Video Article

Spatial Quantification of Drugs in Pulmonary Tuberculosis Lesions by Laser Capture Microdissection Liquid Chromatography Mass Spectrometry (LCM-LC/MS)

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URL: https://www.jove.com/video/57402
DOI: doi:10.3791/57402

Keywords: Medicine, Issue 134, Tuberculosis, drug distribution, quantification, laser capture microdissection, liquid chromatography mass spectrometry, granuloma, necrosis

Date Published: 4/18/2018

Citation: Zimmerman, M., Blanc, L., Chen, P.Y., Dartois, V., Prideaux, B. Spatial Quantification of Drugs in Pulmonary Tuberculosis Lesions by Laser Capture Microdissection Liquid Chromatography Mass Spectrometry (LCM-LC/MS). *J. Vis. Exp.* (134), e57402, doi:10.3791/57402 (2018).

Abstract

Tuberculosis is still a leading cause of morbidity and mortality worldwide. Improvements to existing drug regimens and the development of novel therapeutics are urgently required. The ability of dosed TB drugs to reach and sterilize bacteria within poorly-vascularized necrotic regions (caseum) of pulmonary granulomas is crucial for successful therapeutic intervention. Effective therapeutic regimens must therefore contain drugs with favorable caseum penetration properties. Current LC/MS methods for quantifying drug levels in biological tissues have limited spatial resolution capabilities, making it difficult to accurately determine absolute drug concentrations within small tissue compartments such as those found within necrotic granulomas. Here we present a protocol combining laser capture microdissection (LCM) of pathologically-distinct tissue regions with LC/MS quantification. This technique provides absolute quantification of drugs within granuloma caseum, surrounding cellular lesion and uninvolved lung tissue and, therefore, accurately determines whether bactericidal concentrations are being achieved. In addition to tuberculosis research, the technique has many potential applications for spatially-resolved quantification of drugs in diseased tissues.

Video Link

The video component of this article can be found at https://www.jove.com/video/57402/

Introduction

The ability to spatially resolve and quantify drug levels is a crucial requirement for determining whether anti-tuberculosis drugs reach bacterial subpopulations within pulmonary lesions at sterilizing concentrations[1]. Of particular importance is determining drug penetration into the necrotic core of the lesion (called caseum), which typically contains the highest number of bacilli and may be poorly accessible to drugs due to the absence of vascularization.

Traditional methods to assess lesion penetration, which involve homogenization of excised pulmonary lesions followed by solvent extraction and liquid chromatography mass spectrometry (LC/MS) analysis, are highly sensitive and selective for the drugs of interest. However, these methods offer poor spatial information, limited to the size of the original homogenized tissue. Mass spectrometry-based imaging approaches, such as matrix-assisted laser desorption ionization (MALDI)[2,3], desorption electrospray ionization (DESI)[4] or liquid-enhanced surface extraction[5,6] offer highly spatially-resolved imaging capabilities, but direct quantification can be extremely challenging or impossible due to heterogeneous ion suppression effects and differing extraction efficiencies of analyte from the various cell or tissue types[7]. Additionally, most direct tissue MS imaging approaches are inherently less sensitive than LC/MS due to the lack of chromatographic separation of endogenous species competing for ionization and the lower solvent extraction efficiency of the drug from tissue.

Laser capture microdissection (LCM) combined with LC/MS analysis has been routinely applied to isolate and characterize distinct tissue regions for proteomic studies[8,9] and recently utilized for drug quantification in dosed animal tissue[10]. Here we present an optimized protocol applying LCM combined with LC/MS (LCM-LC/MS) analysis to quantify anti-TB drugs within distinct granuloma compartments. In the laser capture microdissection process, a UV laser is focused through the microscope objective onto the tissue section, which cuts and isolates the desired tissue area by following a path defined by the user. For gravity-assisted LCM (the technique used for this research), the tissue section is mounted onto a thin polymer membrane slide (PET or PEN) and the tissue is captured in a collection tube cap sited below the slide. The drugs are extracted from the excised tissue and quantified using standard LC/MS approaches. The amount of tissue required to be collected is ultimately determined from the expected concentration of the drug present in the tissue and the sensitivity of the LC/MS method. For most analyses of drugs dosed at therapeutic levels and analyzed using a routine triple quadrupole mass spectrometer, 3 million μm^2 (3 mm^2) of tissue surface area is sufficient.

