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Equilibrium ex vivo calibration of homogenized tissue for in vivo SPME quantitation of doxorubicin in lung tissue

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Abstract

The fast and sensitive determination of concentrations of anticancer drugs in specific organs can improve the efficacy of chemotherapy and minimize its adverse effects. In this paper, *ex vivo* solid-phase microextraction (SPME) coupled to LC-MS/MS as a method for rapidly quantitating doxorubicin (DOX) in lung tissue was optimized. Furthermore, the theoretical and practical challenges related to the real-time monitoring of DOX levels in the lung tissue of a living organism (*in vivo* SPME) are presented. In addition, several parameters for *ex vivo/in vivo* SPME studies, such as extraction efficiency of autoclaved fibers, intact/homogenized tissue differences, critical tissue amount, and the absence of an internal standard are thoroughly examined. To both accurately quantify DOX in solid tissue and minimize the error related to the lack of an internal standard, a calibration method at equilibrium conditions was chosen. In optimized *ex vivo* SPME conditions, the targeted compound was extracted by directly introducing a 15 mm (45 μm thickness) mixed-mode fiber into 15 g of homogenized tissue for 20 min, followed by a desorption step in an optimal solvent mixture. The detection limit for DOX was $2.5 \mu\text{g}\cdot\text{g}^{-1}$ of tissue. The optimized *ex vivo* SPME method was successfully applied for the analysis of DOX in real pig lung biopsies, providing an averaged accuracy and precision of 103.2% and 12.3%, respectively. Additionally, a comparison between SPME and solid-liquid extraction revealed good agreement. The results presented herein demonstrate that the developed SPME method radically simplifies the sample preparation step and eliminates the need for tissue biopsies. These results suggest that SPME can accurately quantify DOX in different tissue compartments and can be potentially useful for monitoring and adjusting drug dosages during chemotherapy in order to achieve effective and safe concentrations of doxorubicin.

Keywords

IN VIVO SPME, SPME CALIBRATION, DOXORUBICIN, LUNG, THERAPEUTIC DRUG MONITORING, LC-MS/MS

1. Introduction

The real-time monitoring of intracellular anticancer drug concentrations and their biodistribution in targeted organs aims to enhance the effectiveness of applied chemotherapy while reducing detrimental side effects. Doxorubicin (DOX) is a therapeutically relevant anthracycline drug that exhibits antitumor activity against a variety of solid tumors, including lung, ovarian, and breast cancer [1]. DOX's cytotoxicity mechanism is based on intercalation with DNA, the inhibition of topoisomerase II, and the formation of free radicals, which results in DNA damage, oxidative stress, and, ultimately, the death of the cancerous cells [2]. Despite these advantages, doxorubicin's clinical application is limited due to its tendency to produce severe adverse dose-dependent systemic and local effects. Consequently, different drug delivery systems [3,4] and novel procedures, such as *in vivo* lung perfusion (IVLP) [5], have been introduced in order to reduce DOX's toxicity and to provide a method for safely administering higher doses to targeted organs. However, to precisely assess the concentration level and biodistribution of DOX in different tissue compartments, fast, simple, and sensitive methods need to be implemented in order to avoid insufficient or excessive dosing.

Researchers have used several analytical methods to determine doxorubicin levels in biological samples, including liquid chromatography coupled to mass spectrometry (LC-MS) [6–9], electrophoresis with laser- or LED-induced fluorescence detection [10,11], and UV fluorescence spectroscopy [12]. Furthermore, the use of different sampling/sample-preparation protocols prior to DOX analysis in solid tissues has also been reported [8,13,14]. However, most of these techniques are based on traditional solid-liquid extractions. These traditional methods use large amounts of solvents, are time-consuming, and require the collection of tissue samples. Moreover, the proposed assays are unable to provide spatial resolution as they are performed on biopsy samples collected from terminal parts of the organ

