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Development and validation of a liquid chromatography-tandem mass spectrometry assay for the simultaneous analysis of isoniazid and pyrazinamide in cerebrospinal fluid

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ABSTRACT

For the effective treatment of tuberculosis with first-line anti-tubercular drugs, drug concentrations need to be measured at the site of infection to determine drug exposure. To enable the measurement of the anti-tuberculosis drugs isoniazid and pyrazinamide in the nervous system of patients with tuberculous meningitis, an analytical method was developed and validated for the quantification of these drugs in human cerebrospinal fluid. Samples were prepared by solid phase extraction using Strata-X polymeric extraction plates. The analytes were separated by high-performance liquid chromatography on an Atlantis T3, 100 A, 3 μm, 2.1 mm × 100 mm analytical column with gradient elution, employing a mobile phase that consisted of acetonitrile-methanol-formic acid (50:50:0.1, v/v/v), at a flowrate of 0.25 mL/min. The total run time was 4.5 minutes, and the average retention times of isoniazid and pyrazinamide were 1.1 and 1.3 min, respectively. The analytes and their respective deuterated internal standards were detected on a Sciex API4000 triple quadrupole mass spectrometer applying positive electrospray ionization with multiple reaction monitoring as the detection mode. The method was validated according to the FDA and EMA guidelines. The method was demonstrated to be accurate, reproducible, and robust, showing the necessary sensitivity and specificity for the quantification of isoniazid and pyrazinamide in cerebrospinal fluid. The method was successfully applied to analyze clinical samples from the LASER-TBM and TBM-KIDS clinical studies.

1. Introduction

Tuberculosis meningitis (TBM) is a severe form of extrapulmonary tuberculosis that affects the central nervous system by infecting the protective membranes that enclose the brain and spinal cord (meninges) [1,2] . In 2021, Dodd et al. [3] reported that an estimated 164,000 TBM incidences occurred in 2019, with a mortality rate of 48 %. TBM is currently treated with the standard first-line antitubercular regimen used in pulmonary TB, which includes rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA), and ethambutol (EMB) [4]. Additionally, the WHO recently endorsed an alternative regimen for children in which ethionamide replaces ethambutol as the fourth drug [5].

INH is an antimycobacterial drug used in the treatment of the latent and active stages of tuberculosis. INH is a potent bactericidal agent against *Mycobacterium tuberculosis*, with a minimal inhibitory concentration (MIC) of between 0.1 and 0.7 μM [6,7]. INH acts by inhibiting the key enzymes required by *Mycobacterium tuberculosis* to synthesize components of the bacterial cell wall, causing bacterial death. INH is typically administered orally and undergoes rapid and complete absorption in the intestines, showing a T_{max} of 1–2 h [8,9]. Since its introduction in 1953, INH has been shown to be life-saving in the treatment of TBM, leading to reduction in mortality rates [10]. A phase 3 trial has also demonstrated a link between higher exposure to INH and reduced risk of death [11].

PZA is a first-line tuberculosis drug that plays a role in shortening the duration of tuberculosis therapy by killing extracellular bacteria in the early treatment period [12]. Upon oral administration, PZA is well absorbed from the gastro-intestinal tract, attaining peak plasma concentrations within 2 h. Plasma concentrations generally range from 30 to 50 μg/mL with doses of 20–25 mg/kg. PZA is widely distributed in