This protocol describes the powerful combination of spatial profiling and full quantification offered by LCM-LC/MS, providing absolute drug concentrations within all compartments of TB granulomas. The technique may also be applied to determining drug concentrations in many different diseased tissues providing vital drug discovery and development information.

Protocol

All animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health with approval from the Institutional Animal Care and Use Committee of the NIAID (NIH), Bethesda, MD.

1. Animal Experiments and Tissue Collection

This section of the protocol describes animal procedures and sample collection under Biosafety Level 3 (BSL3) conditions. Detailed protocols of the *Mycobacterium tuberculosis* aerosol infection procedure and drug administration protocols in rabbits have been described previously[11,12].

1. Infect New Zealand White rabbits (male and female at 4 - 5 months old) with *M. tuberculosis* HN878 using a nose-only aerosol system, as previously described[11].
2. Administer the chosen drugs (Ethambutol in the example presented here) via the preferred route and euthanize the animals at 2, 6, and 24 h following administration. First, anesthetize the rabbit by intramuscular injection of Ketamine at 35 mg/kg and Xylazine at 5 mg/kg. Wait for ten minutes and confirm proper anesthetization by pinching the tail and gently touching the eye. If there is no reaction, euthanize by intravenous administration of pentobarbital and phenytoin (see **Table of Materials**) at 1 mL/4.5 kg in 2 mL sterile saline.
   NOTE: These timepoints are optimal to cover the pharmacokinetic profile for Ethambutol and may require adjusting/optimizing for other study drugs.
3. Using forceps, scissors, and/or scalpel, remove the lungs from the chest cavity, resect lung biopsies containing large necrotic granulomas embedded in surrounding uninvolved lung tissue (as described previously[3]). Necrotic granulomas appear beige in color and typically protrude slightly from the surrounding red/pink-colored lung. To facilitate easy cryosectioning, ensure that biopsies are no larger than 2 x 1.5 x 1.5 cm.
4. Using forceps, place the biopsy onto a pre-labeled cryomold tray with the desired cutting surface in direct contact with the base of the tray. After freezing, this will provide a flat surface from which cryosections will be cut.
5. Freeze the biopsy in liquid nitrogen vapor. Fill a styrofoam container to a depth of 2 inches with liquid nitrogen and place a metal wire tube rack. The rack should protrude above the surface of the liquid nitrogen providing a flat surface on which the tissue trays are placed. Place the lid back on the styrofoam container and leave tissues for 10 minutes to fully freeze.
6. Remove the tissue trays, quickly wrap in aluminium film and place in individually labeled resealable plastic bags and seal. Transfer to -80 °C freezer for storage.
   NOTE: Steps 1.1 - 1.6 are performed in BSL3 conditions (including all animal work and handling of infected organs and tissues). Gamma irradiate the lung biopsies at 3 Megarads to enable handling outside of BSL3 containment. Laser capture microdissection on unsterilized tissue may be performed within the BLS3 facility if approved safety protocols are in place. However, the remainder of this protocol describes downstream processing in a BSL-2 facility.

2. Tissue Sectioning

1. Set the cryostat to the desired cutting temperature. Transfer the gamma-irradiated lung biopsy from -80 °C storage to the cryostat and leave for 30 minutes to equilibrate the tissue temperature. Note: -20 to -22 °C is optimal for TB lesion biopsies.
2. Using tweezers, fix the biopsy to the cryostat chuck using a small amount of optimal cutting temperature adhesive (OCT) to adhere the base of the tissue to the chuck. Orient the tissue so that the flat surface (that was in contact with the base of the cryomold) is the exposed surface for cutting. Ensure the OCT does not contaminate the tissue surface, as this may interfere with the subsequent mass spectrometry analysis.
3. Cut three tissue sections at 25 µm thickness and mount onto PET membrane slides. Gently touch the membrane to the tissue section and remove. If too much pressure is applied, the thin membrane may tear.
   1. Avoid excessive handling of the slides prior to mounting as this will result in the PET membrane becoming charged and poor adhesion of the tissue sections. Ensure that the membrane slide is kept at room temperature to enable thaw-mounting and successful adhesion of the tissue to the membrane.
4. Remove the slide from the cryostat and allow to air-dry for 3 minutes. If LCM-LC/MS/MS will not be performed immediately, seal the slide in a small airtight sealable bag and transfer to -80 °C storage until required for dissection.
5. Cut an adjacent section at 10 - 12 µm and thaw-mount on a standard glass slide for Hematoxylin and Eosin (H&E) staining and reference. Additional sections can be cut at this time for other desired histochemistry stains (such as Ziehl-Niellsen for visualizing Mycobacterium tuberculosis (MTB)).

