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Direct Sampling from Human Liver Tissue Cross-sections for Electrophoretic Analysis of Doxorubicin

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Abstract

After chemoembolization of the liver with doxorubicin (Dox), this drug and its metabolites are not homogeneously distributed in this organ. The distribution cannot be easily measured making it difficult to assess how the drug performs in different tissue regions. Here we report a technique for sampling tissue cross-sections that can analyze the contents of micrometer size regions. The tissue cross-sections were from the explanted liver of a hepatocellular carcinoma patient. Samples were directly aspirated from a 5- μ m thick tissue cross-section into a 50- μ m i.d. capillary where the tissue was solubilized with a separation buffer containing sodium dodecyl sulfate. Upon sample dissolution, Dox and natively fluorescent compounds were separated and detected by micellar electrokinetic chromatography with laser-induced fluorescence detection. Sampling reproducibility and recovery were assessed using 10% (w/v) gelatin as tissue-mimic. Sampling from gelatin slices containing Dox revealed a relative standard deviation of 13%, which was comparable to that of sampling from solution. Dox recovery was $82 \pm 16\%$ ($n=5$). When sampling tumor and non-tumor tissue regions, samples could be taken in the same region 100 μ m apart. Atomic force microscopy was used to determine that each sample was 8.4 ± 1.0 pL in volume which made it possible to determine Dox concentrations in the ranges of 0.4 - 1.3 μ M and 0.3 - 0.5 μ M for the samples taken from tumor and non-tumor regions. The results demonstrated the feasibility of sampling, detection and quantification of Dox in micrometer size regions, which could be a useful resource for analyzing the Dox concentration and distribution in highly heterogeneous tissues.

Keywords

direct tissue sampling; tissue cross-section; doxorubicin; micellar electrokinetic chromatography; liver; hepatocellular carcinoma

1. Introduction

The liver is a highly specialized tissue responsible for many vital functions including the metabolism and clearance of xenobiotics, serum protein synthesis and lipid storage.¹ These multiple functions can be performed by the same organ because of its highly heterogeneous nature manifested by the different liver cell types, matrix compositions, and protein activities.¹ This heterogeneity may complicate the interpretation of the effect of treatment of diseases such as hepatocellular carcinoma (HCC) and the underlying cirrhosis. Thus, techniques that make it possible to measure the tissue properties with high spatial resolution are important for elucidating the mechanisms of these diseases, discovering biomarkers and evaluating treatments.^{2, 3} These techniques would be especially helpful for evaluating the efficacy of

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transcatheter arterial chemoembolization (TACE), a treatment that delivers chemotherapy drugs directly to the tumor site by a catheter via the hepatic artery, and uses embolic agents to create an ischemic effect on the tumor.⁴ Since the efficacy of TACE is presumably related to the concentrations of drugs at the tumor site and the adjacent parenchyma, it should be useful to determine these concentrations. These measurements are challenging because drug concentrations are not constant throughout the treated area of the liver. The variations in concentrations may be due to the heterogeneous structure of cancerous and non-cancerous tissue and/or to the method of drug delivery.

Among existing techniques, tissue homogenate analysis cannot reveal the concentrations of drugs at the tumor site and the adjacent parenchyma, which are only several micrometers apart. The spatial distribution information is lost as the homogenization process destroys the organization of the sample. Fluorescence microscopy can provide the spatial distribution of a fluorescent drug but it cannot distinguish between drugs, metabolites, and other native components with similar spectroscopic properties.

New techniques have been developed to directly sample and analyze the distribution of biomolecules in target tissues. Reyzer has reported the use of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) to directly analyze and image an anti-tumor drug SCH 226374 in mouse tumor tissue with resolutions of 200 μm in the x-direction and 400 μm in the y-direction.⁵ However, the background signal from the MALDI matrix interferes with the detection of drugs and metabolites with low molecular weights. Moreover, the discrepancies in the ionization efficiency of drugs and their metabolites due to their different structures, abundances and tissue environments make this method hardly quantitative.

Another technique that allows the direct sampling from tissues with high resolution is laser-capture microdissection (LCM) which uses a laser to selectively remove a small region of interest from a tissue (e.g., 3- μm diameter circle)⁶ for subsequent DNA, RNA or protein analyses.⁷ This sampling method is seldom used to analyze drug metabolites, probably because a large number of cells must be collected to make low abundant metabolites detectable. For example, Drexler reported that 20-30 LCM samples need to be combined for one HPLC-MS analysis of a prodrug and its metabolites.⁸

Oguri and co-workers reported a method for analyzing taurine in rat brain by directly sampling from the rat brain using a capillary followed by on-line pre-concentration, in-capillary derivatization and capillary electrophoresis (CE).⁹ Unlike microdialysis that only samples the fluid in the extracellular space of tissues, this method samples both intra- and extra-cellular taurine. While site-specific, the method is not quantitative as there is no way to control and calculate the volume of tissue sampled.