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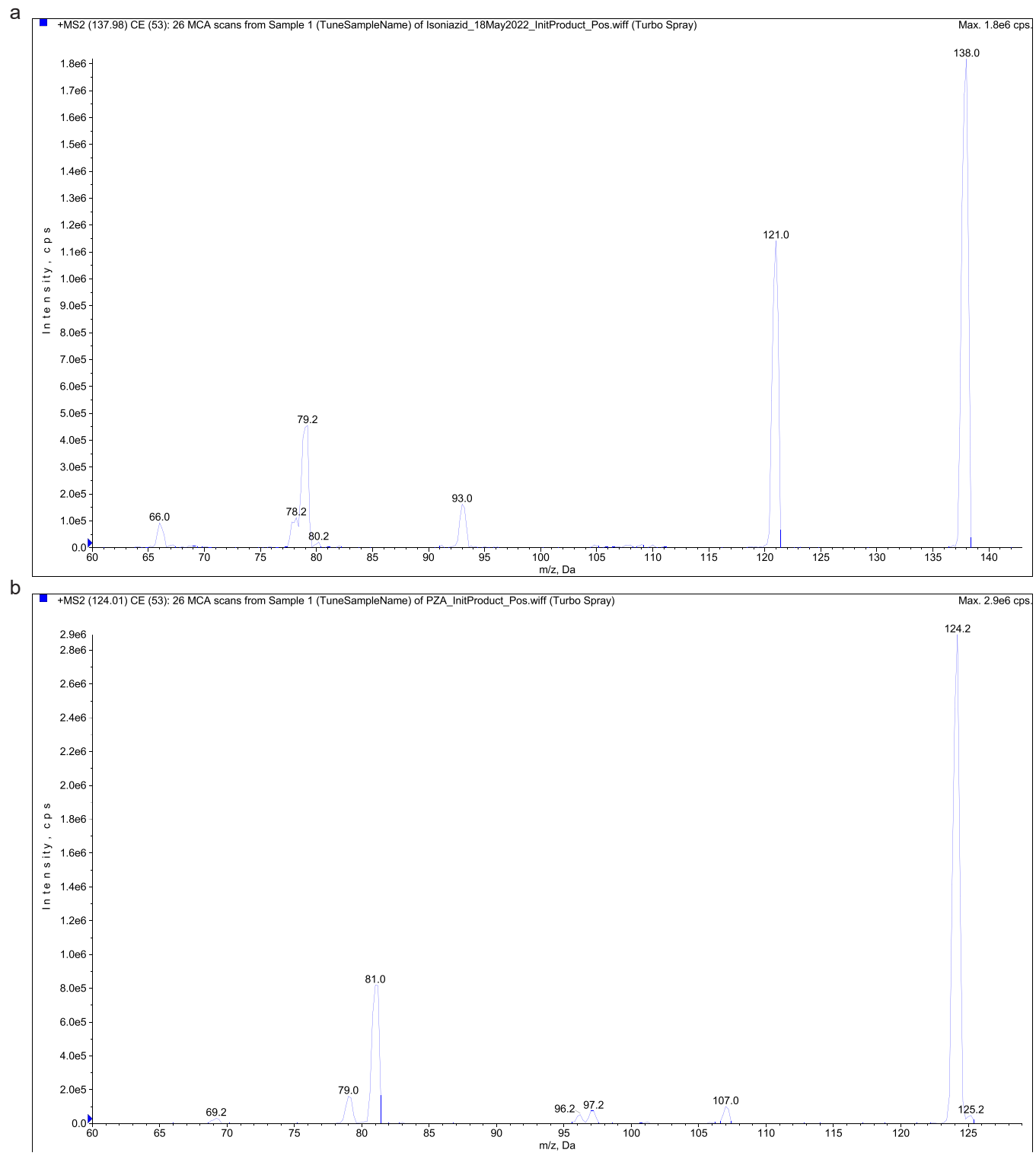


Fig. 1. a: Representative ion spectra showing the precursor (137.8 *m/z*) and fragment ions of isoniazid. b: Representative ion spectra showing the precursor (124.2 *m/z*) and fragment ions of pyrazinamide.

body tissues and fluids including the liver, lungs, and CSF. The drug is active only at a slightly acidic pH, both *in vitro* and *in vivo*, and the mechanism of action is still mostly unknown [13].

INH and PZA are reported to have good penetration into the cerebrospinal fluid (CSF) [14,15]. Peak CSF concentrations of 12.0 μg/mL (± 2.3 μg/mL) were reached for INH after 3–4 hours following a dose of 20 mg/kg in a study conducted in 38 South African children aged 0.3–8.6 years [14]. In a study involving children of ages 0.6–10 years who received a PZA dose of 40 mg/kg, peak CSF concentrations of 37.7 μg/mL (± 15.0 μg/mL) were reported [15].

A review article by Donald [16] summarized publications that reported INH and PZA concentrations in CSF, but descriptions of the bioanalytical methodology used to measure them were not included. Additionally, none of the reported methods used LC-MS/MS as the

detection method, and the studies that measured them are outdated and not stated to be validated [16].