3. Microdissection

1. Remove sealed bag containing the slide from -80 °C storage and allow to reach room temperature for 5 minutes.
   NOTE: If the cold slide is immediately exposed to the laboratory atmosphere, the tissue will become coated with condensation, and the spatial integrity of the drug may be compromised.
2. Turn on the microscope and laser (laser requires 5 - 10 minutes warm up before cutting can commence). Load flat-cap 0.20 mL PCR tubes into the holder.
3. Remove slide from the bag and take an optical image of the tissue section on the PET slide using a flatbed scanner.
4. Place the slide into the slide holder (tissue side facing down) and assign separate collection tubes to specific granuloma regions of interest using the microscope software. Typically, these will be 'uninvolved lung,' 'cellular granuloma,' and 'caseum' (necrotic center), but may vary depending upon the specific pathology of the granuloma/biopsy.

5. Focus on the tissue using the 5X microscope objective. This magnification should provide a good overview of the tissue containing both cellular and necrotic granuloma areas. In the software select the tube designated 'caseum' to move it into position under the tissue.
6. Enter the desired dissection parameters. Typical settings for a 25 µm thick lung section are laser power 30, speed 15, and aperture 35 (arbitrary units). However, these will differ depending upon the microscope used and potential declining power due to the age of the laser.
7. Select the 'free-draw' tool and, using either a mouse or touchscreen pen, outline the desired region for dissection. The surface area of the region will be displayed in the software. Keep selected regions under 500,000 µm^2 (0.5 mm^2) to facilitate easier dissection. Repeat the dissection until 3 million µm^2 (3 mm^2) have been collected in total in the tube cap.
    1. On occasion, the dissected region may remain stuck to the surrounding membrane (for example due to static attraction) and not fall into the collection cap. Remove these regions from the cumulative surface area total by selecting and removing manually within the software.
8. Select the cap for 'cellular lesion' and collect 3 million µm^2 of tissue using the same process as outlined in step 3.7.
9. Select the cap for 'uninvolved lung' and collect 3 million µm^2 of tissue using the same process as outlined in step 3.7. Note that uninvolved lung tissue contains many bronchioles and alveolar spaces. Pay careful attention to exclude these from the defined tissue regions for dissection.
10. Remove the cap holder and carefully unclip, seal and label each tube. Protect the dissected tissues from surrounding air disturbances (such as from air flow disruption from an opening door). Analyze the dissected tissues immediately, or store at -80 °C and thaw prior to processing and LCMS analysis.

4. Extraction and LCMS Analysis

1. Prepare extraction solution of 1:1 acetonitrile/methanol containing Ethambutol d-10 internal standard. When selecting an internal standard, use a stable labeled form of the analyte drug (such as deuterium-labeled EMB used in this demonstration) with sufficient mass shift to avoid isotope cross talk between the analyte drug and standard (usually a minimum of 4 daltons).
   NOTE: Creating standards in homogenate of each respective tissue type is difficult because there is very limited control tissue with which to create homogenate standards. As an alternative to making standards from a spiked homogenate sample, a standard can be created by adding blank tissue and test compound together and extracting. A volume of control tissue in a homogenate that matches the target volume of the study sample tissue sections is combined directly with an amount of the test compound that would be present at a given concentration.
2. Calculate the targeted tissue volume based on the surface area and thickness of the tissue section and determine the necessary dilution factor for the homogenate using the volume of homogenate that will be added to the standard and QC samples. Calculations are illustrated below for a 3 million µm^2 (3 mm^2) target dissected area with a 25 µm thickness and 2 µL volume of homogenate.

