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TITLE:

Validated LC-MS/MS panel for Quantifying 11 Drug-Resistant TB Medications in Small Hair Samples

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SUMMARY:

Current methods of analyzing patients' adherence to complex drug resistant-tuberculosis (DR-TB) regimens can be inaccurate and resource-intensive. Our method analyzes hair, an easily collected and stored matrix, for concentrations of 11 DR-TB medications. Using LC-MS/MS, we can determine sub-nanogram drug levels that can be utilized to better understand drug adherence.

ABSTRACT

Drug resistant-tuberculosis (DR-TB) is a growing public health threat, and assessment of therapeutic drug levels may have important clinical benefits. Plasma drug levels are the current

gold standard assessment, but require phlebotomy and a cold chain, and capture only very recent adherence. Our method uses hair, a matrix that is easily collected and reflective of long-term adherence, to test for 11 anti-TB medications. Previous work by our group shows that antiretroviral drug levels in hair are associated with HIV outcomes. Our method for DR-TB drugs uses 2 mg of hair (3 cm proximal to the root), which is pulverized and extracted in methanol. Samples are analyzed with a single LC-MS/MS method, quantifying 11 drugs in a 16 min run. Lower limits of quantification (LLOQs) for the 11 drugs range from 0.01 ng/mg to 1 ng/mg. Drug presence is confirmed by comparing ratios of two mass spectrometry transitions. Samples are quantified using the area ratio of the drug to the deuterated, ^{15}N-, or ^{13}C-labeled drug isotopologue. We used a calibration curve ranging from 0.001-100 ng/mg. Application of the method to a convenience sample of hair samples collected from DR-TB patients on directly observed therapy (DOT) indicated drug levels in hair within the linear dynamic range of nine of the eleven drugs (isoniazid, pyrazinamide, ethambutol, linezolid, levofloxacin, moxifloxacin, clofazimine, bedaquiline, pretomanid). No patient was on prothionamide, and the measured levels for ethionamide were close to its LLOQ (with further work instead examining the suitability of ethionamide's metabolite for monitoring exposure). In summary, we describe the development of a multi-analyte panel for DR-TB drugs in hair as a technique for therapeutic drug monitoring during drug-resistant TB treatment.

INTRODUCTION:

In the twenty-first century, drug-resistant TB (DR-TB) is an evolving catastrophe for already weak national TB control programs, with confirmed cases doubling in the past 5 years alone, accounting for nearly one-third of all deaths related to antimicrobial resistance globally[1,2]. Successful treatment of DR-TB has conventionally required longer and more toxic second-line regimens than treatment for drug-sensitive TB. Moreover, patients with DR-TB often have significant pre-existing challenges to adherence, which contributed to the emergence of resistance initially[3].

Unlike HIV infection where viral loads can be used to monitor treatment, surrogate endpoints of treatment response in TB are delayed and unreliable on an individual level[4]. Monitoring patient adherence, an important predictor of subtherapeutic anti-TB drug concentration and treatment failure, is also challenging. Self-reported adherence suffers from recall bias and the desire to please providers[5,6]. Pill counts and medication event monitoring systems (MEMS) can be more objective[7] but do not measure actual drug consumption[8-10]. Drug levels in biomatrices can provide both adherence and pharmacokinetic data. Therefore, plasma drug levels are commonly used in therapeutic drug monitoring[11,12]. In the context of drug adherence monitoring, however, plasma levels represent short-term exposure and are limited by significant intra- and inter-patient variability when determining appropriate adherence reference range. "White coat" effects, where adherence improves prior to clinic or study visits, further complicates the ability of plasma levels to provide accurate drug adherence patterns[13].

Hair is an alternative biomatrix that can measure long-term drug exposure[14,15]. Many drugs and endogenous metabolites incorporate into the hair protein matrix from the systemic circulation as hair grows. As this dynamic process continues during hair growth, the amount of drug

deposited in the hair matrix depends on the continuous presence of the drug in circulation, making hair an excellent temporal readout of drug intake. Hair as a biomatrix has the additional advantage of being easily collected without the need for cold chain for storage and shipment compared to blood. Moreover, hair is non-biohazardous, which provides additional feasibility advantages in the field.

Hair drug levels have long been used in forensic applications[16]. Over the last decade, hair antiretroviral (ARV) levels have demonstrated utility in assessing drug adherence in HIV treatment and prevention, to which our group contributed. ARV levels in hair have been shown to be the strongest independent predictors of treatment outcomes in HIV infection[17-21]. To determine whether hair levels of DR-TB patients will have the same utility in predicting treatment outcome, we used LC-MS/MS to develop and validate a method for analyzing 11 DR-TB medications in small hair samples. As an initial assessment of the assay's performance, we measured DR-TB drugs levels in a convenience sample of patients with DR-TB receiving directly observed therapy (DOT) in the Western Cape, South Africa[22].

PROTOCOL:

All patients provided written informed consent prior to hair sample collection. We obtained Institutional Review Board approval from the University of Cape Town and the University of California, San Francisco.

1. Hair sampling

1.1 Obtain written informed consent.

1.2 Use clean scissors to cut approximately 20-30 scalp hair strands from the occipital region as close to the scalp as possible.

1.3 Place tape around the distal side of the hair to indicate directionality. Fold hair sample into an aluminum foil square and store at room temperature. Label the distal end of the hair to avoid possible contamination from additional handling of the proximal end.

1.4 In addition to patient samples, collect "blank hair": a scalp hair sample from someone who has not taken TB medication. Collect a large amount (>30 mg blank hair for each 20 patient samples).

2. Drug extraction

2.1 Label bead tubes. Each patient sample requires one tube. Label 12 tubes from "C0" to "C11", one for each of the 12 calibration points. Label a tube for Low Quality Control, a tube for Medium Quality Control, and a tube for High Quality Control. Lastly, label a tube for Matrix Blank.

2.2 Open the aluminum foil square containing hair sample. If hair sample is longer than 3 cm, cut hair at 3 cm from the proximal end and use that proximal portion for analysis.

2.3 Weigh 2 mg of the hair sample into a bead tube.

2.4 Weigh 2 mg of blank hair into 16 additional bead tubes. These will be used as the calibration points, quality controls, and matrix blank. The tubes will follow the same extraction procedure as the patient samples, aside from being spiked with drug reference standards at levels indicated in steps 2.8 and 2.9.

2.5 Place all of the bead tubes into the homogenizer. Run homogenizer at speed 6.95 m/s. Run for two cycles of 30 s each, with a 15 s rest period in between the two cycles.

2.6 Make internal standard mix and add it to the samples.

2.6.1 Add ~40 mL of methanol to a 50 mL volumetric flask.

2.6.2 In amber glass vials, make the mixes of the internal standards shown in **Table 1**, using methanol as the solvent.

NOTE: Methanol is very volatile. Leave all vials capped during this process to prevent loss due to evaporation.

2.6.3 From those mixes, add the volume shown in **Table 1** to the 50 mL volumetric flask. Then, fill the flask to 50 mL with methanol.

2.6.4 Cap and mix the volumetric flask. Add 500 µL of the mixture to each of the pulverized hair tubes, except for the matrix blank tube. Add 500 µL methanol to the matrix blank tube.

2.7 Make reference standard mixes.

2.7.1 Constitute neat reference standards of the following drugs with methanol to get the concentration shown in the table in step 2.7.2. Only 1 mL of the following concentrations is necessary.