[8,15]. To overcome these issues, solid-phase microextraction (SPME) has been introduced as a minimally invasive technology that both integrates the sampling, extraction, clean-up, and enrichment steps, and also allows for the repeated sampling of different compartments of the tissues and organs [16–19]. Additionally, it is important to highlight the fact that SPME sampling does not require sample collection; instead, the fiber containing the extraction phase is inserted directly into the untreated living tissue. In this way, SPME is a unique technique for *in vivo* drug monitoring because it does not disturb the studied system's chemical equilibria or molecular pathways [17,20,21] as a result of negligible depletion [22]. Fundamentally, the principles of SPME extraction can either be based on the equilibrium established between the analyte concentrations in the biological matrix and the extraction phase, or on extraction under pre-equilibrium conditions. Therefore, the selection of an appropriate calibration model for either *ex vivo* or *in vivo* SPME allows for the determination of a given drug's free concentration. In the specific challenge of tissue sampling, a number of physiological factors, such as blood flow or temperature, may affect accuracy and precision when measuring analyte concentrations in a living organism. The first strategy, equilibrium extraction, was initially implemented by Es-haghi et al [23] for *in vivo* blood pharmacokinetic studies of diazepam in dogs. Essentially, since the matrix studied was a biofluid, the equilibrium was accelerated by adding forced convection and by recirculating the blood at a constant flow. Despite this approach is reliable and practical, it is limited to liquid *in vivo* sampling. The second strategy, diffusion-based calibration, is mainly governed by the mass-transfer rate at pre-equilibrium conditions. With diffusion-based calibration, it should be noted that all physiological and experimental parameters must stay constant to control the thickness of the boundary layer over the extraction time during *in vivo* analysis [16,23–25]. Hence, this approaches usually need the introduction of an internal standard to correct for potential

extraction condition changes during the procedure [25–28]. For instance, Bai et al [16] reported the determination of off-flavor components in live fish using predetermined *ex vivo* sampling rate calibration as well as on fiber standardization. However, it is not always allowed to introduce an exogenous compound -internal standard- in the living system and, consequently, this approach tends to exhibit some weaknesses. Once the free concentration has been determined, it becomes possible to obtain accurate information about the drug's unbound fraction in the analyzed matrix [25,29]. Although the application of *in vivo* SPME sampling in animal models has previously been reported [17,30,31], several theoretical considerations related to challenges associated with *in vivo* SPME studies in humans and other clinical applications have yet to be discussed thoroughly.

Before DOX in a living system can be accurately quantified, multiple steps of method optimization and validation must be performed using external matrices (*ex vivo* SPME). For instance, it is necessary to determine how sterilized SPME probes influence extraction efficiency; the amount of surrogate matrix required for method validation, an appropriate calibration strategy, and how to quantify DOX without internal standard correction. In the present study homogenized lamb's lungs were used as a surrogate matrix in order to develop and validate an *ex vivo* SPME method that can precisely quantitate doxorubicin levels in tissue. Furthermore, several analytical considerations related to the optimized method's ability to achieve fast *in vivo* extraction of DOX and near real-time monitoring of its levels and biodistribution in human lungs during chemotherapy (IVLP) are also discussed.

2. Materials and methods

2.1. Chemicals and materials

The standard of doxorubicin (DOX) was purchased from Toronto Research Chemicals (Toronto, ON, Canada). The standard solution was prepared in water at a concentration of 1000 $\mu\text{g mL}^{-1}$ and stored in a glass vial at -80°C . Fresh working solutions of DOX in water were prepared before each experiment.

Formic acid (FA), sodium chloride, potassium chloride, potassium phosphate monobasic, and sodium phosphate dibasic were purchased from Sigma Aldrich (Oakville, ON, Canada). LC-MS-grade acetonitrile (ACN), methanol (MeOH), and water were purchased from Fischer Scientific (Fair Lawn, NJ, USA). SPME-biocompatible mixed-mode (MM) fibers (coating: 45 μm thickness, 15 mm length) were generously provided by Supelco (Bellefonte, PA, USA). A phosphate-buffered saline solution (PBS) with a pH of 7.4 was prepared according to Reyes-Garces et al [32]. Lamb's lungs, which were used as the surrogate matrix, were purchased in a grocery store, homogenized, and stored at -80°C . Briefly, 250 g of tissue were cut in small pieces and mixed with dry ice. The mixture was added in a blender for homogenization. The homogenized samples were spiked, mechanically agitated for an initial 5 minute period followed by another 2 hours at 500 rpm, and then stored at 4°C overnight in order to bind and properly diffuse the analytes over the tissue. The following day, the samples were again agitated at 500 rpm for 2 hours prior to SPME extraction.

2.2. Pig lung biopsies

Lung biopsies containing DOXO at unknown concentrations were obtained via *in vivo* lung perfusion (IVLP) procedures performed on pigs at the Toronto General Hospital

(Toronto, ON, Canada) and were kept frozen at -80°C until extraction. Before the experiments, the samples were thawed and equilibrated to room temperature. The biopsies corresponded to different IVLP procedures at different sampling time. Sample 1 was taken at the middle of the procedure (2 hours), the concentration of doxorubicin in perfusate was 75 mg/m^2 and the total perfusion time was 4 hours. In sample 2, the biopsy was taken at the end of the procedure after flushing the lungs with perfusion solution without doxorubicin. The concentration of doxorubicin in perfusate was 75 mg/m^2 and the total perfusion time was 2 hours. Finally, Sample 3 was taken at the middle of the procedure (2 hours), the concentration of doxorubicin in perfusate was 150 mg/m^2 and the total perfusion time was 4 hours. This study was approved by the University of Waterloo Office of Research Ethics (AUPP # A-16-07 and ORE # T-1608-5).