$$Target\ Tissue\ Volume = Target\ Dissection\ Area \times Slice\ Thickness$$

$$Target\ Tissue\ Volume = 3{,}000{,}000\ \mu m^2 \times 25\ \mu m \times \frac{1\ \mu L}{1{,}000{,}000{,}000\ \mu m^3} = 0.075\ \mu L$$

$$Homogenate\ Dilution\ Factor = Homogenate\ Volume \div Target\ Volume$$

$$Homogenate\ Dilution\ Factor = 2\ \mu L \div 0.075\ \mu L = 26.67$$

3. Assuming a tissue density of 1 g/mL, prepare the homogenate stock by weighing 50 mg of control tissue and adding PBS buffer to dilute (using the 26.67 homogenate dilution factor calculated in step 4.2, the diluent is 1.283 mL). Homogenize by bead beating lung tissue and PBS buffer for 5 minutes at 1750 rpm on a bead homogenizer.

$$(Homogenate\ Dilution\ Factor - 1) \times Control\ Tissue\ Volume = Diluent\ Volume$$

$$(26.67 - 1) \times 50\ mg \times \frac{g}{1000\ mg} \times \frac{1\ mL}{g} = 1.283\ mL$$

4. Dilute 1 mg/mL drug stocks concentration in 1:1 acetonitrile/water to create standard curve spiking solutions. Determine spiking standard concentrations based on the spike volume and the target tissue volume. The illustrated example is for a 100 ng/mL standard using a 10 µL spike volume.

$$\frac{Target\ Tissue\ Volume\ \times\ Standard\ Concentration}{Spike\ Volume} = Spike\ Concentration$$

$$\frac{0.075\ \mu L \times \frac{1\ mL}{1000\ \mu L} \times \frac{100\ ng}{mL}}{10\ \mu L \times \frac{1\ mL}{1000\ \mu L}} = \frac{0.75\ ng}{mL}$$

5. Remove the tubes containing the microdissected tissues from -80 °C storage and allow to reach room temperature for 5 minutes.
6. Add 10 µL of 1:1 acetonitrile/water solution and 2 µL of PBS buffer to the tubes containing microdissected tissue.
7. For standard curve and quality control tubes, add 10 µL of spiking solution to 2 µL of control lung homogenate.
8. Add 50 µL of extraction solution to each tube.
9. Vortex each tube for 5 minutes, sonicate for 5 minutes and centrifuge at 5000 RPM for 5 minutes to form a pellet of film and tissue in each tube.
10. Transfer 50 µL of supernatant to a 96-well deep-well plate and dilute with an additional 50 µL of deionized water in each well.
11. Perform LC/MS/MS analysis using optimized instrument parameters for Ethambutol and Ethambutol-d10 internal standard (as previously described in detail[12]).
12. Use a dilution factor to correct for the exact amount of tissue dissected for each sample.

$$Dilution\ Factor = Target\ Area \div Actual\ Area$$

5. Method Validation

1. Create a homogenate in control lung tissue by combining 1 part lung, 2 parts PBS, and 3 - 4 steel beads. Beat lung tissue and PBS buffer for 5 minutes at 1750 rpm using a bead homogenizer.
2. Spike the homogenate by adding 10 µL of 1 mg/mL Ethambutol DMSO stock into 990 µL homogenate to create a final concentration of 10,000 ng/mL (10 mg/mL) and vortex for 1 minute.
3. Create a frozen homogenate block by pouring the homogenate into a cryomold and rapidly freezing on dry ice for 5 minutes.
4. Prepare 25 µm thick sections from the homogenate block as described in steps 2.1 - 2.5.
5. Dissect the target tissue area as specified in steps 3.2 - 3.10.
6. Add 10 µL 1:1 acetonitrile/water and 2 µL of PBS buffer to the tubes containing microdissected tissue.
7. Add 50 µL of extraction solution to each tube. Follow steps 4.9 - 4.12 to create a standard curve and determine the drug concentration in the tissue homogenate block.
8. Calculate extraction efficiency using the formula below:

$$Extraction\ Efficiency\ (\%) = \frac{Measured\ Drug\ Concentration\ in\ Homogenate}{Spiked\ Drug\ Concentration\ in\ Homogenate} \times 100$$

Representative Results

An overview of the LCM-LC/MS approach is shown in **Figure 1**. After sterilizing the tissue by gamma-irradiation, all subsequent steps (from tissue sectioning onwards) take place outside of BSL3 conditions. **Figure 2** shows the lesion biopsy sections before and after tissue isolation by LCM. Necrotic and cellular areas of TB lesions can be easily identified and isolated by visual inspection of optical images alone (without the requirement to refer to histologically-stained adjacent tissue sections). The dissection process produces a clean cut with minimal disturbance to the surrounding tissue, and dissecting 3 million µm^2 (3 mm^2) of each region of interest from the lesions takes approximately 1 hour in total.