2.7.2 Add 1768 µL of methanol to a vial, and then add the amounts of reference standard listed in Table 2 to the same vial to get 2 mL final volume. Label this vial “Ref Std Mix 1x”. Vortex.

2.7.3 Spike 100 µL from “Ref Std Mix 1x” into 900 µL methanol in a new vial. Label this vial “Ref Std Mix 10x”. Vortex.

2.7.4 Spike 100 µL from “Ref Std Mix 10x” into 900 µL methanol in a new vial. Label this vial “Ref Std Mix 100x”. Vortex.

2.7.5 Spike 100 µL from “Ref Std Mix 100x” into 900 µL methanol in a new vial. Label this vial “Ref Std Mix 1000x”. Vortex.

2.8 Spike the calibration curve tubes by adding the amount of Ref Std Mix described in **Table 3**.

2.9 Create QC mixes and spike quality control tubes.

2.9.1 Label 5 vials “QC-A” through “QC-E”.

2.9.2 Add the following amounts of methanol to the five labeled vials:
QC-A: 990 µL
QC-B: 940 µL
QC-C: 950 µL
QC-D: 980 µL
QC-E: 950 µL

2.9.3 Using the 1 mg/mL drug stocks created in step 2.7.1, add 10 µL to the specific vials listed below. For the BDQ and CLF stocks, which are at 0.5 mg/mL, add 20 µL to the vials listed below.
QC-A: PTH
QC-B: EMB, CLF, BDQ, PTM
QC-C: INH, LFX, LZD, MFX, PZA
QC-D: PTH, EMB
QC-E: CLF, BDQ, PTM

NOTE: Some drugs are present in multiple mixes.

2.9.4 Label a vial as “QC-A df100”. Dilute 10 µL of QC-A into 990 µL methanol.

2.9.5 Label a vial as “Low QC stock”. Add 1832 µL methanol to this vial. Add the amounts of QC mixes detailed below:
QC-A df100: 80 µL
QC-B: 8 µL
QC-C: 80 µL

2.9.6 Label a vial as “Mid QC stock”. Add 760 µL methanol to this vial. Add the amounts of QC mixes detailed below:
QC-A df100: 800 µL
QC-B: 40 µL
QC-C: 400 µL

2.9.7 Label a vial as “High QC stock”. Add 1376 µL methanol to this vial. Add the amounts of QC mixes detailed below:
QC-D: 160 µL
QC-E: 320 µL

MFX, 1mg/mL stock: 16 µL
INH, 1mg/mL stock: 32 µL
LFX, 1mg/mL stock: 32 µL
LZD, 1mg/mL stock: 32 µL
PZA, 1mg/mL stock: 32 µL

2.9.8 Spike 10 µL Low QC stock into the Low QC bead tube.

2.9.9 Spike 10 µL Mid QC stock into the Mid QC bead tube.

2.9.10 Spike 10 µL High QC stock into the High QC bead tube.

2.10 Place all tubes in hot shaker for 2 h at 37 °C. Shaking should be slow enough that the water does not splash up on to the tubes.

2.11 Remove tubes from shaker. Transfer liquid from bead tubes into new microcentrifuge tubes. Label these microcentrifuge tubes in the same way.

2.12 Add 500 µL methanol to the old tubes. Cap and vortex.

2.13 For a second time, transfer liquid from the bead tubes into the corresponding microcentrifuge tube. It is okay to transfer pulverized hair. This will eventually be centrifuged out.

2.14 Centrifuge the microcentrifuge tubes for 10 min at 2,800 x *g*.

2.15 Carefully remove the liquid and transfer it into new centrifuge tubes with corresponding labels. Be careful not to disturb or transfer the hair pellet.

2.16 Evaporate the liquid in the centrifuge tubes to dryness at 32 °C.

2.17 Reconstitute the samples by adding 200 µL of mobile phase A (HPLC-grade water with 1% formic acid) to the dry tubes. Vortex.

2.18 Transfer the liquid to amber vials with 250 µL inserts.

3. LC-MS/MS preparation

3.1 Make one liter of mobile phase A (HPLC-grade water with 1% formic acid) by adding some HPLC-grade water to a one-liter volumetric flask. Then add 10 mL of >95% formic acid to that flask, and then fill to the line with HPLC-grade water.

3.1.2 Make one liter of mobile phase B (acetonitrile with 0.1% formic acid) by adding some acetonitrile to a one-liter volumetric flask. Then add 1 mL of >95% formic acid to that flask, and

then fill to the line with acetonitrile.

3.2 Install a 2 x 100 mm column with 2.5 µm particle size and 100 Å pore size with polar endcapped, ether-linked phenyl beads fully made of porous silica in the column compartment. Ensure that column also has manufacturer recommended guard cartridge installed.

3.3 Open the data acquisition software and double-click Hardware Configuration. Highlight **LCMS** and click **Activate Profile**.

3.3.1 Click **New Sub-Project**, or, if other sub-projects already exist, click **Copy Sub-Project**. Name the sub-project.

3.3.2 Click **New Document**. Double-click **Acquisition Method**. Click **Mass Spec** within the Acquisition method window.

3.3.3 Change the **Scan Type** dropdown to **MRM (MRM)**. Make sure **Polarity** is set to **Positive**.

3.3.4 Click **Import List** and select the .csv file **MDR-TB LCMS method transitions.csv** that is included in Supplemental Materials.

3.3.5 Scroll down and set the **Duration** to 16.751 min. The appropriate cycle time and number of cycles will auto-populate.

3.3.6 In left sidebar, click **Integrated Valco Valve**. Make sure that position name for step 0 is A. In **Total Time (min)** column, type in 0.4 in the first row and 13 in the second row.

3.3.7 In the **Position** column, set row one to B and row two to A.

3.3.8 In left sidebar, click **Binary Pump**. Set the gradient and flow rate table according to **Table 4**.

3.3.9 In left sidebar, click **Autosampler**. Change injection volume to 10 µL. Click Temperature control enabled and set to 4 °C.

3.3.10 In left sidebar, click **Column Compartment**. Set both right and left temperatures to 50 °C.

3.3.11 Close and save method.

3.4 Create batch by clicking **New Document** and selecting **Acquisition Batch**. Type in a set name and select the newly created method from the dropdown bar.

3.4.1 In a spreadsheet, create a batch that follows this order: calibration curve, quality controls, patient samples, calibration curve, quality controls, patient samples, calibration curve, quality controls. Add solvent blank injections at the start and end of the run, as well as before and after

the calibration curve, quality controls and patient samples. Put at least eight solvent blank injections after injections of the calibration curve and high quality control vial in order to reduce analyte carryover.

NOTE: More solvent blank injections may need to be added depending on column age.

3.4.3 In the column adjacent to the sample names, type in the appropriate autosampler position for the corresponding vial.

3.4.2 Click **Add Set**. In the pop-up window, type in the number of samples in the batch.

3.4.3 Copy and paste sample names and vial locations from the spreadsheet to the newly created batch.

3.4.4 Go to the **Submit** tab. Click **Submit** button.

3.5 Equilibrate system by inserting solvent line A into mobile phase A and solvent line B into mobile phase B. Open the purge valve on the binary pump.