In this paper, we report a novel direct tissue analysis method with 100-micrometer spatial resolution to analyze the tissue distribution of an anti-cancer drug, doxorubicin (Dox), in liver tissue cross-sections after TACE treatment. Using this method, less than 10 picoliters of tissue were directly sampled from either tumor or adjacent non-tumor regions in a liver cross-section using an etched-tip capillary (50 μm i.d. and about 100 μm o.d. after hydrofluoric acid (HF) etching). Dox and other fluorescent species in the sample were subsequently separated and detected using micellar electrokinetic chromatography with laser-induced fluorescence detection (MEKC-LIF), a separation technique that has been used to analyze Dox and its metabolites with a 61 zeptomole limit of detection (LOD) of Dox.¹⁰ Further development of this sampling technique would be highly relevant for evaluating drug distributions and presumably the efficacy of established TACE treatments.

2. Material and methods

2.1 Chemicals and Reagents

Doxorubicin hydrochloride was a generous gift from Meiji Seika Kaisha Ltd. (Tokyo, Japan). Doxorubicinol was from Qvantas Inc. (Newark, DE, USA). Sodium borate decahydrate, sodium hydroxide (NaOH) and *o*-phosphoric acid were from Fisher Scientific (Fair Lawn, NJ, USA). Sodium dodecyl sulphate (SDS), hydrochloric acid (HCl), hydrofluoric acid (HF), potassium phosphate dibasic, chloroform and isopropanol were from Mallinckrodt (Phillipsburg, NJ, USA). Fluorescein was from Invitrogen (Carlsbad, CA, USA). γ -cyclodextrin (γ -CD) was from TCI America (Portland, OR, USA). Optimal Cutting Temperature compound (O.C.T.) was from Sakura Finetek (Torrance, CA, USA). Dimethyl sulphoxide (DMSO) was from Honeywell (Muskegon, MI, USA). 10 $\times$ phosphate-buffer saline (10 $\times$ PBS containing 1.37 M NaCl, 14.7 mM KH₂PO₄, 78.1 mM Na₂HPO₄, and 26.8 mM KCl) was from EMD Chemicals (Gibbstown, NJ, USA). Methanol and ethanol were from Pharmco-AAPER (Brookfield, CT, USA).

The tissue homogenization buffer consisted of 100 mM phosphate buffer (pH = 3.8) and the MEKC buffer was 10 mM borate and 10 mM SDS (pH = 9.3) (BS-10 buffer) or 50 mM borate, 40 mM SDS and 20 mM γ -CD (pH = 9.3) (BS-CD buffer). All the buffers were made using 18 M Ω water purified from a Millipore water purification system (Millipore, Billerica, MA) and the pH was adjusted with 0.1 M HCl or NaOH.

Stock solutions of Dox, doxorubicinol and fluorescein were prepared in methanol at a concentration of 1×10^{-3} M and kept at -20 °C until use.

2.2 Preparation of tissue sample and gelatin tissue-mimics

After explant, human liver tissue specimens were stored in O.C.T. in a Cryomold (Sakura Finetek, Torrance, CA, USA) at -80 °C. Cross-sections 5 μ m in thickness were obtained by cutting tissue specimens in a cryostat (Leica CM1850, Leica Microsystems, Bannockburn, IL, USA) at -20 °C. The cross-sections were mounted on glass slides (M6146, Cardinal Health, IL, USA) and then frozen at -80 °C; they were brought to room temperature for direct tissue sampling and subsequent MEKC analysis.

For bulk tissue analysis, Dox and its metabolites were extracted using liquid-liquid extraction.¹¹ A 40 mg tissue sample was homogenized in 200 μ l homogenization buffer by 50 strokes in an ice-chilled Dounce homogenizer (0.00025" clearance, Kontes Glass, Millville, NJ, USA). Dox and its metabolites were extracted using a 200 μ l DMSO, chloroform and isopropanol mixture (1:60:30, v/v/v) and then subjected to centrifugation at 1000g to separate the different phases. The aqueous phase and emulsification were removed, and the organic phase was dried under a nitrogen flow, reconstituted in 200 μ l methanol and diluted 25 times in BS-10 buffer prior to MEKC analysis.

Gelatin tissue-mimics containing Dox were made by dissolving different amounts of gelatin powder (type A, MP Biomedicals, Solon, OH, USA) into boiling water to create different concentrations (10%, 15% and 20%, w/v). When adding the gelatin powder, the mixture was stirred with a magnetic stirring bar until all the powder had dissolved. Dox stock solution was then added into the gelatin solution to a final concentration of 10 μ M. After the mixture was degassed by sonication and centrifugation (1000g for 10 min), it was poured into a Cryomold and allowed to solidify at 4°C in a refrigerator for at least 1 h. The gelatin slices (5 μ m in thickness) were cut in the same way as the tissue cross-sections and kept at -20 °C before MEKC analysis.