The extraction efficiency and stability of the LCM-LC/MS method was assessed using Ethambutol (EMB)-spiked lung homogenate (**Table 1**). Complete extraction of the drug was observed, and no drug stability issues were detected through the dissection and extraction process. The LCM-LC/MS extraction and quantification protocol was further validated by comparing to established LC/MS quantification methods applied to the same tissues. Due to the inherent heterogeneity of necrotic pulmonary TB lesions and the poor spatial specificity offered by standard tissue homogenization, we validated the LCM-LC/MS method by directly comparing drug concentrations in uninvolved lung analyzed by both analytical techniques (due to its relatively high tissue homogeneity). **Table 2** shows the drug concentrations in uninvolved lung from biopsies taken from three Ethambutol-dosed rabbits as evaluated by: 1) homogenizing 25 µm thick tissue sections and analyzing by standard LC/MS, and 2) LCM-LC/MS analysis of uninvolved lung areas taken from an adjacent 25 µm thick tissue section. The data shows that two approaches produce consistent quantification data, demonstrating the suitability for routine spatial quantification.

We have applied LCM-LC/MS to spatially-quantify many existing and novel anti-TB drugs within pulmonary lesions. **Figure 3A** shows example data from MTB-infected rabbits dosed steady-state with Ethambutol. LCM-LC/MS enabled full quantification of the drug resolved within caseum, cellular lesion, and uninvolved lung tissue areas. EMB was observed to penetrate well into the lesion and reach sterilizing concentrations within the necrotic caseum. The corresponding MALDI-MS image of EMB distribution acquired from an adjacent tissue section is shown in **Figure 3B**. The qualitative MALDI-MS images correlates well with the quantitative LCM-LC/MS data with lower drug concentrations detected in the necrotic caseum in comparison to the cellular rim. LCM-LC/MS data was validated by direct comparison to tissue homogenates analyzed by standard LC/MS.

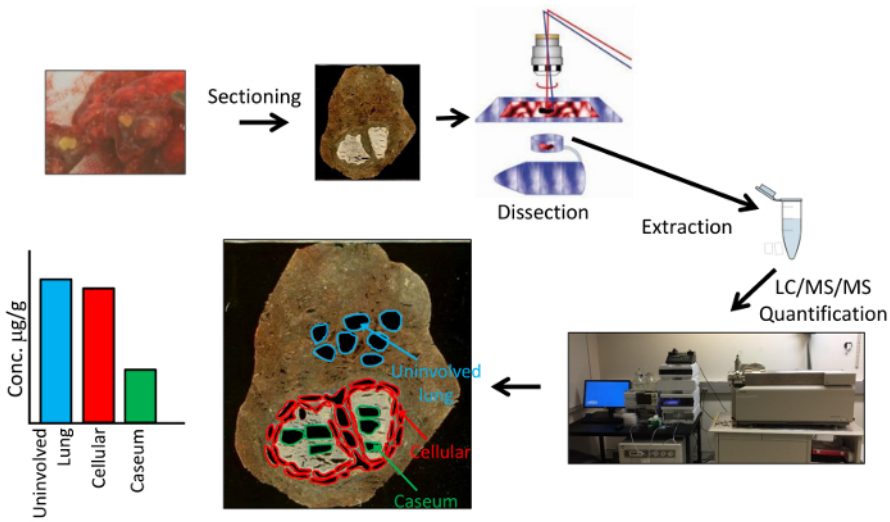

Figure 1: Schematic of the LCM-LC/MS process. Rabbit lung biopsies containing necrotic lesion along with surrounding uninvolved lung are collected and frozen. Cryosections are cut onto thin PET membranes and areas of uninvolved lung, cellular, and caseous lesion are dissected and isolated for quantification by LC/MS. Adapted from Zimmerman *et al.*[12] Please click here to view a larger version of this figure.