We provide a detailed description of a fully validated LC-MS/MS bioanalytical method, employing mixed-mode solid phase extraction to quantify INH and PZA in human cerebrospinal fluid. The validation was performed according to the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines [17, 18]. The reported bioanalytical method provides a sensitive and specific tool for application in clinical studies designed to measure INH and PZA concentrations in CSF for pharmacokinetic and drug monitoring studies, at the site of infection.

2. Materials and methods

2.1. Reagents and chemicals

Reference grade isoniazid (97.1 % purity), pyrazinamide (100 % purity), and their stable isotope labeled internal standards isoniazid-d4 (98.3 % purity) and pyrazinamide-^{15}N-d3 (98.0 % purity) were purchased from Toronto Research Chemicals (Toronto, Canada). LC-MS grade water was produced in-house using the Millipore Synergy S water purification system supplied by Merck (Darmstadt, Germany). LC-MS grade methanol and acetonitrile were purchased from Honeywell (Charlotte, NC, USA), and LC-MS grade formic acid was purchased from Merck (Darmstadt, Germany). High-purity grade dimethyl sulfoxide (DMSO) and ammonium bicarbonate were purchased from Sigma-Aldrich (Burlington, MA, USA) and Acros Organics (Geel, Belgium), respectively.

2.2. Preparation of calibration standards, quality controls, and internal standards working solution

Stock solutions of INH and PZA in methanol at concentrations of 2.00 mg/mL and 5.00 mg/mL, respectively, were used to prepare a range of working solutions (WSs) in methanol by volumetric serial dilution. Calibration standards (STDs) and quality controls (QCs) were then prepared by spiking 30 µL of the appropriate WS into 870 µL blank cerebrospinal fluid (CSF). In this way, nine INH STDs (15.0, 11.3, 5.63, 2.57, 1.28, 0.642, 0.321, 0.160, and 0.0586 µg/mL), four INH QCs (12.0, 6.00, 0.150, and 0.0586 µg/mL), nine PZA STDs (60.0, 45.0, 22.5, 10.3, 5.14, 2.57, 1.28, 0.642, and 0.234 µg/mL), and four PZA QCs (48.0, 24.0, 0.600, and 0.234 µg/mL) were prepared. Aliquots (120 µL) of the STDs and QCs were stored at −80 °C. An internal standard (ISTD) WS was prepared by adding 20 µL of 1 mg/mL INH-d4 and 40 µL of 1 mg/mL PZA-^{15}N-d3 stock solutions to 940 µL methanol. Then, 200 µL of the ISTD WS was spiked into 19.98 mL 50 mM ammonium bicarbonate to obtain final concentrations of 0.200 µg/mL and 0.400 µg/mL for INH-d4 and PZA-^{15}N-d3, respectively. The ISTD WS was freshly prepared on the day of extraction.

2.3. Sample preparation and extraction procedure

CSF samples were thawed at room temperature and sonicated for 5 minutes. A volume of 50 µL of each sample was added to 150 µL ammonium bicarbonate (50 mM) containing the internal standards. Following vortex mixing for 30 seconds and sonication for 2 minutes, the analytes were extracted by solid phase extraction on a Strata-X 33 µM 30 mg/well plate (Phenomenex) using a Biotage 96-well plate positive pressure system. The solid phase media was conditioned with 500 µL acetonitrile followed by 500 µL 50 mM ammonium bicarbonate. The prepared samples were then loaded and washed twice with 500 µL water to remove unretained compounds. Excess water was removed by applying maximum gas flow. The bound analytes were eluted into collection plates with 100 µL acetonitrile-formic acid (100:0.1, v/v), followed by 200 µL methanol-acetonitrile-formic acid (50:50:0.1, v/v/v). The collection plate was then placed into the autosampler for injection of the samples into the LC-MS/MS system.

Table 1
Compound-dependent parameters for the multiple reaction monitoring detection of isoniazid, pyrazinamide, and their respective internal standards.