The elastic moduli of liver tissue and gelatin tissue-mimics (1 cm in length, 0.8 to 1 cm in width and 0.98 to 1.63 mm in thickness) were measured with a Micro Bionix® testing system (MTS Systems, Eden Prairie, MN, USA). The elastic modulus (E), defined as the ratio of stress to strain, was calculated as

$$E = \frac{\text{stress}}{\text{strain}} = \frac{F}{A_0} \times \frac{\Delta L}{L_0} \quad (1)$$

where F is the force applied to tissue or gelatin, A_0 is the original cross-sectional area through which the force is applied, L_0 is the original length of tissue or gelatin, and L is the change in length when the force is applied. Thus, E can be determined from the slope of the linear region in a stress (F/A_0) versus strain ($\Delta L/L_0$) plot.

2.3 Capillary preparation and direct tissue sampling

The polyimide coating at the injection end of a fused silica capillary (50 μm I.D and 150 μm O.D, Polymicro Technologies, Phoenix, AZ, USA) was burned with a small flame and then the outer wall was etched by immersing the tip of the capillary into HF for 5 min. In order to protect the inner walls of the capillary from etching, water was flushed through the capillary (3 mL/h) with a syringe pump connected at the other end of the capillary. Figure 1 shows the tip of a capillary before (A) and after (B) etching.

In order to select the region to be sampled, tissue cross-sections or gelatin slices were observed with a Nikon Eclipse TE300 microscope (Nikon, Huntley, IL, USA) using 10 $\times$ or 40 $\times$ objectives. Figure 1C illustrates the steps of direct sampling. First, a capillary was x-y positioned over the spot to be analyzed using a micromanipulation system (MX100L, Soma Scientific, Irvine, CA, USA) as previously described.¹² The capillary was then lowered carefully with a hydraulic micromanipulator (MW1, Soma Scientific) until contact with the tissue cross-section or gelatin slice was detected (Figure 1C, i). The capillary was then lowered 5 μm further into the sample (Figure 1C, ii) and a negative pressure of 7.6 kPa was applied for 2 s to aspirate the sample into the capillary (Figure 1C, iii).

2.4 Atomic force microscopy (AFM) of tissue cross-sections after direct sampling

After direct sampling, the surface topology of eight sampled regions on a tissue cross-section was mapped in the tapping mode with a Digital Instruments Nanoscope III Multimode AFM (Digital Instruments (DI), Santa Barbara, CA, USA) to determine the volume of tissue taken in each sampling. The AFM topological image of each sampling spot was processed with Image J software (NIH) to determine the area of the spot and the average intensities of both sampled and neighboring regions. The average intensity (I) was then converted into height (h) using the calibration curve provided by the DI software:

$$I = (33.7 \pm 0.4) \times h - (11.8 \pm 0.3); \quad (R^2 = 0.97) \quad (2)$$

The volume of tissue sampled (V) in each spot was calculated as

$$V = A \times \Delta h \quad (3)$$

where A is the spot's area and Δh is the average height difference between the sampled and the neighboring regions.

2.5 MEKC analysis of Dox in directly sampled tissue

After a tissue sample was aspirated into a capillary, the capillary was brought into the vial containing a fluorescein (internal standard) solution to inject (aspirate) this solution at 7.6 kPa for 2 s. Then the capillary was brought into the vial with BS-CD buffer and MEKC of the tissue sample was performed in a home-built instrument equipped with post-column LIF detection, previously described.¹³ The gelatin tissue mimics were sampled and analyzed in the same way as tissue samples, except the separation buffer was BS-10. The SDS in the separation buffers was important as solubilizing agent making it possible to release analytes and other fluorescent compounds that were then separated under a +400 V/cm electric field.

A sheath flow cuvette encased the detector end of the capillary. The last 2-mm of this end had the polyimide coating burned off to reduce the background fluorescence caused by this material. As fluorescent analytes migrated out from the capillary, they were excited at 488 nm with an argon ion laser (JDS Uniphase, San Jose, CA, USA). Fluorescence was collected at a 90° angle with respect to the laser beam by a 60× microscope objective (Universe Kogaku, Inc., Oyster Bay, NY, USA). A 505 nm long-pass filter (505 AELP, Omega Optical, Brattleboro, VT, USA) and a 1.4 mm pinhole were used to reduce light scattering. As previously reported, a dual channel fluorescence detector was used.¹⁴ The fluorescence spectral range was split by a 580 nm short-pass dichroic mirror (CP-RR-580, CVI Laser, Albuquerque, NM, USA) and then the fluorescence from Dox was selected by a 635 ± 27.5 nm band-pass filter (XF3015, Omega Optical, Brattleboro, VT, USA) while the fluorescein fluorescence was selected by a 535 ± 17.5 nm band-pass filter (XF3007, Omega Optical, Brattleboro, VT, USA). Fluorescence at these two separate spectral regions was then detected in separate photomultiplier tubes (PMT) (Hamamatsu, Bridgewater, NJ, USA) biased at 1000 V. The PMT outputs were sampled at 10 Hz and processed with a home-written Labview program (National Instruments, Austin, TX, USA).