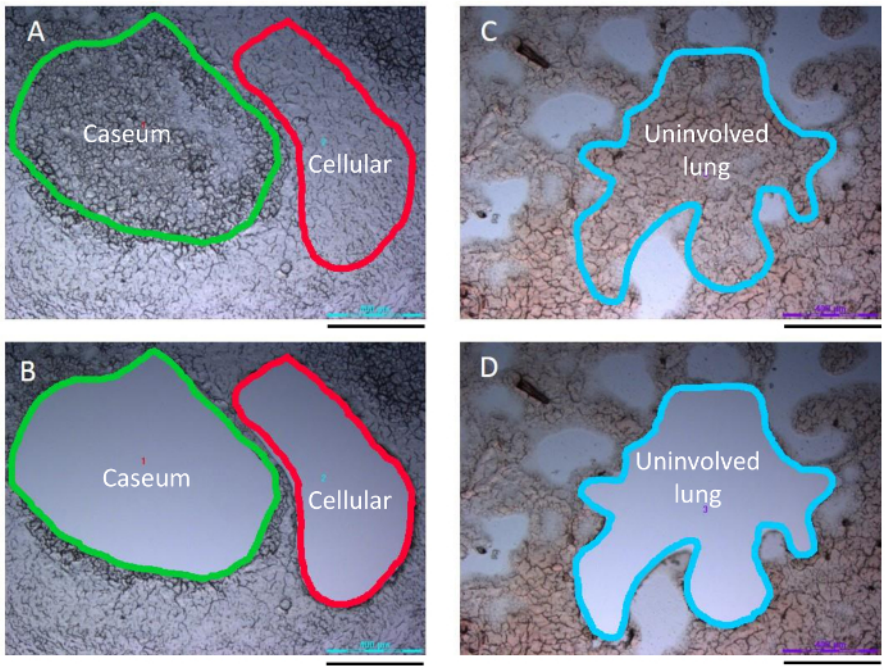

Figure 2: Lesion (A,B) and uninvolved lung (C,D) as they appear when viewed using the microscope before (A,C) and after (B,D) microdissection. Caseous lesion areas appear darker and 'cracked' in the optical image scan (A, green outline). Cellular lesion is lighter in color and more solid in structure (A, red outline). Uninvolved lung should be sampled at least 5 mm away from the lesion border and appears red/pink in color (C, cyan outline). Scale bars (black, blue, and purple) = 400 µm. Note that only solid areas of uninvolved lung should be selected to avoid including alveolar spaces and bronchioles in the total surface area of tissue collected (as shown in in D). Please click here to view a larger version of this figure.