3.5.1 Set solvent composition to 50% B at 4 mL/min flow rate. Turn binary pump on.

3.5.2 After 5 min, decrease flow to 0.3 mL/min. Close the purge valve. Check for any leaks.

3.5.3 In the software, press **Equilibrate** on the top toolbar. Set time to >5 min, press **OK**.

3.5.4 After the instrument has equilibrated, the modules in the bottom right of the window will appear green. Check that pressure has stabilized, and then start the batch by clicking **Start Sample**.

4. Data analysis

4.1 After the batch is completed, open the quantitation software. Click the wand icon to create a new **Results** table.

4.1.1 Click **Browse** to navigate to the appropriate folder, and then highlight the data file and click the right-pointing arrow to move the data into the **Selected** area. Click **Next**.

4.1.2 Select **Create New Method** and click **New**. Input new quantitation method name and press **Save** and then **Next**.

4.1.3 Select the first injection of the middle calibration point. Press **Next**.

4.1.4 Tick mark all the transitions of the internal standards in the **IS** column.

4.1.5 For the quantifier transitions for the reference standards, select the corresponding IS in the **IS Name** column. Click **Next**.

4.1.6 Scroll through the transitions to assure that the automatically selected retention time is accurate. Make sure that **Gaussian Smoothing** is set to 1.5. All other default settings can remain as is (i.e., Noise Percentage 100%, Baseline Sub. Window 2.00 min, Peak Splitting 2 points).

NOTE: If wanted, modify automatic integration parameters at this point. Since these parameters change based on instrument setup, we have not included ours here.

4.1.7 Click **Finish** to apply the quantitation method to the batch.

4.2 Click the top left **Displays the peak review** button to view chromatograms. Navigate through the transitions using the left sidebar. Scroll through each injection of every quantifier transition and manually integrate the correct peak if necessary.

4.2.1 To manually integrate a peak, click on the **Enable manual integration mode** button, zoom into the chromatogram by clicking and dragging along the x- or y-axis, and then draw a line from one baseline to the other baseline, defining the peak. **Figure 3** shows two chromatograms: one that has INH, and therefore has been manually integrated, and another that does not have INH.

NOTE: All injections must be integrated using the same parameters. Peak width can provide a guideline for adhering to these parameters, but sometimes peak width will differ. To quantify a peak, the retention time must be within ±0.15 min of the expected retention time for that analyte (as defined by the reference standard peaks), qualitatively confirmed as having the expected quantifier to qualifier ratio (as shown in **Figure 2**), and have a signal-to-noise ratio of greater than 10.

4.3 In the **Sample Type** column, set the calibration curve injections (with the exception of the blank calibration curve injections) to **Standard**. Set the quality control injections to Quality Control. Leave the remaining injections as **Unknown**.

NOTE: This will be set across all transitions.

4.4 In the **Actual Concentration** column, type in the concentrations found in **Table 5** for all calibration curve and quality control injections.

4.5 Click the second from top left **Displays the calibration curve** button. Click the **Regression** button.

4.6 Set **Weighting Type** to **1/x** and press **OK**.

4.7 Validate the calibration curve and quality control samples to assure that the batch ran

successfully.

4.7.1 For each quantifier reference transition (not internal standard transitions), look at each calibration curve injection accuracy (in the **Accuracy** column). At least two-thirds of the calibration points must have an accuracy within 80-120%.

4.7.2 For calibration points far outside of the expected accuracy, the injection may be an outlier. Exclude outliers if their calculated concentration is more than two standard deviations away from the other two injections of that vial. Clicking the "**et peak to 'not found** button above each chromatogram.

4.7.3 Check that the R-value displayed above the calibration curve is >0.975.

4.7.4 Check that all quality control injections have an accuracy within 80-120%.

4.8 If all above conditions are satisfied, the batch has passed, and samples can be quantified. Click **Edit** in the toolbar, then click **Copy entire table**. Paste the table in a spreadsheet.

4.9 Take the average of the calculated concentration of the two sample injections to determine the reported concentration of each sample.

REPRESENTATIVE RESULTS:

An illustration of a chromatogram with confirmed levels of all 11 DR-TB drugs is shown in **Figure 1**. The retention time for each analyte can change when using different instruments and columns, so the exact retention time should be determined individually.

The Extracted Ion Chromatograms (EICs) for one particular drug (isoniazid, INH) in one of the calibrators (blank hair sample spiked with DR-TB drug reference standards) are shown in **Figure 2**. The quantifier and qualifier transitions are used to qualitatively confirm the presence of the drug, as the ratio between area of quantifier and area of qualifier remains constant across samples. The internal standard is also monitored to ensure that each sample injection is normalized.

For purposes of demonstration, we analyzed a convenience sample of 15 hair samples among a total study population of 96 patients taking DR-TB drugs under DOT conditions from Western Cape, South Africa. **Table 6** presents representative levels of DR-TB drugs across the lowest and highest levels measured for each analyte. Although data for 15 patient samples are presented, each analyte did not have 15 levels reported because each patient is on a different combination of DR-TB medications. None of the patients were on prothionamide, and only a single patient was taking pretomanid.

FIGURE AND TABLE LEGENDS:

Figure 1. An illustration of a representative chromatogram showing peaks of the 11 analytes in the DR-TB method (EMB= ethambutol; INH= isoniazid; PZA= pyrazinamide; ETH= ethionamide; PTH= prothionamide; LFX= levofloxacin; MFX= moxifloxacin; LZD= linezolid; PTM= pretomanid; BDQ= bedaquiline; CLF= clofazimine). Because the sensitivity of the method for each analyte is different, INH, LZD, LFX, MFX and LZD were spiked at 20 ng/mg hair while BDQ, CLF, EMB, ETH, PTH and PTM were spiked at 2 ng/mg hair.

Figure 2. Two Extracted Ion Chromatograms (EICs) from an injection of calibration point 9 (C9), isoniazid (INH) at 20 ng/mg. The top EIC shows both the INH quantifier transition (blue, labeled INH-2) and the INH qualifier transition (red, labeled INH-3). The bottom EIC shows response of INH-d4, the internal standard (IS) used to quantify INH.

Figure 3. Screenshots of the process of quantitation. The top portion is a partial sample list showing injection data for one analyte (INH, isoniazid) across 12 calibration points (labeled C0-C11), three QC levels, and six samples. Bottom left portion is the calibration curve, ranging from 0.5 ng/mg –100 ng/mg. Opaque blue dots are calibration points. Transparent blue squares are quality control points. The R-value is shown in the top left (0.99722) with weighting 1/x. The two chromatograms in the bottom right illustrate a sample with INH (top chromatogram) and a sample without INH (bottom chromatogram).

Table 1. Concentration and amount of each internal standard to add to a 50 mL volumetric flask.

Table 2. Amount of each drug reference standard to add to "Ref Std Mix 1" vial.

Table 3. Amount of each Ref Std Mix intermediate to add to the 12 calibration points.

Table 4. The flow rate and mobile phase gradient used for each injection.

Table 5. Final concentration of analytes in each calibration point.

Table 6. Representative levels of drugs measured in 15 patients taking DR-TB medications under DOT. The limit of detection (LOD), lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) of the method for each drug are given for comparison.

DISCUSSION:

We report here the protocol for the method we developed and validated for quantifying 11 anti-TB medications utilized in the treatment of DR-TB in small hair samples using LC-MS/MS. No other method for quantifying these 11 drugs in hair has been previously developed, validated and published. Our method can quantify sub-nanogram levels of drugs in only 20-30 hair strands of approximately 3 centimeters (cm) in length (~2 mg) and has already been validated[22]. The low weight of hair analyzed means that patients involved in the study can participate discreetly and potentially return for repeat testing without fear of exposing bald scalp. We have previously published data on the association between DR-TB drug levels in hair

and DR-treatment outcomes[23]. Therefore, the development and validation of this multi-analyte panel method represents a significant advance in the field of DR-TB therapeutic drug monitoring.