Prior to MEKC analysis, the capillary was conditioned with sequential flushes of 0.1 mM NaOH, water, 0.1 mM HCl, water and separation buffer for 30 min each using 150 kPa pressure at the inlet. The same sequence, but running for 2 min each, was carried out between samplings of different tissue regions. The separation buffer was replaced every 2 h with a fresh one to avoid electrolyte depletion and buffer contamination. Prior to sample analysis, the instrument was aligned by maximizing the response to 5×10^{-10} M fluorescein that continuously flowed through the detector while applying a +400 V/cm electric field.

2.6 Determination and quantification of Dox

The mobilities of the peaks in the electropherograms of tissue samples taken directly from tissue cross-sections were corrected using the mobility of an internal standard, fluorescein. The corrections were done according to the procedure described by Li, *et al.*¹⁵ that uses the expression

$$t_{\text{corrected},x} = \left[\frac{1}{t_x} - \left(\frac{1}{t_{\text{fluorescein}}} - \frac{1}{t_{\text{fluorescein}}} \right) \right]^{-1} \quad (4)$$

where $t_{\text{corrected},x}$ is the corrected migration time of an analyte x in tissue samples and t_x is its observed migration time; $t_{\text{fluorescein}}$ is the migration time of fluorescein used as an internal standard and $t_{\text{fluorescein}}$ is the migration time of fluorescein in the reference run with Dox or doxorubicinol standards. The corrected mobility of the analyte x was calculated by

$$\mu_{\text{corrected},x} = \frac{L^2}{V \times t_{\text{corrected},x}} \quad (5)$$

where L is the length of the capillary, and V is the separation voltage. The corrected mobility of the analyte x was compared to that of Dox or doxorubicinol standards. The Student's t test was used to test the null hypothesis "there is no significant difference between the mobilities of Dox or doxorubicinol standards and those of the analytes in the tissue sample" using open-source statistical software "R". The null hypothesis was rejected when the p -value (p) < 0.02 (98% confidence level).

The amount of Dox in each tissue sample was determined according to the calibration curve:

$$A_{\text{Dox}} = (4.8 \pm 0.1) \times 10^{16} m_{\text{Dox}} + (0.028 \pm 0.006) \quad (R^2=0.99) \quad (6)$$

where A_{Dox} is the peak area of Dox and m_{Dox} is the number of moles of Dox injected. The linear range of the curve is from 2.9×10^{-18} to 5.7×10^{-16} mol (corresponding to 5×10^{-9} to 1×10^{-7} M Dox with 5.7 nL sample volume). The LOD ($S/N=3$) of Dox is estimated to be $(2.00 \pm 0.03) \times 10^{-18}$ mole ($n = 3$) as determined from the peak intensity resulting from injecting 5.7×10^{-17} mol Dox. Compared to previous reports¹⁰, the LOD in BS-CD buffer is about 30 times higher than in BS-10 buffer because of 1) the addition of γ -CD increases the background noise and 2) the longer Dox migration time in BS-CD buffer that results in broader less-intense Dox peaks.

Doxorubicinol was also detected in some instances. As the integrated fluorescence intensities of Dox and doxorubicinol over the spectral band-pass of the 635 ± 27.5 nm filter were nearly identical (18 fluorescence units (FU) versus 17 FU, respectively),¹⁶ the amounts of doxorubicinol in the tissue samples were also calculated according to Equation 6.

The amounts of Dox in the tumor and non-tumor regions of a tissue cross-section were compared using statistic software "R". The null hypothesis "there is no difference in the amounts of Dox between the tumor region and the non-tumor region" was tested using the Student's t test and rejected when $p < 0.02$.

2.7 Safety considerations

The study using human subjects was approved by the University of Minnesota Institutional Review Board (Study Number: 0508M72944). Protective clothing and surgical gloves were worn when handling liver tissues. All liver specimens were autoclaved before disposal.