	Isoniazid	Isoniazid-d4	Pyrazinamide	Pyrazinamide,^{15}N-d3
Precursor ion (*m/z*)	138.0	142.1	124.2	128.1
Product ion (*m/z*)	79.2	125.3	97.2	84.0
Dwell time (ms)	50.0	50.0	90.0	90.0
Declustering potential (V)	51.0	60.5	38.0	55.0
Entrance potential (V)	10.0	10.0	10.0	10.0
Collision energy (eV)	21.0	21.0	25.0	23.0
Collision cell exit potential (V)	8.00	8.00	14.0	14.0

2.4. Instrumentation and chromatographic conditions

Chromatographic separation of INH, PZA, and their ISTDs was achieved on an Atlantis T3, 2.1 mm × 100 mm, 3 µm analytical column (Waters Corp) at a column temperature of 30 °C using an Agilent 1260 Infinity II high performance chromatograph. A gradient was applied using 0.1 % formic acid in water as solvent A and 0.1 % formic acid in methanol-acetonitrile (1:1, v/v) as solvent B. Solvent flows and ratios used during the runtime are summarized in the table imbedded in Fig. 1.

For detection of the analytes, a Sciex API 4000 triple quadrupole mass spectrometer was coupled to the chromatograph through an electrospray ionization source. Optimal ionization of the two analytes in the positive mode was achieved at a capillary voltage (Ion Spray Voltage) of 4500 V and a source temperature of 550 °C. The nebulizer gas, turbo gas, and curtain gas were set at 55, 60, and 30 arbitrary units, respectively. Detection of the analytes was based on multiple reaction monitoring (MRM) acquisition, employing collision induced dissociation of the analyte precursor ions in the collision cell, with the collision gas set at 10 arbitrary units. The analyte specific settings of the ionization source and the collision cell, with which optimal intensities of the monitored precursor and fragment ions were achieved, are indicated in Table 1.

2.5. Bioanalytical method validation

The guidelines of the FDA and the EMA for the validation of quantitative bioanalytical methods were used to validate the analytical method [17,18].

2.5.1. Accuracy and precision

Accuracy and precision were assessed over the calibration ranges of 0.0586–15.0 µg/mL and 0.234–60.0 µg/mL for INH and PZA, respectively, over three independent validation runs. The nine STDs were analyzed in duplicate in each validation batch and freshly prepared QCs at high, medium, low, and lower limit of quantification concentrations (QCH, QCM, QCL, and LLOQ) were analyzed in six-fold. The analysis of one of the validation batches was performed by a different analyst, and a different analytical column of the same specification was used for analysis of one of the batches, to assess the robustness of the method.

Observed concentrations were compared to nominal concentrations to assess the accuracy of the method expressed as %Accuracy, whereas the precision was assessed by calculating the ratio of the standard deviation of the observed values to their mean value, expressed as a percentage of coefficient of variation (CV(%)). For acceptance of both STDs and QCs, the accuracy was required to be $\leq$ 15 % of the nominal

concentrations over the calibration range, but ≤ 20 % at the LLOQ, whereas the CV(%) was required to be ≤ 15 % over the calibration range, but ≤ 20 % at the LLOQ.

2.5.2. Dilution integrity

An additional QC (QC-Dil) was prepared at twice the QCH concentration (24.0 μg/mL and 96.0 μg/mL for INH and PZA, respectively) to demonstrate the accuracy of dilution of unknown samples with concentrations above the upper limit of quantitation (15.0 μg/mL and 60.0 μg/mL for INH and PZA, respectively). A 5× dilution was performed prior to extraction by diluting 20 μL of QC-Dil sample with 80 μL of blank CSF. An additional partial volume dilution, using 10 μL of the QC-Dil sample and 40 μL of blank CSF, was validated to allow for dilution reanalysis of samples with low volumes. The accuracy and precision criteria for QCs were applied for the acceptance of dilution integrity (% difference ≤ 15 % of nominal concentrations and CV(%) ≤ 15 %).

2.5.3. Sensitivity and specificity

For this assessment, six different CSF lots were used to prepare QCs at the LLOQ concentrations of 0.0586 μg/mL and 0.234 μg/mL for INH and PZA, respectively. These were then extracted with and without ISTDs. The same six CSF lots were additionally extracted to prepare blank samples (without analytes but with ISTDs) and double blank samples (without analytes and ISTDs).

Unprocessed chromatograms of respective LLOQ samples (extracted with ISTDs) and blanks (extracted with ISTDs) from the same matrix lots were overlayed to determine the sensitivity at the LLOQ. The individual and the mean S/N ratios had to be ≥ 5 to confirm adequate sensitivity.