Hair requires different homogenization techniques than those required with liquid biomatrices. Pulverization of hair strands allowed efficient access of extraction solvent to analytes in the hair matrix. Thus, one important feature of our method is the quick and easy extraction process of drugs from hair using the pulverized samples. Incubation time during the extraction process is only two h, due to the large accessible surface area of pulverized hair, and there is no clean up step, due to the small sample size (2 mg). Care must be taken, though, to limit drug degradation during the extraction process. The protocol uses a two-cycle pulverization, with a 45 s cooling period in between the cycles. This process avoids overheating and potentially degrading the drugs in the hair.

Unlike many hair analyses for drugs of abuse, this method does not use a washing step. DR-TB drugs come in capsule or tablet form, limiting possible sources of external contamination and the subsequent need to wash hair prior to analysis. Future studies could analyze wash solvent from DR-TB patient hair to assess external contamination.

Although hair pulverization promotes efficient drug extraction, it has its own limitations. Our laboratory has found that if hair is pulverized in the bead ruptor and left at room temperature, the concentration of some of the 11 drugs decreases over weeks and months. This may be due to the large surface area of the pulverized hair exposed to the atmosphere that can promote oxidation and other degradation reactions. If a stability study of the drugs in hair is desired, hair can be cut with scissors into small segments of <1 cm, homogenized by hand, and then left at room temperature for weeks or months during the stability study. When this cut hair is pulverized on the day of analysis, we have not observed any significant drug degradation over time. Hence, in performing the described protocol, we recommend that hair be pulverized on the day it is extracted. Likewise, all drug mixes below 10 µg/mL concentrations should be prepared on the day of extraction.

No previously published methods are available to assess the suitability of the linear dynamic ranges (LLOQ-ULOQ) we established for each TB drug in the multi-analyte method. However, the convenience sample of hair samples from Western Cape, South Africa, indicates the suitability of the linear dynamic range of this method. With the exception of ethionamide, pretomanid, and prothionamide, more than 95% of the drug levels we measured in these patients are within the linear dynamic range of each analyte. Only one patient was taking pretomanid (which was detected), and no patients were taking prothionamide. For ethionamide, we hypothesize that the drug may not deposit to the hair matrix well, as our LOD is 0.01 ng/mg hair (or 10 pg/mg hair) and yet only one of the eight patients taking ethionamide has levels greater than 0.02 ng/mg hair. Further examination is warranted to determine the pharmacokinetics of different TB drugs in hair. For example, a potential alternative for monitoring drugs like ethionamide is to develop a method targeting their metabolite(s) instead. We have made a similar observation for delamanid, a novel DR-TB medication, which was

initially part of this panel. A method targeting delamanid's metabolite is currently in the process of being validated in our laboratory, because the metabolite is found in higher concentrations than the parent drug. The same procedure can be performed for ethionamide. The drug concentrations in **Table 6** are presented as a group because the individual results and clinical outcomes are not the focus of this method paper. Individual assessment of this group of patients has been published elsewhere[23].

The patients contributing small hair samples for the demonstration study were administered a variety of drug regimens via DOT in an inpatient setting, and all regimens were documented according to nursing records during the inpatient period. However, as is common among DR-TB patients, previous, poorly documented drug regimens had also been administered prior to their inpatient stay. This led to detection of drugs in patient hair that were not noted on their inpatient records. Therefore, we could not use these samples to determine specificity of the method, as we could not determine if these samples were truly false positives. Instead, we tested hair from patients who were not taking DR-TB drugs. No DR-TB drugs were detected in these samples, indicating that the method is specific.

Although our method demonstrates the utility of using hair in measuring DR-TB drugs, hair analysis has its own set of limitations. Because hair is a solid matrix, spiking of drug reference standards during method validation does not allow for the standards' full integration into the matrix as with urine and blood. Thus, recovery assessment is limited to detection of drug after spiking onto the solid matrix, and not actual retrieval from the matrix. Likewise, because hair is an alternative matrix that is still being explored for testing, no readily available reference ranges for medications are available to assess method suitability. More pharmacokinetic studies on the incorporation of drugs into hair will be useful to further understand the utility of hair drug levels in adherence monitoring. Finally, the proper collection of hair samples at field sites has its own unique challenges. While collection and storage of hair samples requires fewer resources than other biomatrices, care must be taken to identify the distal and proximal ends of any hair strands longer than 2 cm. Longer hair strands may have different drug concentrations along the strand, depending on medication use over time. Proper labeling allows for analysis of specific segments of the strands; in the case of our method, the three centimeters of hair closest to the scalp was used to determine the most recent data on medication adherence. Proper labeling requires training and quality assurance procedures at the sites.

In summary, we have developed the first multi-analyte panel for analyzing TB medications used for DR-TB via LC-MS/MS in small hair samples. Given the feasibility of collecting and storing hair in resource-limited settings, our method represents a potentially significant advance in the field of TB therapeutic drug monitoring. Objective measures of drug exposure that take into account both adherence and individual pharmacokinetic variability may provide early indication of ineffective treatment regimens, thereby aiding both individual treatment as well as limiting community transmission of DR-TB[24].