2.3. SPME method development

2.3.1. SPME procedure

The optimization of the SPME procedure was performed in PBS solution and used homogenized lamb's lungs as a surrogate matrix. Before use, the fibers were sterilized via steam autoclaving (Market Forge, Steamatic STM-E type C, VT, USA) at 121°C for 40 min. Next, the sterile fibers underwent preconditioning in $300\ \mu\text{L}$ of a acetonitrile/water mixture (80:20, v/v) for 60 min at 1500 rpm agitation. This was followed by 20 min of extraction under static conditions in either $0.3\ \text{mL}$ of standard PBS solution or $15\ \text{g}$ of homogenized lamb's lung spiked with DOX. After sampling, the fibers were cleaned with a Kimwipe to remove any remaining matrix components from the coating, and they were then put through an additional cleaning stage consisting of vortex agitation in $300\ \mu\text{L}$ of nanopure water for 10 sec. Finally, the final extracts for LC-MS/MS analysis were obtained by immersing the fibres

in 300 μL of acetonitrile/water (80:20, v/v) with 0.1% formic acid for 60 min desorption at 1500 rpm agitation.

2.3.2. Extraction time profile

Extraction time profiles of DOX were performed to determine the kinetic of extraction, equilibration time, and optimum extraction time for *ex vivo/in vivo* SPME sampling from lung tissue. Sterile fibres were used to perform extractions from 15 g of homogenized lamb's lung spiked with DOX at a concentration of 25 $\mu\text{g}\cdot\text{mL}^{-1}$. Extractions were performed in triplicate at intervals of 5, 10, 15, 20, 30, 60, and 120 min. The preconditioning, rinsing, and desorption steps were performed using the same methods and conditions described above.

2.3.3. Method validation

Three different homogenized lamb's lungs were used for the validation experiments. The first validation step was to evaluate the method's specificity. Hence, SPME was applied to homogenized blank lamb's lungs (blank samples) to test for potential interferences in DOX retention time. The limit of quantification (LOQ) was calculated as the concentration at which the signal-to-noise ratio (S/N) was ≥ 10 and the accuracy and precision are better than 80-120% and 20%, respectively. Precision and accuracy were determined in three replicates at three validation levels: 10, 25, and 50 $\mu\text{g}\cdot\text{mL}^{-1}$. The method's precision was given as a percentage of the relative standard deviation (RSD), and the method's accuracy was given as a percentage of the bias against the nominal concentration. The validation point concentrations were back-calculated using three separated calibration curves from different lung samples in order to also prove the inter-animal reproducibility. Linearity was evaluated at 6 different

DOX concentrations, with independent triplicates ranging between 2.5 to 50 $\mu\text{g}\cdot\text{mL}^{-1}$. Extraction efficiency was expressed in mass units, while recovery was presented as a percentage expressing the amount of analyte extracted from the total sample by the coating. For instrumental calibration, the DOX standard was prepared by directly diluting the analyte standard stock solution in desorption solvent. Relative matrix effect was measured as the %RSD of the calibration curve slopes for the three different lungs.

2.4. Ex vivo SPME sampling for determination of DOX level from intact (non-homogenized) and homogenized pig lung samples

Ex vivo sampling was conducted using three non-homogenized lung biopsies containing DOX. Pig lung biopsies were obtained from IVLP experiments performed at the Toronto General Hospital. Static extraction was performed by inserting the fibers into intact tissue for 20 min. The experimental details of the SPME methodology are described above. After extraction, the lung samples were homogenized and the entire SPME procedure was repeated in order to compare extraction efficiency between the non-homogenized and homogenized tissue. If the extract concentration of DOX was over the reported linear range, they were diluted to fit in the matrix matched calibration curve. It must be noted that the linearity was fully assessed until 2000 $\mu\text{g}\cdot\text{g}^{-1}$.

