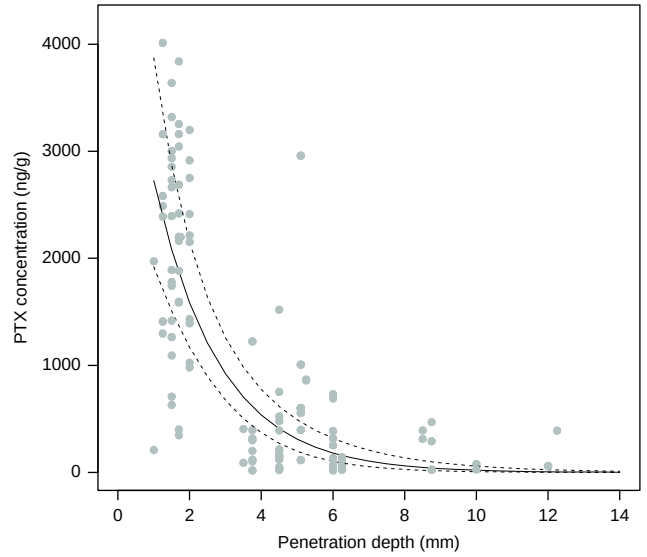

Fig. 5. The PTX penetration profile in tumour tissue post IPEC administration of 30 mg of Taxol®. The circles represent measured PTX concentrations as a function of penetration depth, whereas the solid and dashed lines represent the mean predicted PTX tumour tissue concentration, based on our published PKPD model [20], as well as the 95% confidence interval on this prediction.

analysis is beyond our control. In our tumour tissue harvesting protocol (Fig. 1) tumours roughly weighing a few hundred mg are partitioned in smaller subsections weighing approximately 50 mg. One part of this tumour tissue sample was used in the original analysis (25 mg), whilst the other part was used one month later in the reanalysis. To our opinion, due to the heterogeneous distribution of PTX, characterised by an exponential decrease in PTX concentrations as a function of penetration depth (Fig. 5) within the tumour tissue, we were unable to collect tumour tissue samples that were similar in PTX concentrations, hence explaining the high variability observed.

Apart from considering these results as a way of quantifying measurement uncertainty, one could consider this experiment as an extension to our earlier conducted stability study. For the robustness experiment, tumour tissue samples which contained PTX were stored for 1 month at −80 °C and therefore provide complementary information with regards to PTX stability. To test whether significant degradation had occurred in tumour tissue during storage, we used a paired sample *t*-test at the 5% level of significance. The resulting *p*-value of 0.679 indicates that no evidence is found in favour of significant degradation, which is in agreement with our earlier findings in bovine muscle tissue.

3.3. Intratumoural distribution of PTX post IPEC dosing

As a proof of concept for our developed methodology, Fig. 5 provides details with regards to the analysis of tumour tissue samples harvested in an in-vivo experimental animal protocol described elsewhere [20]. In short, animals were treated with PTX by IPEC administration of Taxol®, and afterwards, tumours were harvested at different times post IPEC administration. PTX concentrations were determined for >100 tumour tissue samples, using our developed method. The results from these analyses confirmed the suitability of our proposed methodology to address the question regarding the degree of and variability in tumour penetration of PTX post IPEC administration.

The full pharmacokinetic results from this study are beyond the scope of this publication and are reported in detail elsewhere [20]. However, using the developed PKPD model, it was estimated that the half-width (similar to the half-live in plasma pharmacokinetics) of PTX tumour penetration was 1.37 mm. This means that, for every 1.37 mm increment from tumour's surface towards tumour's centre, the PTX concentration was reduced with 50%. In other words, coming back to our hypothesis with regards to the high degree of variability observed in our robustness experiment, tumour tissue samples collected only 0.5 mm apart (with respect to tumour's centre) will, on average, show a 22.3% difference in PTX concentration due to the exponential penetration profile.

To our opinion, this explains why our robustness experiment showed such high variability between results from both analyses. Moreover, these results show that the pre-analytical phase, in this application, has a significant impact on the robustness of the analytical methodology and that the way forward, in this type of applications where the focus is on studying tissue penetration, is to further miniaturise the sample collection protocol.

4. Conclusion

This study presents the development and validation of an assay to be used in the determination of PTX in tumour tissue samples. Sufficient evidence is provided to gain insights in the performance of the developed method. Nevertheless, future efforts should be directed to improve the robustness of the methodology with respect to PTX determinations in tumour tissue samples originating from in-vivo experiments. In our opinion, the pre-analytical phase, consisting of the harvesting and subsectioning of the tumours holds room for improvement. One way of addressing this problem, in our opinion, is the further minimisation of the harvested tumour samples so that samples that are harvested in close proximity to each other show less variable tissue composition and PTX concentrations.

Through the use of an enzymatic tissue digestion protocol, we minimised matrix effects whilst simultaneously maximising recovery. Furthermore, sample handling was minimised as compared to the mechanical homogenisation, providing the analyst with an easy, less labour intensive, sample preparation protocol. Finally, using automatic pipettes and/or robotic based instrumentation this sample preparation could easily be adapted to accommodate sample preparation in a high-throughput fashion.

As a concluding proof-of-concept, we used our enzymatic tissue digestion protocol to analyse samples from an experimental animal trial with rats being administered PTX through IPEC.

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