3. Results and Discussion

3.1 Assessing the reproducibility of direct tissue sampling

The reproducibility of the sampling procedure is crucial to quantifying the Dox in each sample taken from tissue cross-sections. To evaluate the reproducibility of this sampling method, samples taken from different spots in a homogenous gelatin slice (5 μ m in thickness) containing Dox were analyzed by MEKC-LIF. Gelatin was used as a tissue-mimic because it is the denatured form of collagen, the main component of tissue extracellular matrices, and is the major determinant of tissue mechanical properties.¹⁷ The elastic modulus of a given tissue, a parameter describing the deformation of tissue under stress, determines the ease of sampling the tissue. The elastic moduli of 10%, 15% and 20% gelatin were measured and summarized

in Supporting information (SI), Figure S-1 and Table S-1. The elastic modulus of 10% gelatin was found to be close to that of HCC tissues, so this concentration of gelatin was used to prepare tissue mimics and to assess the reproducibility of direct sampling.

Figure 2A shows the image of a 10% gelatin slice after 6 samplings using an etched-tip capillary, and the corresponding electropherograms are shown in Figure 2B. The peak areas and amounts of Dox are tabulated in Table 1. The darker parts (indicated by arrows in Figure 2A) of the sampling spots were the result of the capillary tip deforming the gelatin due to contact during the sampling and were therefore excluded when measuring the areas of the sampling spots. The first sampling (electropherogram not shown) was discarded as an outlier by a Q-test at 99.5% confidence level. This is not surprising since when performing the MEKC analysis of Dox, the capillary wall needs to be conditioned by several injections of Dox standard or sample to improve electropherogram reproducibility.¹⁸ The relative standard deviation (RSD) of the peak areas of Dox in the next 5 samplings (spots 2 to 6) was 13% (Table 1), which is comparable to the RSD (11%) of 5 injections of 1×10^{-7} M Dox solution (results not shown), which were intercalated between samplings from the gelatin slice.

The reproducibility of direct sampling from a gelatin slice using a normal capillary (150 μm o.d.) is more than twice as low (RSD = 29%, results not shown). This is because the capillary wall compressed or destroyed the gelatin upon contact, which “squeezed” Dox solution out of the gelatin. When this occurred, in addition to the Dox in the gelatin, Dox solution was also introduced into the lumen of the capillary, artificially increasing the intensity of the observed Dox peak. Thus, by etching the tip of a capillary, these detrimental effects were avoided and the reproducibility of the sampled volume improved and was comparable to that of samplings from solution.

3.2 Surface topology after sampling

The surface topologies of tissue cross-sections after sampling were also measured by AFM to determine the volume of tissue sampled in each sampling spot (Figure 3). The volumes were calculated according to Equations 2 and 3. Although the shapes of the holes after sampling were irregular, the RSD of the volumes of tissues sampled was 12% (see Table 2), which matches well with the RSD of Dox amount (13%; Table 1) detected by MEKC-LIF for samples taken from gelatin slices containing Dox. The difference in sampling volumes mainly comes from the areas of the sample spots (RSD = 12%, Table 2), while the RSD of the average thickness of the tissue sampled (4.5%, Table 2) is relatively small.

Under the conditions described here, the direct sampling technique does not remove the 5- μm thick tissue section. On average, the removed section was 1.67 μm thick (Table 2). This means that only 33% of the tissue from the sampled area was removed (i.e. 8.4 pL). Although it is beyond the scope of this report, the remaining material could be re-sampled for further analyses.

In order to estimate the Dox recovery from a given tissue cross-section we made use of the analysis of gelatin sections. We calculated the total amount of Dox in the volume of sampled gelatin as,

$$\text{Amount of Dox in sampled gelatin} = V \times [\text{Dox}] = s \times d \times [\text{Dox}] \quad (7)$$

where V is the volume of the gelatin sampled, $[\text{Dox}]$ is the concentration of Dox in gelatin (i.e., 10 μM), s is the sampled area (i.e. average = $9.5 \times 10^3 \mu\text{m}^2$, Table 2) and d is the thickness of the gelatin removed from the cross-section (i.e. average = 1.67 μm). Thus, the average amount of Dox in the sampled gelatin volumes is 1.6×10^{-16} moles Dox. From the Dox amounts

detected by MEKC-LIF (1.3×10^{-16} moles, Table 2), the average recovery of Dox in gelatin is $(82 \pm 16) \%$ ($n = 5$).