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DISCLOSURES

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Figure 1

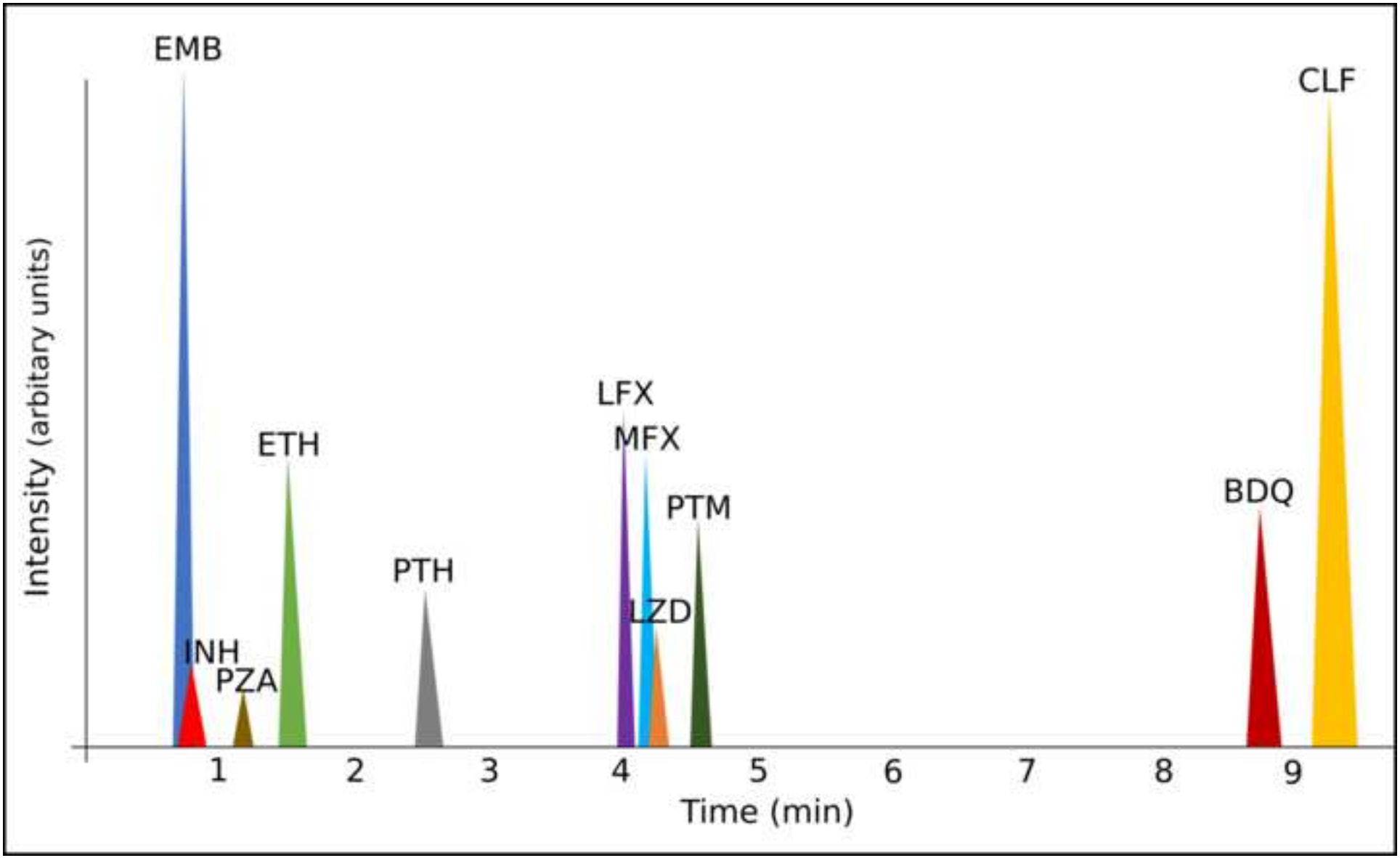

Figure 2

Click here to access/download;Figure;fig 2.png

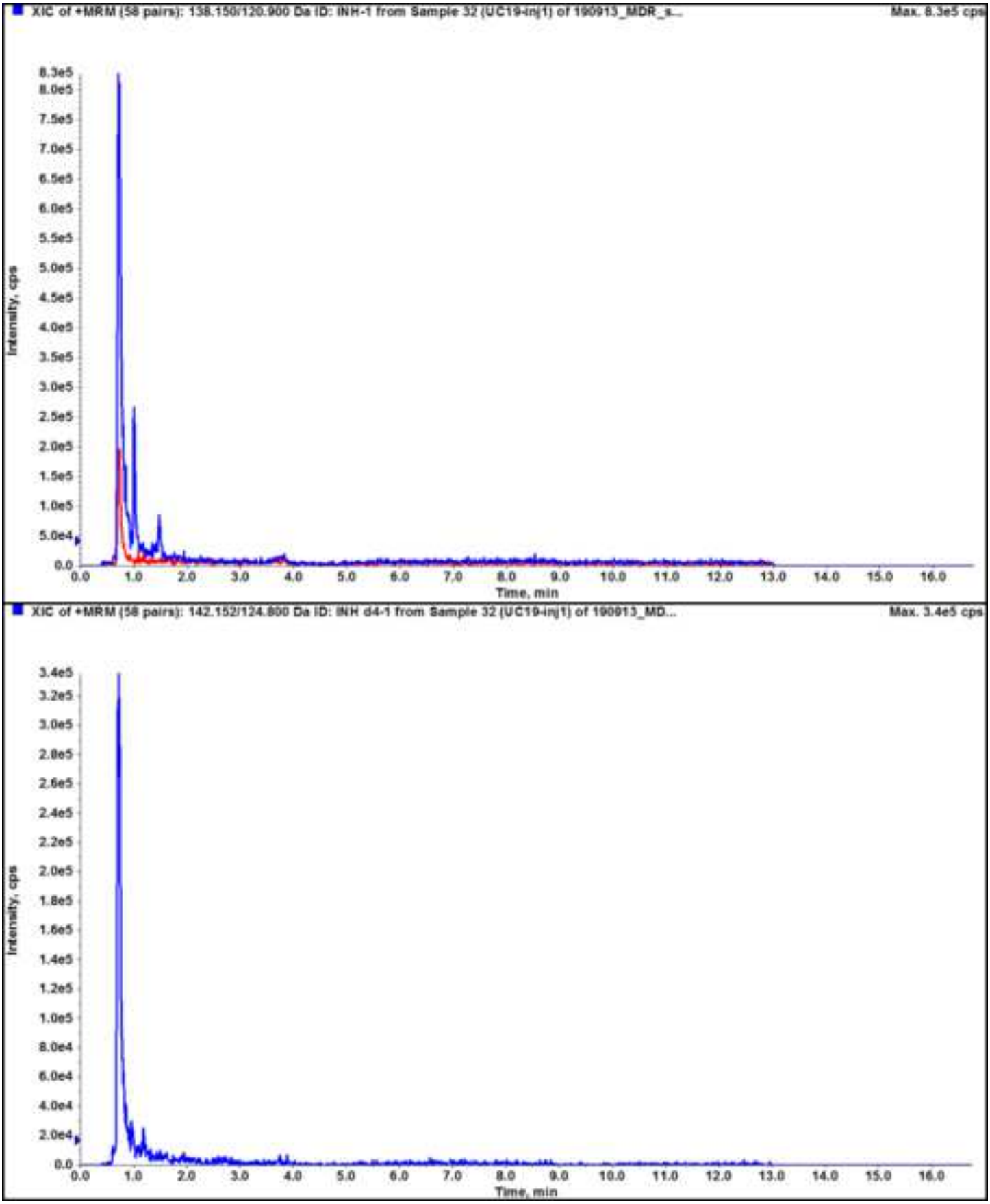

Figure 3

Click here to access/download;Figure;fig 3.png

Sample Name	Component Name	Calculated Concentration	Accuracy	Actual Concent	Signal / Noise	Area	IS Area	Area Ratio	Retentio Time	Start Time	End Time	Total Width	IS Total Width	Integration Type	Sample Type	IS Actual Concent	IS Name	Concentra Ratio	Acq. Method Name	Acquisition Date & Time
Solvent Blank-2	INH-2	4.545	N/A	N/A	31.5	1.901e5	1.568e5	1.214	0.64	0.51	0.80	0.29	0.23	Baseline	Unknown	1.00	INH-d4-2	N/A	TB Synergi 44-DLM_ETH_LZD1	9/13/2019 3:14:31 PM
Matrix Blank-1	INH-2	2.578	N/A	N/A	11.3	2.119e4	3.015e4	0.702	0.76	0.65	0.79	0.14	0.16	Valley	Unknown	1.00	INH-d4-2	N/A	TB Synergi 44-DLM_ETH_LZD1	9/13/2019 3:32:37 PM
C0	INH-2	N/A	N/A	N/A	N/A	N/A	4.953e5	N/A	N/A	N/A	N/A	N/A	0.28	N/A	Unknown	1.00	INH-d4-2	N/A	TB Synergi 44-DLM_ETH_LZD1	9/13/2019 3:50:45 PM
C1	INH-2	N/A	N/A	0.0500	N/A	N/A	4.187e5	N/A	N/A	N/A	N/A	N/A	0.27	N/A	Standard	1.00	INH-d4-2	0.05	TB Synergi 44-DLM_ETH_LZD1	9/13/2019 4:08:52 PM
C2	INH-2	N/A	N/A	0.1000	N/A	N/A	4.454e5	N/A	N/A	N/A	N/A	N/A	0.24	N/A	Standard	1.00	INH-d4-2	0.10	TB Synergi 44-DLM_ETH_LZD1	9/13/2019 4:26:58 PM
C3	INH-2	N/A	N/A	0.2000	N/A	N/A	5.158e5	N/A	N/A	N/A	N/A	N/A	0.28	N/A	Standard	1.00	INH-d4-2	0.20	TB Synergi 44-DLM_ETH_LZD1	9/13/2019 4:45:03 PM
C4	INH-2	0.526	105.12	0.5000	21.8	7.502e4	4.485e5	0.167	0.73	0.63	0.84	0.16	0.28	Manual	Standard	1.00	INH-d4-2	0.50	TB Synergi 44-DLM_ETH_LZD1	9/13/2019 5:03:09 PM
C5	INH-2	0.964	96.41	1.0000	30.5	1.369e5	4.868e5	0.281	0.73	0.65	0.84	0.20	0.22	Baseline	Standard	1.00	INH-d4-2	1.00	TB Synergi 44-DLM_ETH_LZD1	9/13/2019 5:21:14 PM
C6	INH-2	2.259	112.97	2.0000	41.3	2.409e5	3.894e5	0.619	0.72	0.65	0.90	0.26	0.23	Baseline	Standard	1.00	INH-d4-2	2.00	TB Synergi 44-DLM_ETH_LZD1	9/13/2019 5:39:19 PM
C7	INH-2	4.424	88.48	5.0000	50.2	5.604e5	4.735e5	1.183	0.73	0.64	0.90	0.26	0.30	Baseline	Standard	1.00	INH-d4-2	5.00	TB Synergi 44-DLM_ETH_LZD1	9/13/2019 5:57:26 PM
C8	INH-2	10.936	109.36	10.0000	68.2	1.181e6	4.101e5	2.879	0.73	0.63	0.89	0.28	0.27	Baseline	Standard	1.00	INH-d4-2	10.00	TB Synergi 44-DLM_ETH_LZD1	9/13/2019 6:15:35 PM
C9	INH-2	23.616	118.08	20.0000	81.3	2.789e6	4.505e5	6.192	0.72	0.65	0.88	0.24	0.24	Baseline	Standard	1.00	INH-d4-2	25.00	TB Synergi 44-DLM_ETH_LZD1	9/13/2019 6:33:43 PM