2.5. Solid-liquid extraction (SLE)

In order to validate the SPME methodology against the gold standard technique, solid-liquid extraction, the samples were analyzed using a slightly modified version of an SLE method that was developed and validated by Kümmerle et al. [14]. The tissue was

homogenized by mixing it with dried ice in a mortar. Then, 0.1 g of lung tissue was weighted and mixed with 400 μL of an ACN/water mixture (40:60 v/v) with 0.1 % FA. The mixture was vortexed for 30 sec, which was followed by agitation for 10 min at 1500 rpm. After this step, the samples were centrifuged for 10 min at 15000 g while the temperature was kept constant at 4°C. The supernatant was then collected and analyzed by LC-MS/MS. When the extracts corresponded, they were diluted to fit the instrumental calibration curve. For quantitation, a matrix matched calibration curve in the range within 2.5-50 $\mu\text{g}\cdot\text{g}^{-1}$ was prepared following exactly the same sample preparation steps than the samples.

2.6. LC-MS/MS analysis

Experiments were performed on an LC-MS/MS system consisting of a UHPLC with a Thermo Dionex UltiMate 3000 autosampler that was coupled via heated electrospray (H-ESI) to a TSQ Quantiva triple quadrupole mass spectrometer (Thermo Scientific, San Jose, UA). Chromatographic separation was performed using a C18 column (Kinetex Phenomenex, 4.6 mm \times 150 mm, 3 μm particle size). The flow rate was set at 500 $\mu\text{L}\cdot\text{min}^{-1}$. Mobile phase A consisted of water with 0.1% formic acid, and mobile phase B consisted of acetonitrile with 0.1% formic acid. The solvent gradient was set at a starting condition of 90% A from 0 to 1.0 min and declined linearly to 10% A from 1.0 to 6.0 min. Finally, the solvent composition was reconstituted to 90% of A and held there until 10.0 min for re-equilibration. The total run time was 10 min at 20°C, and the total injection volume was 10 μL . The H-ESI conditions were 1200 V for spray voltage, and 45, 13, 1 arbitrary units for sheath gas, auxiliary gas, and sweep gas, respectively. The vaporizer temperature was set at 358°C, and the ion transfer tube temperature was set at 350°C. Regarding MS detection, all analyses were performed in

positive ionization mode. The monitored transition for DOX was 544.1 to 361.0, which was achieved using a collision energy of 24.2 V. Furthermore, the dwell time was set at 50 msec, the RF lens was set at 66 V, and the CID gas level was set at 2 mTorr. The resolution of both the first and third quadrupole was 0.7 (FWHM), and the analyte was tuned in the instrument via external infusion of the standard at a concentration of $1 \mu\text{g}\cdot\text{mL}^{-1}$. Under these conditions, the instrumental LOD for DOX was 0.01 ng. MS data were processed with XCalibur software v.4.0. (Thermo Fisher Scientific, San Jose, USA).

3. Results and discussion

3.1. Development of SPME method

An *in vivo* SPME method for extracting and monitoring DOX levels in human lungs during administration via IVLP requires the development of a robust procedure under laboratory conditions (*ex vivo* SPME). To this end, several parameters that could potentially influence the extraction efficiency during *ex vivo/in vivo* studies (e.g., selection of optimal preconditioning and desorption solvents, fiber sterilization, and sample volume) were evaluated. Comprehensive optimization that considers all conceptual aspects related to SPME and *in vivo* conditions is crucial in evaluating the methodology's validity for further human studies.

The application of SPME in live human tissue requires a sterile environment, including the use of sterile fibers. However, the efficient extraction of a drug via such fibers has not been explored vis-a-vis biocompatible mixed mode fibers. Therefore, prior to the standard SPME protocol, the fibers were sterilized by steam autoclaving for 40 min. After sterilization, the autoclaved fibres were used to extract DOX from a PBS solution spiked with

doxorubicin ($10 \mu\text{g}\cdot\text{mL}^{-1}$), and the results were compared to those of the typically used non-autoclaved fibers. As can be seen in Fig.1a, the autoclaved fibers provided about a 75% better extraction efficiency than the non-autoclaved fibers. The t-test comparison between both conditions give significant differences ($p=0.02$). One hypothesis for this result is that the autoclaving step is a more exhaustive conditioning procedure. This hypothesis is plausible, as a more exhaustive conditioning procedure will lead to the activation of more of the fibre's surface area and, consequently, greater coating extraction capacity.