3.3 Determination of Dox in liver tissue

Endogenous fluorescent species in liver specimens (SI, Figure S-2) and Dox fluorescent metabolites may interfere with the determination and quantification of Dox in liver tissue. The BS-10 buffer which is normally used to separate Dox and its metabolites¹⁰ is unable to resolve Dox from its major metabolite, doxorubicinol, and from other endogenous fluorescent species found in the tissue samples (results not shown). We previously reported that a buffer containing 50 mM SDS, 50 mM borate, 20 mM γ -CD is able to separate Dox and doxorubicinol,¹⁹ and this buffer was used as a starting point for selecting a suitable buffer for optimization. By varying the concentrations of SDS, it was found that buffer containing 40 mM SDS, 50 mM borate and 20 mM γ -CD (pH = 9.3) was sufficient to separate Dox, doxorubicinol and endogenous fluorescent species in bulk extracts from a liver administered with Dox via TACE (Figure 4, Trace a). Similarly, spiking a tissue extract from an untreated liver confirmed that no endogenous fluorescent species co-migrated with Dox, (SI, Figure S-3).

The ability to distinguish Dox (Peak 3) from doxorubicinol (Peak 2) and other fluorescent species was confirmed by analyzing tissue extract prepared in bulk (Figure 4, Trace a), spiking an aliquot of the extract with Dox standard (Figure 4, Trace b) or with doxorubicinol standard (Figure 4, Trace c) and comparing the electropherograms obtained before and after spiking. This analysis confirmed that the separation was adequate to distinguish Dox from doxorubicinol (when present) and from other fluorescent species. It is worth noticing that there were two peaks in the doxorubicinol standard (Figure 4, Trace c, Peak 2 and 2'), believed to correspond to two stereoisomers,¹⁹ while only one doxorubicinol peak (Figure 4, Trace a, Peak 2) was observed in the tissue sample. This suggests the stereospecificity of carbonyl reductase, which is the enzyme that catalyzes the conversion of Dox to doxorubicinol in the liver.²⁰

In direct tissue sampling, it is impractical to spike samples with Dox or doxorubicinol standards to determine their peaks in electropherograms. An alternative method is to compare the mobility of a peak to that of Dox and doxorubicinol standards. In this method, the reproducibility of the mobility is crucial. However, it is known that sample components alter the observed mobilities between runs. Here we used fluorescein as an internal standard (detected in the 535 ± 17.5 nm range) to correct the migration times (Equation 4) and consequently correct the observed mobilities (Equation 5) of the peaks of the tissue samples (detected in the 635 ± 17.5 nm range). After this correction, the reproducibility of the mobilities of the peaks improved and the Student's *t* test confirmed that the selected peaks in the tissue sample were Dox ($p = 0.82$ at 98% confidence level, c.f. SI, Figure S-4 and Table S-2).

3.4 Dox in tumor and non-tumor tissue regions

Similar to sampling from gelatin tissue mimics (c.f. Figure 2), we monitored direct tissue sampling by bright field microscopy. Figure 5 shows a tissue cross-section before (A), during (B) and after sampling (C) and the corresponding electropherogram (D). This procedure was effective for selecting the region to be sampled (Figure 5A) and to examine the quality of the region after taking a sample (Figure 5C). We then demonstrated that corrections to mobilities were feasible when a sample taken from tissue cross-sections and the internal standard solution were introduced separately into the separation capillary. Green fluorescence peaks are shown in the upper trace and include those of native fluorescent species in the tissue as well as the internal standard, fluorescein. Red fluorescence peaks are shown in the lower trace and include those of Dox and native fluorescent species in the tissue. Mobility correction made it possible to correctly identify the presence of Dox in the sampled tissue regions.

The tumor and non-tumor regions in a tissue cross section were determined based on the tissue morphologies. The vasculature pattern in the non-tumor region was more regular, while the tumor region showed more irregular vasculature as well as extensive necrosis due to Dox treatment. These features are consistent with those observed by enhancement of the differences between the tumor and non-tumor regions after hematoxylin and eosin (H&E) staining (SI, Figure S-5).

Sampling in both non-tumor and tumor regions as close as ~ 100 micrometers apart was feasible (Figures 6A and 6B, respectively). Despite the proximity of the three spots in each sampled region, the electropherogram for each spot was successfully obtained (Figures 6C and 6D, respectively). The amounts of Dox in each sampling spot were calculated according to the calibration curve (Equation 6) and are summarized in Table 3. The amounts of Dox in the tumor region are more widely dispersed than in the non-tumor region, from 3.3 to 10.6 amoles versus 2.7 to 4.3 amoles, respectively (Table 3). Assuming that the average volume of the tissue sampled is 8.4 ± 1.0 pL (Table 2), the concentrations of Dox in tumor and non-tumor regions are 0.4 to 1.3 μM and 0.3 to 0.5 μM , respectively. It is not surprising to observe larger variations in the tumor region since tumor vasculature heterogeneity and the irregular organization of the cellular environment may lead to such variations. Furthermore, the sampled volume is equivalent to ~ 2 to 3 parenchyma liver cells (3.8 pL/cell),²¹ since the drug accumulation is expected to be different in each cell, variations are expected even within a given region. Overall, the observed heterogeneity in Dox levels points to the importance of measuring drug contents in various tumor regions as it may reveal regions in which the drug does not reach therapeutic levels.