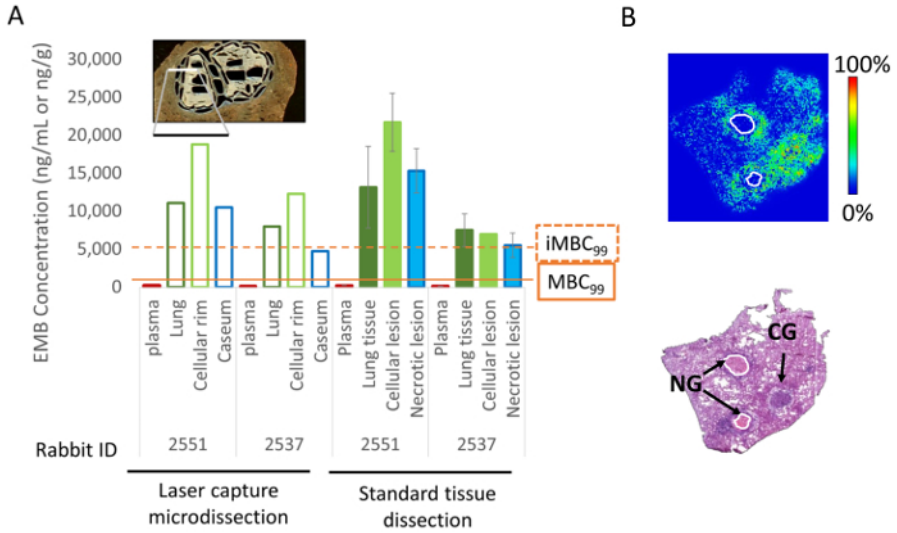

Figure 3: Example LCM-LC/MS dataset from lesion biopsies taken from two rabbits dosed with 100 mg/kg EMB for 7 days. (A) Favorable penetration of the drug into all lung and lesion compartments was observed. Drug concentrations quantified by LCM-LC/MS (hollow bars)/MS were in strong agreement with those quantified from homogenized dissected lesions by standard LC/MS (solid bars, mean ± standard deviation, n = 3 - 8). The minimum concentrations required to kill 99% of extracellular replicating bacilli (MBC_{99}) and 99% of intracellular bacilli in macrophages ($iMBC_{99}$) are indicated. **(B)** Top panel: MALDI-MS image showing the distribution of EMB $[M+H]^+$ ion (*m/z* 205.193) within an adjacent tissue section. Note that the MALDI-MS image suggests poor penetration of EMB into the caseum due to the present drug concentrations being below the lower limit of detection (LOD) of the technique. However, the spatial specificity and superior LOD of LCM-LC/MS show the drug is reaching sterilizing concentrations within all lesion compartments including the caseum. Lower panel: Hematoxylin & Eosin stained tissue section directly adjacent to the section used for MALDI MSI. Cellular (C) and necrotic granulomas (NG) were present in the tissue. The caseum core is outlined in white. Adapted from Zimmerman *et al.*[12] Please click here to view a larger version of this figure.

Dissected area (μm^2)	Measured EMB conc. (ng/g) (n = 3)	EMB recovery (%) (n = 3)
3 million	10633 (±404)	106 (±4)
5 million	10057 (±1132)	101 (±11)
10 million	10563 (±1128)	105 (±11)

Table 1: Extraction efficiency of the LC-LC/MS method for quantifying EMB in lung. Complete recovery of EMB was observed in the EMB spiked lung homogenate dissected tissues for all evaluated tissue volumes.

Rabbit ID	LC/MS EMB (ng/g)	LCM-LC/MS EMB (ng/g)	Difference (%)
899	2910	3320	14
904	2010	1870	-7
911	2150	2370	9

Table 2: Comparison of EMB quantification in uninvolved lung biopsies from 3 dosed rabbits by LC/MS and LCM-LC/MS. Equivalent drug concentrations were detected by both methods and no loss of signal due to degradation or extraction during the LCM-LC/MS process was observed.

Discussion

Spatially-resolved quantification of drugs within pulmonary TB lesions is required to determine whether drug exposure reaches sterilizing concentrations to bacterial populations residing within the different lesion compartments. The LCM-LC/MS method described here enables absolute quantification of anti-TB drugs within all lesion compartments, including the bacteria-rich caseum, using only 1 - 3 tissue sections in total. Traditional tissue homogenization and LC/MS approaches for drug quantification in tissue often lack the spatial specificity to resolve specific lesion compartments and even when it is possible, there is significant potential to cross contaminate cellular and necrotic lesion areas during the manual extraction process.

The LCM-LC/MS approach has several key advantages to mass spectrometry-based imaging technologies. Primary among these are the fully quantitative capabilities of the method and the additional sensitivity of the LC/MS analysis due to resolving drug from interfering/suppressing endogenous species via chromatographic separation. However, MALDI-MSI provides more detailed spatial information regarding drug distribution. As both MALDI-MSI and LCM-LC/MS/MS require the same sample preparation steps to generate frozen tissue sections, the two techniques can be performed in tandem on the same tissue biopsy providing full quantification alongside highly-detailed imaging of drug distribution. An example of the requirement for higher analytical sensitivity is shown in **Figure 3**. Only very low levels of EMB signal were detected in the central necrotic granuloma region of the MALDI MS image (shown in **Figure 3B**), suggesting caseum penetration of the drug is poor. However, due to the superior limit of detection of LCM-LC/MS the drug was clearly demonstrated to reach sterilizing concentrations within that compartment and the drug concentration present was predominantly undetectable by MALDI (**Figure 3A**).

Tissue staining protocols are typically used prior to LCM for proteomic applications to enable easy identification of tissue areas and the cell populations located within. Routine histochemical stains such as H&E are not compatible with drug quantification, as the many solvent washing steps involved would delocalize the drug from the tissue section. Adjacent cut sections can be stained with H&E and used as a guide for microdissection. However, this is not usually necessary, as the lesion compartments can be optically resolved under the standard LCM brightfield microscope (**Figure 2**).

