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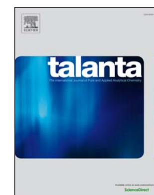

Equilibrium *ex vivo* calibration of homogenized tissue for *in vivo* SPME quantitation of doxorubicin in lung tissue

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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 $\mu g\, g^{-1}$ 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.

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

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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 $\mu g\ mL^{-1}$ 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-Garcés 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 min period followed by another 2 h 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 h 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 h), the concentration of doxorubicin in perfusate was 75 mg/m^2 and the total perfusion time was 4 h. 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 h. Finally, Sample 3 was taken at the middle of the procedure (2 h), the concentration of doxorubicin in perfusate was 150 mg/m^2 and the total perfusion time was 4 h. 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 µL 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 mL of standard PBS solution or 15 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 µL of nanopure water for 10 s. Finally, the final extracts for LC-MS/MS analysis were obtained by immersing the fibers in 300 µL of acetonitrile/water (80:20, v/v) with

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