In one of six samples (spot 3, tumor, Table 3 and Figure 3D) the main metabolite of Dox, doxorubicinol, was detected and estimated to be of 2.5×10^{-18} mole. At first, the absence of doxorubicinol in the rest of the samples is perplexing since doxorubicinol was $\sim 41\%$ of the Dox contents in the tissue bulk measurements (Figure 4). However, given the average Dox amounts in the tumor and non-tumor regions (6 and 3 amole, respectively, Table 3) that gives an estimate of 2.4 and 1.2 amole of doxorubicinol based on the bulk measurement. These doxorubicinol amounts are comparable or lower than the LOD for doxorubicinol (2.0 amole). Moreover, carbonyl reductase, the enzyme that catalyzes the conversion of Dox to doxorubicinol may unevenly distribute through out the tissue cross-section, resulting in heterogeneous distribution of doxorubicinol.

In an ideal TACE treatment, it is expected that more Dox would accumulate in tumor than in non-tumor region as the drug is delivered to the tumor site directly through a catheter. However, when comparing the Dox amounts in the tumor and non-tumor regions (c.f. Table 3), they are not significantly different ($p = 0.39$ at 98% confidence level). Clearly, direct tissue sampling is promising as a tool to evaluate the distribution of the drug in resected livers, but several future developments are needed: (i) use of focal point drug delivery methods, (ii) knowledge of the distance between the point of drug delivery and the spot in the tissue from which the sample is taken, and (iii) extensive sampling defined by a grid that includes sufficient sample points in both tumor and non-tumor regions. The tissue samples used here were obtained from a liver treated with the segmental arterial delivery of drug, which is too general to provide a focal point for determining the center of drug administration. In the newer drug delivery method, drug-eluting beads, the center of drug administration should be easier to define and address point (i) above. Detailed book keeping of tissue sectioning so that the relative position of a section with respect to the center of drug administration is not routine at tissue procurement facilities, but could be implemented as needed (c.f. point (ii) above). Lastly, it is difficult to estimate the number of samples that may be needed to define a grid that overlays a reliable representation of the spatial drug distribution in tumor and non-tumor regions (point (iii) above). A next practical goal would be to define the radial distribution along a line with origin

at the point of delivery and that spans both tumor and non-tumor regions. For example, 82 samples ($\beta = 0.05$, $\alpha = 0.02$), 250 μm apart, will be needed to statistically evaluate differences in the Dox contents of tumor versus non-tumor regions over 2 cm of the tissue cross section. With our current technologies these measurements would be time prohibitive and would require development of conditions that preserve the tissue cross section during the sequence of measurements. Clearly, the next step would be to increase the sampling and analysis throughput. This may be feasible by using capillary arrays that simultaneously sample several tissue regions similar to other reports in which two or more capillaries are used to sample simultaneously more than one single cell.^{22,23}

4 Conclusions

Here we demonstrated the feasibility of detecting and quantifying Dox in a 100- μm -diameter region of a tissue cross-section by direct sampling from the cross-section followed by MEKC-LIF analysis. Estimation of the sampled volume by atomic force microscopy made it possible to calculate Dox concentrations in the sampled regions. This method has a spatial resolution of about 100 μm , with an attomole LOD, and is reliable as the reproducibility of the sampling method is comparable to that of sampling from solution. The method has great potential as a technology for evaluating the efficacy of TACE treatment and guiding future research to improve such treatment by establishing the tissue distribution map of Dox. This method also has potential use in determining the tissue distribution of other fluorescent biomolecules and xenobiotics in the liver or other highly heterogeneous tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Details relevant to direct tissue sampling

The tip of a capillary before (A) and after (B) HF etching. (C) Diagrams illustrating the position of a capillary before (i), at (ii) and after (iii) sampling from a tissue cross-section or gelatin slice.

Figure 2.

Direct sampling from a 5 m thick gelatin slice. (A) Bright field image of a gelatin slice after six direct samplings. (B) Electropherograms of spots 2-6; those of spots 3-6 are x and y offset for clarity. Gelatin was sampled at 7.6 kPa for 2 s with an etched-tip capillary. Separations were performed in a 48.5 cm long, 50 μm i.d. fused silica capillary at 400 V/cm in BS-10 buffer. Analytes were excited at 488 nm and fluorescence was detected at 635 ± 27.5 nm.

Figure 3. Tapping-mode AFM images. Eight regions showing samplings from a 5-μm thick tissue cross-section. Tissues were sampled at 7.6 kPa for 2 s with an etched-tip capillary. AFM images were obtained with 1 Hz scan rate.

Figure 4.