Specificity was evaluated by comparing double blanks (extracted without analyte and ISTD) against LLOQ samples (extracted without ISTDs) and blanks (extracted with ISTD) for potential endogenous interference at both the analyte and ISTD retention times. Specificity was considered acceptable if an analyte response in the double blank samples was ≤ 20 % of the analyte response in the LLOQ samples, and an ISTD response was ≤ 5 % of the ISTD response in the blank samples.

2.5.4. Carry-over

Carry-over was assessed by placing double-blank samples immediately after the highest standard. Peaks attributed to carry-over had to be ≤ 20 % of the analyte response at LLOQ, whereas carry-over at the ISTD retention time had to be ≤ 5 % of the mean ISTD response at the working concentration.

2.5.5. Crosstalk

This experiment was conducted to assess the potential crosstalk between the MRM acquisition channels of the analytes and their respective ISTDs. The assessment is specifically relevant when using isotope-labeled ISTDs, as their isotopic impurity can contribute to the analyte MRM channel. ULOQ samples, extracted without ISTDs, were used to assess the contribution to the respective ISTD's MRM channels at the appropriate retention times of the ISTDs. Potential crosstalk caused by the ISTDs was assessed at the working concentrations (as blank samples) of the respective ISTDs (200 ng/mL and 500 ng/mL for INH-d4 and PZA-^{15}N-d3, respectively) in their analytes' respective MRM channels, at their appropriate retention times. For crosstalk/contribution to meet acceptance criteria, the interference from ISTDs had to be ≤ 20 % of the analyte response at LLOQ, and the contribution of the analyte ULOQ to the ISTDs MRM channel had to be ≤ 5 % of the mean ISTD response.

2.5.6. Matrix effects, recovery, and process efficiency

Matrix effects (ME) and recovery (RE) assessments were performed using six different CSF lots, whereas process efficiency (PE) was assessed using pure injection solution.

The ME experiment assesses the effects that co-extracted matrix compounds have on the ionization of analytes and ISTDs (ion suppression or ion enhancement). The extracted double blanks were spiked at QCH, QCM, and QCL concentrations and at the working concentration of the ISTD, in accordance with the method published by Matuszewski [19]. A simple linear regression was generated for each matrix lot using analyte/ISTD peak area ratios. The slope variability (CV(%)) over the six lots had to be < 5 % for matrix effects to be regarded as negligible.

The RE was assessed by comparing the results of the analysis of prepared QCH, QCM, and QCL, to the results obtained from the analysis of the ME QCs (double blank post-spiked with analytes and ISTDs), expressed as percentage recoveries. Although recovery is not expected to be 100 %, it must be reproducible over the three QC levels (CV(%) ≤ 15 %).

The PE compares the response of the instrument between the matrix extracted samples and neat injection solution spiked samples to assess the effect of extraction recovery and the ionization effect of matrix components. The repeatability (CV(%)) across all three QC levels had to be ≤ 15 %.

2.5.7. Concomitant medication effect

Medications that are commonly co-administered with INH and PZA for the treatment of TB were assessed for potential effects on the specificity of the method, to demonstrate the performance of the method in the anticipated study samples. QCH and QCL samples containing these medications were compared to those without concomitant medication. The concentrations of the concomitant medications in the matrix were based on the expected C_{max} values of these medications when administered therapeutically. The comparison was expressed as %difference using QCs without concomitant medications as references. The effects of the concomitant medication on the specificity of the method were considered acceptable if the difference of concentrations of QCs with and without concomitant medication was ≤ 15 %, and the CV(%) was ≤ 15 %. Additionally, the specificity of the method was assessed by extracting double blank and blank samples using matrix containing the concomitant medications, and monitoring for any chromatographic peaks at the retention times of the analytes.