C10	INH-2	42.653	85.31	50.0000	83.3	4.211e6	3.875e5	11.140	0.73	0.65	0.87	0.22	0.25	Baseline	Standard	1.00	INH-d4-2	50.00	TB Synergi 44-DLM_ETH_LZD1	9/13/2019 6:51:47 PM
C11	INH-2	95.834	95.83	100.0000	115.6	1.102e7	4.471e5	24.995	0.72	0.65	0.90	0.26	0.26	Baseline	Standard	1.00	INH-d4-2	100.00	TB Synergi 44-DLM_ETH_LZD1	9/13/2019 7:09:51 PM
Solvent Blank-3	INH-2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Unknown	1.00	INH-d4-2	N/A	TB Synergi 44-DLM_ETH_LZD1	9/13/2019 9:33:24 PM
Matrix Blank-2	INH-2	14.078	N/A	N/A	14.1	3.461e4	9.382e3	3.687	0.74	0.69	0.84	0.17	0.14	Baseline	Unknown	1.00	INH-d4-2	N/A	TB Synergi 44-DLM_ETH_LZD1	9/13/2019 9:51:30 PM
QC low-1	INH-2	2.293	114.67	2.0000	34.7	1.805e5	3.035e5	0.628	0.73	0.69	0.78	0.15	0.26	Manual	Quality Control	1.00	INH-d4-2	2.00	TB Synergi 44-DLM_ETH_LZD1	9/13/2019 10:09:39 PM
QC mid-1	INH-2	10.361	103.61	10.0000	68.2	1.048e6	3.834e5	2.734	0.73	0.64	0.87	0.24	0.28	Baseline	Quality Control	1.00	INH-d4-2	10.00	TB Synergi 44-DLM_ETH_LZD1	9/13/2019 10:27:43 PM
Solvent Blank-4	INH-2	N/A	N/A	N/A	11.8	3.341e4	N/A	N/A	0.62	0.56	0.69	0.13	N/A	Baseline	Unknown	1.00	INH-d4-2	N/A	TB Synergi 44-DLM_ETH_LZD1	9/13/2019 11:48:32 PM
QC high-1	INH-2	66.954	83.69	80.0000	84.0	8.053e6	4.619e5	17.470	0.72	0.65	0.87	0.20	0.22	Baseline	Quality Control	1.00	INH-d4-2	80.00	TB Synergi 44-DLM_ETH_LZD1	9/14/2019 12:06:40 AM
Solvent Blank-5	INH-2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Unknown	1.00	INH-d4-2	N/A	TB Synergi 44-DLM_ETH_LZD1	9/14/2019 1:58:48 AM
UC19-sy1	INH-2	7.741	N/A	N/A	61.8	8.701e5	4.252e5	2.046	0.73	0.65	0.91	0.27	0.24	Baseline	Unknown	1.00	INH-d4-2	N/A	TB Synergi 44-DLM_ETH_LZD1	9/14/2019 2:16:55 AM
UC21-sy1	INH-2	N/A	N/A	N/A	N/A	N/A	2.491e5	N/A	N/A	N/A	N/A	N/A	0.28	N/A	Unknown	1.00	INH-d4-2	N/A	TB Synergi 44-DLM_ETH_LZD1	9/14/2019 2:35:01 AM
UC24-sy1	INH-2	N/A	N/A	N/A	N/A	N/A	3.307e5	N/A	N/A	N/A	N/A	N/A	0.20	N/A	Unknown	1.00	INH-d4-2	N/A	TB Synergi 44-DLM_ETH_LZD1	9/14/2019 2:53:09 AM
UC39-sy1	INH-2	2.669	N/A	N/A	32.9	1.877e5	2.868e5	0.726	0.74	0.64	0.89	0.21	0.23	Baseline	Unknown	1.00	INH-d4-2	N/A	TB Synergi 44-DLM_ETH_LZD1	9/14/2019 3:11:17 AM
UC63-sy1	INH-2	2.190	N/A	N/A	36.4	1.679e5	3.126e5	0.601	0.73	0.66	0.88	0.20	0.35	Baseline	Unknown	1.00	INH-d4-2	N/A	TB Synergi 44-DLM_ETH_LZD1	9/14/2019 3:29:24 AM
UC72-sy1	INH-2	6.679	N/A	N/A	64.4	6.405e5	3.664e5	1.770	0.73	0.65	0.87	0.22	0.24	Baseline	Unknown	1.00	INH-d4-2	N/A	TB Synergi 44-DLM_ETH_LZD1	9/14/2019 3:47:30 AM
Solvent Blank-6	INH-2	4.535	N/A	N/A	39.5	4.063e5	3.354e5	1.211	0.65	0.50	0.80	0.31	0.30	Baseline	Unknown	1.00	INH-d4-2	N/A	TB Synergi 44-DLM_ETH_LZD1	9/14/2019 4:05:36 AM