Optimization of the sectioning and microdissection steps is crucial for successful lesion quantification. During development of the method, we evaluated two different membrane materials, polyethylene terephthalate (PET) and polyethylene naphthalate (PEN), as substrate for mounting the tissue sections for microdissection. PEN membranes suffered from increased static charging in comparison to PET membranes and approximately 30% of all dissected tissue areas were lost due to static attraction between the dissected tissue region and the membrane. For this reason, PET membranes were chosen for future dissections due to the significantly reduced static attraction observed (only 5% of regions dissected from PET membranes were lost during the LCM process).

The stability of drugs within tissue sections is an important consideration when designing an LCM-LC/MS study. During the sectioning and dissection process, the tissue sections are exposed to lab temperature and light for a period of at least 1 hour. Other issues to consider include the extraction efficiency of the drug from the tissue sections, as there is no homogenization step involved (extraction is only performed by vortex mixing and sonication). In our experience, the extraction does not suffer from the lack of tissue homogenization due to the high extraction solvent to tissue ratio used and the thinness of the tissue sections dissected. Our observations are in agreement with a previously-described online LCM-LC/MS method developed to quantify propranolol in microdissected tissue sections of brain and liver, where complete extraction of drug from tissue was observed from 20 µm and 40 µm thick sections[10]. We validated the efficiency of drug extraction and the drug stability during the LCM process by directly comparing lung tissue concentrations (from the same rabbit and lung lobe) analyzed by LCM-LC/MS with concentrations determined by standard tissue homogenization and LC/MS (an example is shown in **Table 1**). This comparison allows us to determine whether any change of signal is occurring between the two methodologies.

The density of dissected tissue regions is also an important consideration when quantifying drug levels based upon the tissue surface area or volume. This is of particular concern for lung and lesions biopsies, in which the uninvolved lung tissue areas have an overall lower density than cellular and caseum (less tissue covering the same relative surface area) due to the presence of multiple open bronchioles and alveolar spaces. The effects of this difference in density can be mitigated by carefully drawing around the open spaces to avoid including them within the cumulative surface area of collected tissue (as shown in **Figure 2**).

An additional limitation is that the presented LCM-LC/MS method only quantifies total drug concentration within the isolated tissue (in common with all tissue homogenization, solvent extraction, and LC/MS quantification approaches) and does not resolve protein-bound drug concentrations from unbound fractions. Microdialysis is an alternative approach for quantifying unbound drug within tissue and enables accurate quantification of free drug concentrations reaching extracellular populations of bacteria[13]. However, the technique would be best applied as a complementary approach, as it lacks the spatial specificity of LCM-LC/MS and only quantifies soluble drug levels within tissue extracellular fluid, not the intracellular content.

We have combined, for the first time, LCM and LC/MS to spatially quantify antibiotics at the site of tuberculosis infection. The methodology is tremendously powerful since it can be applied to any small molecule drug used in any disease state. Indeed, we have recently quantified an antifungal drug candidate in a mouse model of abdominal candidiasis[14]. Differential drug partitioning into heterogeneous tumor compartments (including necrotic cores) is a primary concern in the treatment of cancer, and a critical area of research in cancer drug discovery[15]. LCM-LC/MS is ideally suited to approach these questions. Furthermore, LCM-LC/MS can be used for biomarker discovery to quantify metabolic, lipidomic and proteomic changes occurring in tissue regions and cell populations during disease pathogenesis.

Disclosures

The authors have nothing to disclose.

Acknowledgements

We thank Paul O'Brien, Marizel Mina and Isabella Freedman for animal experiments, Jacquie Gonzalez and Danielle Weiner from NIH/NIAID for help with gamma irradiation of rabbit tissues prior to laser capture microdissection and Jansy Sarathy for manuscript thoughts and advice. This work was supported by funding from the Bill and Melinda Gates Foundation (OPP1174780) and NIH shared instrumentation grant 1S10OD018072. We thank Eliseo A. Eugenin for providing access to the Leica LMD 6500 microscope and sharing expertise and advice. The purchase, and ongoing support of, the LMD 6500 was funded by The National Institute of Mental Health grant, MH096625, the National Institute of Neurological Disorders and Stroke, NS105584, PHRI funding (to E.A.E) and GSK contributions (to E.A.E).

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