In SPME method development, it is crucial to select an appropriate combination of solvents for coating preconditioning prior to sampling and for desorbing the analyte from the coating after sampling, as this can greatly influence extraction efficiency. The mixtures tested for preconditioning were MeOH/water (50:50, v/v), ACN/water (80:20, v/v), and ACN/MeOH/water (40:40:20, v/v/v). Fig. 1b shows the area of DOX recovered from PBS as a function of the preconditioning strategy. The ACN/water mixture (80:20, v/v) provided higher efficiency in comparison to the MeOH/water mixture ($p=0.04$) and comparable results to the ACN/MeOH/water. Nevertheless, considering that the ACN/water mixture gave the lowest %RSD, the latter was selected as the optimal preconditioning mixture. Next, desorption solvent solutions were tested to find one that could completely desorb the analyte from the coating. Various combinations of solvents, including those used in the preconditioning step, were evaluated, with the mixtures containing ACN and water providing the best results (data not presented). Finally, two ACN/water mixtures with ratios of 50:50 (v/v) and 80:20 (v/v) were tested to determine the optimal desorption solvent. After 60 min extraction in PBS, the 80:20 (v/v) ACN/water mixture proved to provide better desorption efficiency than the 50:50 (v/v) ACN/water mixture (Fig.1c). Furthermore, adding FA at a concentration of 0.1% (v/v) to the optimum desorption solution significantly improved both

the amount of analyte recovered and the %RSD. The latter was corroborated by a F-test which gave significant differences between variances ($P=0.03$). Furthermore, these experiments allowed us to select the 80:20 (v/v) ACN/water solution with 0.1% FA as the optimum final mixture, as it demonstrated less than 1% carryover on the fiber after the first desorption step (Fig. 1d). This improvement can be explained as a function of the pH wherein the acidity constant (pK_a) shifts in the presence of solvents [33–35]. Hence, although the DOX amine group's pK_a is 8.2 in pure water [33,36,37], it shifts towards a more acidic pK_a in a mixture with high ACN content. For instance, given that DOX has an amine pK_a of 7.21 in a solution with an ACN composition of 50% (v/v) [33], its pK_a will be even more acidic in the optimal desorption solution (ACN/H₂O, 80:20, v/v). Therefore, the analyte will be desorbed in its neutral form, which reduces efficiency due to the desorption mixture's high polarity. On the other hand, adding 0.1% FA to the desorption mixture keeps DOX fully charged and promotes efficient desorption from the coating to the solution.

3.2. Considerations for *in vivo* SPME

This section details the practical and fundamental considerations involved in selecting the most feasible surrogate matrix. In addition, this section identifies and details the *ex vivo* SPME method that most closely reflects the *in vivo* SPME extraction conditions. One of the most important features of SPME under equilibrium conditions is the independence of the volume of the sampled tissue, but this assumption is only valid when the product between the fiber's constant and volume ($K_{fs} \cdot V_f$) is much lower than the volume of the sample (V_s) ($K_{fs} \cdot V_f \ll V_s$) [22,25]. Given this, it is crucial to select the optimal amount of homogenized tissue for DOX extraction for two reasons: first, it enables the independence of the tissue volume's influence on extraction efficiency; second, it enables extraction from the external matrix to be comparable to sampling a whole organ or living system. As it is impossible to collect enough

human lung tissue for this kind of experiment, lamb's lung, which was relatively easy to access, was selected as the optimal surrogate matrix for *ex vivo* studies. As shown in Fig.2, homogenized lung tissue with DOX at a concentration of $25 \mu\text{g}\cdot\text{mL}^{-1}$ in the range within 0.7 g to 30 g does not evidence a clear increasing. In addition, an ANOVA study of the experimental points gave no statistical differences within the range. However, in order to use a representative amount of tissue, 15 g of the homogenized surrogate lung was selected for *ex vivo* studies to reproduce an *in vivo* sampling.

Next, a calibration method under equilibrium conditions, independent of the hydrodynamic variables, was selected. It is critical to note that some blood-flow-related convection is generated when the fiber is inserted into a living system, which promotes faster extraction than under static conditions. Thus, using the static *ex vivo* extraction as a reference, it becomes apparent that equilibrium is always reached faster during *in vivo* extraction. This phenomenon is attributable to internal convection in the living system, which results in shorter equilibration times in real samples. Given this, the extraction time profile was obtained in static conditions for three lamb lungs using three independent autoclaved fibers for each time point. As indicated in Fig. 3, 20 min was the minimum extraction time required to reach equilibrium extraction, to attain maximum SPME sampling sensitivity. Furthermore, under equilibrium conditions the error in the determination decreased dramatically—more precisely, $\%RSD \approx 14\%$ for inter-sample analysis (see Fig. 3). This is mainly attributable to the fact that, under equilibrium conditions, the only important factor is the coating's affinity with the free analyte concentration, which remains practically invariable over different samples[22]. In contrast, shorter extraction times (below 15 min) appeared to be linear, diffusion-based extractions. In these extractions, several factors in the analyzed matrix, such as blood flow, fluid content, and tissue consistency, may have affected and modified the