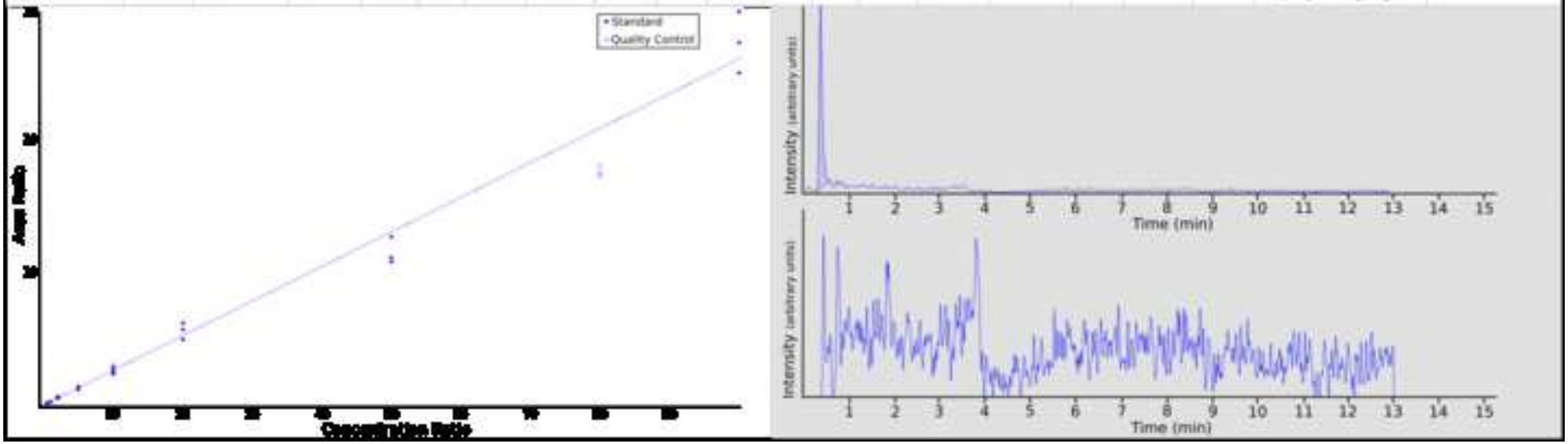

Figure 1 svg


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Figure 3 svg


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Table 1

Drugs in each mix	Concentration of each drug in mix	Volume of mix added to 50mL vol. flask
Mix 1: CLF-d7, EMB-d4	10 µg/mL	40 µL
Mix 2: LFX-d8, PTH-d5	10 µg/mL	10 µL
Mix 3: BDQ-d6, LZD-d3, MFX 13C-d3, OPC (IS for PTM)	10 µg/mL	20 µL
Mix 4: PZA 15N-d3	10 µg/mL	200 µL
Mix 5: INH-d4	10 µg/mL	100 µL

Table 2

Drug	Stock concentration	Volume added
BDQ	0.5 mg/mL	8 µL
CLF	0.5 mg/mL	8 µL
EMB	1 mg/mL	4 µL
PTH	1 mg/mL	4 µL
PTM	1 mg/mL	4 µL
INH	1 mg/mL	40 µL
LFX	1 mg/mL	40 µL
LZD	1 mg/mL	40 µL
MFX	1 mg/mL	40 µL
PZA	1 mg/mL	40 µL

Table 3

Label name	Vial drawn from	Volume added
C0	N/A	0 µL
C1	Ref Std Mix df1000	5 µL
C2	Ref Std Mix df1000	10 µL
C3	Ref Std Mix df1000	20 µL
C4	Ref Std Mix df100	5 µL
C5	Ref Std Mix df100	10 µL
C6	Ref Std Mix df100	20 µL
C7	Ref Std Mix df10	5 µL
C8	Ref Std Mix df10	10 µL
C9	Ref Std Mix df10	20 µL
C10	Ref Std Mix df1	5 µL
C11	Ref Std Mix df1	10 µL

Table 4

Total Time (min)	Flow Rate (µL/min)	A (%)	B (%)
0	450	95	5
0.3	450	95	5
2.3	450	0	100
5	550	0	100
11	550	0	100
11.1	550	95	5
13	450	95	5
16.75	450	95	5

Table 5

Calibration point	Actual concentration of BDQ, CLF, ETH, EMB, PTH, PTM (ng/mg)	Actual concentration of INH, LFX, LZD, MFX, PZA (ng/mg)
C0	0	0
C1	0.005	0.05
C2	0.01	0.1
C3	0.02	0.2
C4	0.05	0.5
C5	0.1	1
C6	0.2	2
C7	0.5	5
C8	1	10
C9	2	20
C10	5	50
C11	10	100

Table 6

Drug	LOD (ng/mg hair)	LLOQ (ng/mg hair)	ULOQ (ng/mg hair)
Bedaquiline	0.005	0.05	10
Clofazimine	0.005	0.05	10
Ethambutol	0.005	0.05	10
Ethionamide	0.01	0.01	10
Isoniazid	0.05	0.5	100
Levofloxacin	0.1	0.5	100
Linezolid	0.1	0.5	100
Moxifloxacin	0.05	0.5	100
Pretomanid	0.005	0.05	10
Prothionamide	0.002	0.01	10
Pyrazinamide	0.05	1	100

Sample values (ng/mg hair) Samples: UC-04, UC-08, UC-11, UC-16, UC-25, UC-36, UC-69, UC-83, UC-89, UC-90, UC-91, UC-104, UC-105, UC-108, UC-109
0.21, 0.38, 0.56, 0.86, 0.90, 1.04, 1.29, 2.15, 2.29, 5.64
0.37, 0.61, 1.84, 2.20, 2.90, 3.41, 3.90, 6.03, 8.25, 10.66, 11.01
0.04, 0.05, 0.25, 0.42, 0.43, 0.5, 0.68, 0.92, 0.95, 1.01, 1.53, 1.54, 9.76
<LOD, <LOD, 0.01, 0.01, 0.01, 0.02, 0.02, 0.17
<LOD, <LOD, 0.12, 0.26, 0.84, 0.94, 1.36, 2.88, 4.03, 4.04, 9.14
8.01, 8.42, 15.37, 24.41, 39.45, 42.12, 56.15, 75.58, 119.96
0.87, 1.09, 3.51, 5.51, 7.80, 9.21, 15.68, 18.32, 19.13, 21.22
0.35, 0.49, 1.58, 1.59, 6.23, 7.06, 13.14, 17.37, 21.72, 55.88, 86.64
0.57
1.14, 1.74, 1.86, 3.21, 5.94, 11.39, 12.36, 12.71, 12.85, 14.38, 16.13, 44.17, 69.66