MEKC-LIF analysis of bulk liver tissue extract. **Trace a:** tissue extract only. **Trace b:** tissue extract spiked with Dox. **Trace c:** tissue extract spiked with doxorubicinol. Peak 2 and 3 are Dox and doxorubicinol, respectively; Peak 1 and 4 are endogenous fluorescence species in the tissue. Traces b and c are y-axis offset by 0.15 and 0.30 A.U. for clarity. Separations were performed in BS-CD buffer in a 39.2 cm long, 50 μm i.d. fused silica capillary at 400 V/cm after 3 s hydrodynamic injection of sample at 7.6 kPa. Analytes were excited at 488 nm. Detection of fluorescein (internal standard) was at 535 ± 17.5 nm (not shown) and red fluorescence (shown here) was at 635 ± 27.5 nm.

Figure 5.

Direct tissue sampling from a tissue cross-section and MEKC-LIF analysis. Bright field images of the tissue cross-section before (A), during (B) and after (C) sampling using an etched-tip capillary and the corresponding electropherogram (D). Experimental conditions were the same as in Figure 4 except a 45.7 cm long capillary was used for separations and samples were injected for 2 s.

Figure 6.

Direct tissue sampling from the non-tumor and tumor regions of a tissue cross-section and their respective MEKC-LIF analyses. Bright field images of the tissue cross-section after 3 samplings from non-tumor (**A**) and tumor (**B**) regions and corresponding electropherograms (**C**: non-tumor, **D**: tumor). The electropherograms of spots 1 and 2 are y-axis offset by 0.2 and 0.1 A.U. For clarity, the traces have been x-axis offset to align the internal standard, fluorescein (labeled with "+"). Peaks labeled with "*" and "#" are assigned to Dox and doxorubicinol after mobility corrections with Equations 4 and 5. Experimental conditions were as described in Figure 5.

Table 1Reproducibility of Dox in direct samplings from a 5 μm thick gelatin slice as determined by MEKC-LIF

	Peak area ¹	Dox amount ² ($\times 10^{-16}$ mol)	Sampling area (μm^2) ³
Spot 2	6.07	1.25	1.02×10^4
Spot 3	5.19	1.08	9.96×10^3
Spot 4	6.28	1.30	8.93×10^3
Spot 5	6.14	1.27	9.69×10^3
Spot 6	7.41	1.54	8.56×10^3
Average $\pm$ SD	6.2 ± 0.8	1.3 ± 0.2	$(9.5 \pm 0.7) \times 10^3$

¹ Peak areas were measured from the Dox electropherograms (Figure 2B) obtained by MEKC-LIF.

² Dox amounts were calculated according to Equation 6.

³ Sampling areas (demarked regions in Figure 2A) were measured by image J.

Table 2Volume of tissue sampled after direct sampling from a 5 μm thick tissue cross-section determined by AFM

	Δh (μm) ¹	Sampling area (μm^2) ²	Average sampling volume (pL) ³
Spot 1	1.71	4.50×10^3	7.72
Spot 2	1.67	5.43×10^3	9.06
Spot 3	1.59	3.96×10^3	6.28
Spot 4	1.61	5.00×10^3	8.07
Spot 5	1.61	5.61×10^3	9.03
Spot 6	1.76	4.92×10^3	8.64
Spot 7	1.63	5.66×10^3	9.21
Spot 8	1.78	5.11×10^3	9.12
Average $\pm$ SD	1.67 ± 0.07	$(5.0 \pm 0.6) \times 10^3$	8.4 ± 1.0

¹ Δh were calculated using Equation 2 from the average intensities of the sampled and neighboring regions measured by Image J.

² Sampling areas (demarked regions in Figure 3) were measured by Image J.

³ Sampling volumes were calculated according to Equation 3

Table 3Corrected mobilities and amounts of Dox in tumor and non-tumor regions of a 5- μ m thick tissue cross-section

	Tumor		Non-tumor	
	Dox		Dox	
	$\mu_{corrected}^1$	Amount²	$\mu_{corrected}$	Amount
Spot 1	2.082	10.6	2.079	4.3
Spot 2	2.098	3.3	2.086	3.3
Spot 3	2.068	4.0	2.097	2.7
Average $\pm$ SD	2.08 $\pm$ 0.02	6 $\pm$ 4	2.09 $\pm$ 0.01	3.4 $\pm$ 0.8
Standard (n=3) ³	2.08 $\pm$ 0.01	-	2.08 $\pm$ 0.01	-

¹The mobility ($\times 10^{-4}$ cm²/V·s) was corrected and calculated according to Equations 4 and 5.

²The Dox amount ($\times 10^{-18}$ mole) was calculated from Equation 6.

³Data correspond to the average of triplicate injections; variation is represented as the standard deviation.