amount of extracted drug. The interaction of these factors could potentially increase errors in determining the %RSD \approx 40% values for inter-sample analysis. Kinetic calibration approaches also need to be excluded from the current study for the following reasons: (1) the lack of the ability to use internal standard correction for DOX extraction during *in vivo* studies in living humans (e.g. any compound can be neither administered nor loaded in the fiber as internal standard) [16,38]; and (2) they allow for the potential instability of experimental conditions during the IVLP (e.g. perfusate flow, leakage, medical interventions). This instability makes it impossible to perfectly control the variables that generate the boundary layer [29,39] required in the diffusion-based model.

Moreover, during the development of the SPME method for the quantitation of a drug in a solid tissue, the obvious physical differences between intact and homogenized tissues must be considered/taken into account. Fig. 4 shows the amount of DOX extracted from intact tissue and homogenized tissue for two different human lung samples (biopsies). As can be seen, there are no statistical differences between the two extractions at two different concentration levels under the aforementioned equilibrium conditions.

To summarize, extraction at equilibrium conditions is the most reliable strategy because it prevents matrix conditions from influencing SPME's quantification capabilities in the absence of an internal standard. Since it takes several hours to administer doxorubicin in human studies [5], a sampling time of 20 min ensures that alternations in the level of the drug during chemotherapy can be monitored, and that a near-real-time profile of DOX biodistribution in the lung tissue throughout IVLP can be provided. Moreover, due to the minimal invasiveness of SPME probes vis-a-vis the living system, sampling can be performed several times in different parts of the lung without requiring the collection of terminal parts of the organ (tissue biopsy). Thus, the total analysis time, from the sampling step to the

assessment of the drug concentration, will be much shorter in comparison to the conventional steps of sample preparation (SLE) and analysis.

3.3. Validation of SPME method

The developed SPME method was validated, and a detailed description of the experiments can be found in the experimental section of the manuscript. In order to assess the described method's carryover, LOQ, linear dynamic range, accuracy, and precision, homogenized lung samples were spiked with doxorubicin at concentrations ranging from 2.5 to 50 $\mu\text{g}\cdot\text{g}^{-1}$. It is important to highlight that, despite the extendibility of the linear dynamic range, this is the optimum condition for the further development of clinical research applications in humans. The procedure was successfully validated for three different lamb lungs. Besides good linear dynamic range within 2.5 and 50 $\mu\text{g}\cdot\text{g}^{-1}$ ($R^2 \geq 0.99$) and an LOQ of 2.5 $\mu\text{g}\cdot\text{g}^{-1}$, acceptable average precision (RSD < 14.7%) and excellent accuracy (≈ 103.2) were also attained in the evaluated concentration range (see Table 1). Moreover, very good results in terms of relative matrix effects were obtained given that the slopes of the calibration curves constructed for the three different lamb lungs exhibited relative standard deviation (RSD) values of about 11%. The above results demonstrate that an *ex vivo* surrogate matrix model under equilibrium extraction conditions is suitable for the *in vivo* sampling and quantitation of DOX from human lungs during chemotherapy.

3.4. Application of developed method for determination of DOX level in real lung samples

The optimized *ex vivo* SPME method was applied to quantify doxorubicin in real pig lung samples collected in different IVLP experiments at different time-points. The values for

the total DOX concentrations were calculated based on the assumption that the lamb lung used in the method optimization process had the same binding properties for the drug as a lung. First, non-homogenized lung samples were subjected to SPME sampling. Since the recovery of the micro-extractive methodology was below 0.01% (negligible depletion), the same tissue samples were then homogenized and SPME sampling was performed from prepared homogenates. Next, the quantitative results obtained by SPME in intact tissue and homogenized lung tissue containing DOX were compared to the results from the extraction performed using the traditional SLE method. Table 2 lists the concentrations attained using three different sampling approaches, and, as can be seen, no significant differences were observed. These results prove that SPME provides the same quantitation capabilities as regular SLE methods, while eliminating the need for sample homogenization or additional sample preparation steps. As can be evidenced from Table 2, the procedure number 3, which uses a dose of doxorubicin of 150 mg/m^2 , gave the highest tissue concentration in contrast to the procedure number 1 when the perfusate concentration was 75 mg/m^2 . It must be highlighted that despite this method was applied to biopsies, it could also be applied to *in vivo* sampling reducing dramatically the invasiveness of the procedure.