Name of Material/Equipment	Company	Catalog Number	Comments/Description
2 mL injection vials	Agilent Technologies	5182-0716	
250 uL injection vial inserts	Agilent Technologies	5181-8872	
Bead ruptor 24	OMNI International	19001	
Bead ruptor tubes (2 mL bead kit, 2.8mm ceramic, 2 mL microtubes)	OMNI International	19628	
Bedaquiline	Toronto Research Chemicals	B119550	
Bedaquiline-d6	Toronto Research Chemicals	B119552	
Clofazimine	Toronto Research Chemicals	C324300	
Clofazimine-d7	Toronto Research Chemicals	C324302	
Disposable lime glass culture tubes	VWR	60825-425	
Ethambutol	Toronto Research Chemicals	E889800	
Ethambutol-d4	Toronto Research Chemicals	E889802	
Ethionamide	Toronto Research Chemicals	E890420	
Ethionamide-d5	ClearSynth	CS-O-06597	
Formic acid	Sigma-Aldrich	F0507-100mL	
Glass bottles	Corning	1395-1L	
Hot Shaker	Bellco Glass Inc	7746-32110	
HPLC	Agilent Technologies	Infinity 1260	
HPLC grade acetonitrile	Honeywell	015-4	
HPLC grade methanol	Honeywell	230-1L	
HPLC grade water	Aqua Solutions Inc	W1089-4L	
Isoniazid	Toronto Research Chemicals	I821450	
Isoniazid-d4	Toronto Research Chemicals	I821452	
LC column, Synergi 2.5 um Polar RP 100 A 100 x 2 mm	Phenomenex	00D-4371-B0	
LC guard cartridge	Phenomenex	AJ0-8788	
LC guard cartridge holder	Phenomenex	AJ0-9000	
LC-MS/MS quantitation software	Sciex	Multiquant 2.1	

Levofloxacin	Sigma-Aldrich	1362103-200MG
Levofloxacin-d8	Toronto Research Chemicals	L360002
Linezolid	Toronto Research Chemicals	L466500
Linezolid-d3	Toronto Research Chemicals	L466502
Micro centrifuge tubes	E&K Scientific	695554
Moxifloxacin	Toronto Research Chemicals	M745000
Moxifloxacin-13C, d3	Toronto Research Chemicals	M745003
MS/MS	Sciex	Triple Quad 5500
OPC 14714	Toronto Research Chemicals	O667600
Pretomanid (PA-824)	Toronto Research Chemicals	P122500
Prothionamide	Toronto Research Chemicals	P839100
Prothionamide-d5	Toronto Research Chemicals	P839102
Pyrazinamide	Toronto Research Chemicals	P840600
Pyrazinamide-15N, d3	Toronto Research Chemicals	P840602
Septum caps for injection vials	Agilent Technologies	5185-5862
Turbovap LV evaporator	Biotage	103198/11

Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Editorial comments:
The manuscript has been modified and the updated manuscript, **60861_R1.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

1. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol steps (including headings and spacing) in yellow that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted 2.75 pages of the protocol. We have also included all of the highlighted portions at the very end of the manuscript, after the references, in a "consolidated protocol", for ease of reading.

2. Please use greek characters for SI unit prefixed, e.g. use 'µL' instead of 'uL'.

We have made this change.

3. Please use h, min, s for time units.

We have made this change.

Reviewers' comments:
Reviewer #4:

Manuscript Summary:
The article JoVE60861R1 describes an analytical method for the determination of 11 drug-resistant TB medications in hair samples by LC-MSMS. The method is quick, simple and sensitive, achieving limits of quantification between 0.01 and 1 ng/mg, depending on the medication, in just 2 mg of hair.

Major Concerns:

My main concern about this methodology is that has been already published in another journal,

as indicated by the authors (Reference 22 in the manuscript). What is the difference between that published method and the present one?

Novelty is not a requirement of JoVE papers as highlighted by the editor above.

In the current protocol, the analytical procedure is described in detail, but there is no information about the validation. What parameters were evaluated? What were the results?

We mention in the first paragraph of the Discussion that the validation is described elsewhere and provided the reference where the validation results were provided.

I noticed that the hair samples were not washed/decontaminated before the extraction procedure. Medications can also be present in the sweat, and contaminate the hair sample. This type the contamination may affect the timeline reflected by the hair analysis (sweat reflects recent exposure of days). The authors should add a comment about this.

We have added a third paragraph in the Discussion section addressing this point, and noting that future studies could investigate external contamination of TB patient hair samples.

Minor Concerns:
What LC-MSMS instrument (model, manufacturer) was employed?
What chromatographic column was (model, manufacturer) used?

As per the Grammar section of the Jove Instructions for Authors, company and brand names are not allowed in the body of the paper. They are listed in the Table of Materials.

Reviewer #5:

Manuscript Summary:
This manuscript provides a clear and explicit new methodology for the simultaneous quantification in hair of 11 anti-tuberculosis medications that are used to treat drug-resistance disease. The data are well presented and the protocol is also very detailed.

Major Concerns:
N/A

Minor Concerns:

My only main concern in the Results and the Discussion would be as follows:

1. Please expand on the detection of some drugs in patients who were not supposed to be given those medications? Any potential for a false positive?

We have changed the paragraph to more clearly demonstrate our process of unsuccessfully testing specificity in the original sample set (due to prior unrecorded regimens) and switching to

hair from patients who had not taken DR-TB drugs. We recently submitted a paper detailing this specificity study that has yet to be accepted.

2. Since anti-TB therapy is usually DOT in many clinical settings, please provide some additional discussion about the utility of hair concentrations to monitor adherence. Perhaps they could be particularly useful to quantify drug concentrations in persons newly diagnosed with TB and establish is previous household-driven therapy has been provided (ie between family or community members), which could predict the likelihood of MDR TB upon diagnosis?

Directly Observed Therapy has various implementations according to setting, and in conventional practice does not literally indicate medically observed pill consumption on a day-to-day basis (please see Metcalfe PlosMed 2015; https://journals.plos.org/plosmedicine/article?id=10.1371/journal.pmed.1001877*). We changed the final paragraph to raise some clinically relevant benefits of improved drug monitoring.*

•ÈÀID	Q1 Mass (D	Q3 Mass (D	Time (msec	DP (volts)	CE (volts)	CXP (volts)
BDQ d6	563.2	64.1	2	61	69	10
BDQ-quant	557.2	58.1	2	51	65	8
BDQ-qualif	557.2	329.9	2	51	31	26
CLF d7	481.2	429.9	2	201	59	36
CLF-quantit	474.2	432	2	11	45	32
CLF-qualifie	474.2	430	2	11	55	28
EMB d4	209	120.1	2	41	19	10
EMB-quant	205	116	2	46	19	10
EMB-qualif	205	44	2	46	35	6
ETH d5	172.2	145	15	41	25	10
ETH-quanti	167	140	15	61	25	12
ETH-qualifi	167	107	2	61	35	10
INH d4	142.2	82.9	2	51	37	8
INH-quanti	138.2	79	2	71	37	8
INH-qualifi	138.2	120.9	2	71	19	10
LFX d8	370.2	265.1	2	26	37	18
LFX-quantit	362.3	220.9	2	82	47	21
LFX-qualifie	362.1	318	2	81	25	22
LZD d3	341.2	297	2	36	25	24
LZD-quanti	338.2	296	15	91	10	25
LZD-qualifi	338.2	235.1	2	91	10	27
MFX 13CD3	406.2	368.1	2	61	35	26
MFX-quant	402.3	364	2	41	35	26
MFX-qualif	402.3	260	2	41	45	18
OPC 14714	459.3	176	2	20	39	14
PTH d5	186.3	125	2	56	41	10
PTH-quanti	181	154	2	36	27	12
PTH-qualifi	181	121	2	36	33	10
PTM-quant	360	175	2	40	27	12
PTM-qualif	360	201	2	40	25	12
PZA 15ND3	128.1	84	2	55	22	14
PZA-quanti	124.1	54	2	81	27	10
PZA-qualifi	124.1	81	2	76	27	10