In addition, as the ultimate goal is to attain precise assessments of DOX levels in different tissue compartments during *in vivo* sampling in the operating room, the developed method offers a promising solution that, to the best of our knowledge, cannot be achieved by any other technology at present.

4. Conclusions

An analytical system based on *ex vivo* SPME sampling hyphenated to LC-MS/MS method was developed to accurately and precisely quantitate the level of the anticancer drug, doxorubicin, by direct sampling from lung tissue samples. The *ex vivo* SPME method was

validated for homogenized lamp lung samples used as the surrogate matrix. It exhibited excellent linearity ($R^2 \geq 0.99$) within the range of 2.5-50 $\mu\text{g}\cdot\text{g}^{-1}$, a LOQ of 2.5 $\mu\text{g}\cdot\text{g}^{-1}$, good precision (RSD<14.7%) and accuracy (≈ 103.2), and relative low matrix effect values of about 11%. Besides, the method was validated analyzing pig lung biopsies taken from doxorubicin IVLP procedures. The concentrations reported, were cross-validated against a well-established LSE-LC-MS method. Due to the fact that *ex vivo* SPME extractions in lung tissue reach equilibrium, the entire procedure can be performed without the use of hydrodynamic controls and internal standard correction, enabling the methodology to be implemented in *in vivo* SPME sampling. However, it is important to highlight that the equilibration time strongly depends on the affinity constant. Thus, higher affinities increase equilibration times, which will lead to diminish the practicality of the stated approach. The optimized *ex vivo* SPME method will facilitate near to real-time monitoring of the concentration and biodistribution of DOX in the lung tissue of a living organism (*in vivo* SPME) during the treatment of cancer metastases in lungs (IVLP). Hence, SPME can be a powerful tool for therapeutic drug monitoring in clinical studies, because it can provide precise information about the delivery of the drug to the specific sites in the organ while also ensuring that the level of a drug in a patient's tissue is safe. Such an approach may become an important part of personalized oncological treatment. Further experiments will focus on analyzing DOX in lung samples and perfusion solutions using the above described extraction method. However, these experiments will attempt to circumvent the chromatographic step, which will dramatically reduce the total sample preparation and analysis time to less than 20 minutes [40,41].

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Figures

Figure 1. A) Comparison of extraction efficiency of doxorubicin between autoclaved and non-autoclaved fibers. Preconditioning and desorption of fibers was performed in ACN/water (80:20, v/v) and ACN/ water (80:20, v/v) with 0.1% FA (n=5). B) Optimization of SPME preconditioning step using three preconditioning solutions: MeOH/ water (50:50, v/v), ACN/

water (80:20, v/v), and ACN/MeOH/ water (40:40:20, v/v/v) (n=5). C) Optimization of SPME desorption step of SPME using two desorption solutions (ACN/ water) ratios: 50:50 (v/v) and 80:20 (v/v) (n=5). D) First and second desorption performed with and without the addition of 0.1% FA to the optimum desorption solution (ACN/water, 80:20, v/v). Extraction of DOX at a concentration of $10 \mu\text{g}\cdot\text{mL}^{-1}$ was performed from 300 μL of PBS for 10 min at 1500 rpm with the use of 15 mm length MM fibers (n=5).

Figure 2. Comparison of the amount (ng) of DOX extracted from different amounts of homogenized lung tissue at a concentration of $25 \mu\text{g}\cdot\text{g}^{-1}$ by four independent replicates; 20 min static extraction from tissue was performed using 15 mm long autoclaved MM fibers (n=3).

Figure 3. Overlapping of three static extraction time profiles of doxorubicin at $25 \mu\text{g}\cdot\text{g}^{-1}$ obtained from three different lamb lung samples (15 g of homogenized tissue). Each experimental point represents the average of 9 different and independent experiments from three lungs (3 fibers per lung per point). Conditions of extraction and analysis are detailed in the experimental section.

Figure 4. Comparison of extraction efficiency between intact (non-homogenized) and homogenized lung tissue for two different pig lung biopsies (1 gram of tissue) after in vivo lung perfusion (IVLP) experiment with the administration of different doses of DOX: $150 \text{ mg}/\text{m}^2$ (Sample 1) and $75 \text{ mg}/\text{m}^2$ (Sample 2). Static extraction was performed for 20 min using 15 mm long autoclaved MM fibers (3 fibers per assay). For desorption and analysis conditions please see the experimental section.

Table 1. Figures of merit for DOX quantitation using three homogenized lamb lung samples as a surrogate matrix. Least square linear regression has been applied to fit the experimental data points.

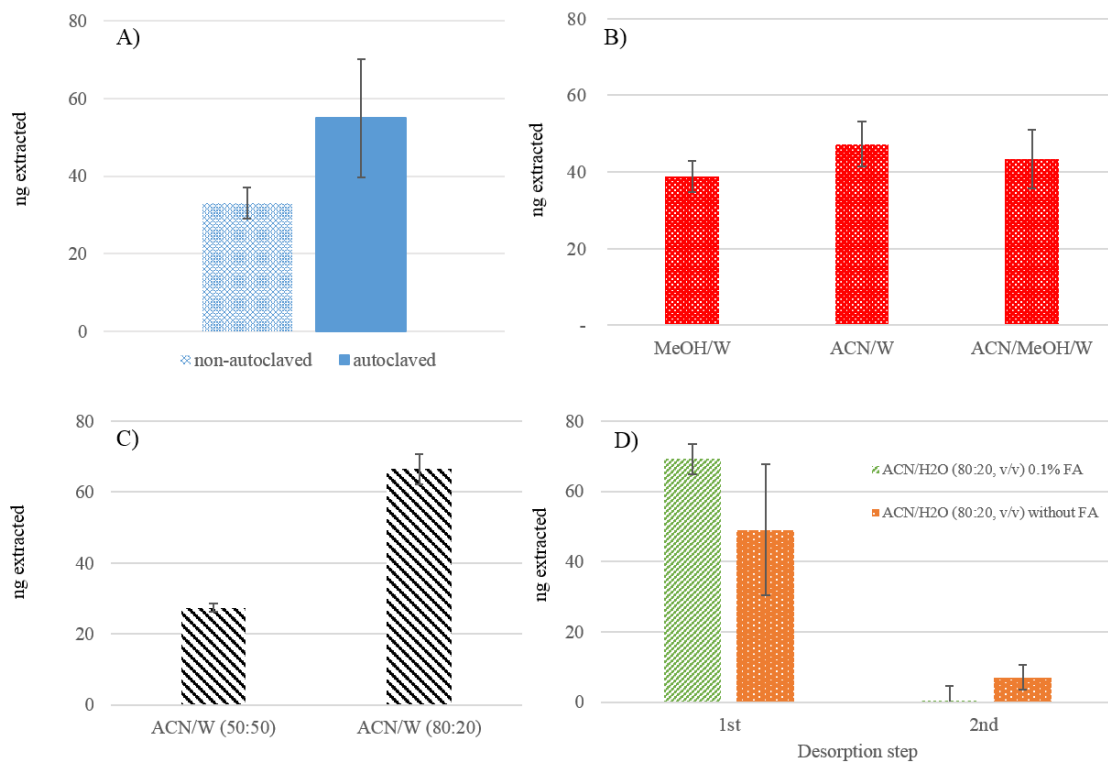
Sample	slope	intercept	R ²	LOQ	Accuracy (n=3)			Precision (n=3)		
					10 µg·g ⁻¹	25 µg·g ⁻¹	50 µg·g ⁻¹	10 µg·g ⁻¹	25 µg·g ⁻¹	50 µg·g ⁻¹
Lung 1	0.0229	-0.06	0.9991	5	113.0	100.6	112.8	20.1	12.1	18.4
Lung 2	0.0271	0.0077	0.9982	5	92.4	102.1	100.1	6.2	5.8	18.3
Lung 3	0.0222	-0.008	0.9989	5	106.3	102.6	99.2	21.5	10.9	19.3

Table 2. Comparison of the quantitation capabilities of SPME probes in intact pig lung samples against ex vivo SPME homogenized samples and solid-liquid extraction. Lung tissue samples (n=3) were collected at the end of IVLP experiment with the administration of different doses of DOX. Sample 1: 75 mg/m², sample 2: 75 mg/m² but post blank perfusion and, sample 3: 150 mg/m². Three technical replicates per sample were performed. sd – standard deviation.

	Intact tissue (µg·g ⁻¹ ± sd)	Homogenate (µg·g ⁻¹ ± sd)	LSE (µg·g ⁻¹ ± sd)
Sample 1	167 (± 54)	170 (± 38)	165 (± 16)
Sample 2	n/d	n/d	n/d
Sample 3	530 (± 49)	519 (± 83)	539 (± 7)

HIGHLIGHTS

- A minimum invasive method for repeated sampling of doxorubicin from lung tissue was developed.
- The SPME fiber is directly inserted in the tissue for minimally invasive extraction.
- Extraction under equilibrium conditions without the use of internal standard correction.
- SPME provides rapid and accurate information about drug level in different organ areas.



Accepted manuscript