2.5.8. Stability assessments

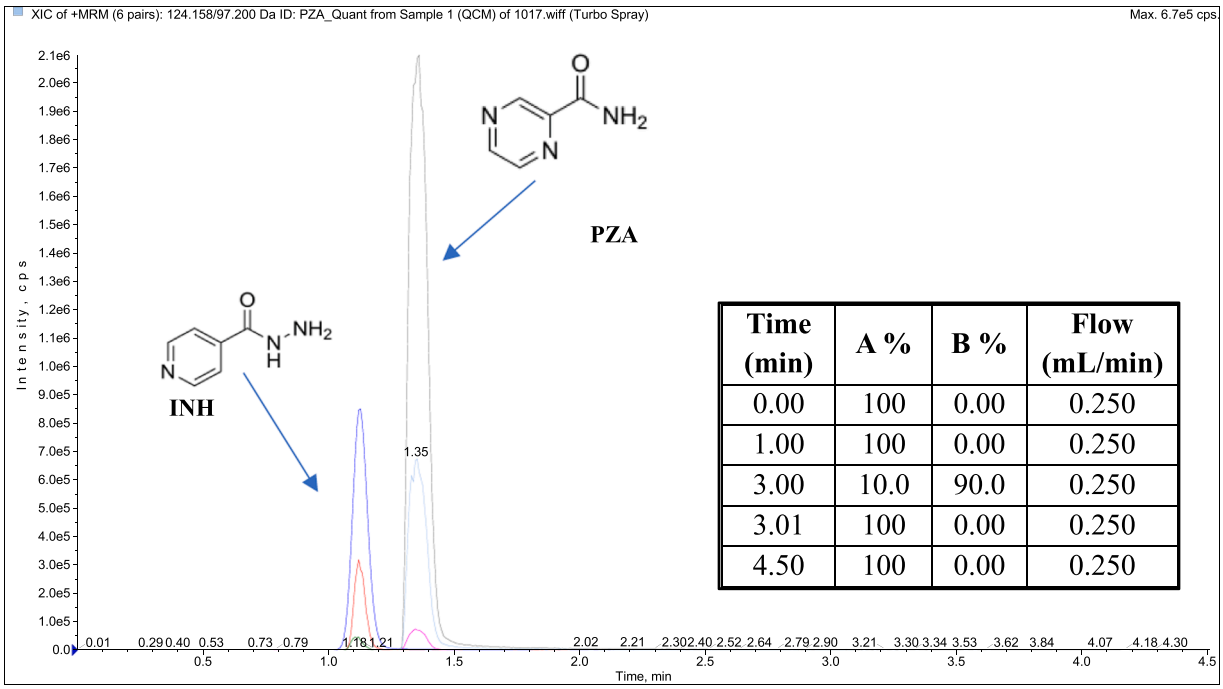
The stability assessments were conducted to ensure adequate chemical stability of the analytes during the expected storage and handling conditions of the analytical process.

2.5.8.1. Stock and working solutions stability. Aliquots of verified (accurate) stock solutions of both analytes were placed at room temperature, on crushed ice, and at 4 °C for specific periods to assess short term stability of the analytes in the stock solutions. Long-term stability of the analytes in stock solutions was assessed at −80 °C. The assessments were based on the spectrophotometric comparison of the test stock solutions, by diluting triplicates of the solutions in methanol (1:100 dilutions) and recording the absorbance at 260 nm in an Agilent Cary 60 ultraviolet-visible light spectrophotometer. The absorbance of the test solutions was compared to that of freshly prepared stock solutions (reference).

Short term stability of the analytes in working solutions was assessed at room temperature, on crushed ice, and at −20 °C for 6 h, whereas long-term stability of analytes in these solutions was assessed at −80 °C for 26 days. Triplicate dilutions of the highest and lowest analyte concentrations in these solutions were prepared by adding 10 μL of working solutions to 290 μL of injection solution (0.1 % formic acid in methanol-acetonitrile (50:50, v/v), and were analyzed in duplicate by LC-MS/MS. The resultant analyte peak areas were compared to those of freshly prepared working solutions.

The analytes in both stock solutions and working solutions were deemed to be stable when the differences between those subjected to different conditions and the freshly prepared solutions were ≤ 10 %.

2.5.8.2. Long-term matrix stability. The stability of the analytes in the matrix was assessed at −80 °C, which was the storage condition for



Time (min)	A %	B %	Flow (mL/min)
0.00	100	0.00	0.250
1.00	100	0.00	0.250
3.00	10.0	90.0	0.250
3.01	100	0.00	0.250
4.50	100	0.00	0.250

Fig. 2. Representative chromatogram showing the chromatographic separation of isoniazid and pyrazinamide on an Atlantis T3 3 μm 2.1 mm × 100 mm analytical column with gradient elution as indicated in the inset table, using 0.1 % formic acid in water (solvent A) and 0.1 %formic acid in methanol-acetonitrile (1:1 (v/v)) (solvent B).

study samples. Using a freshly prepared standard curve, the analyte concentrations were determined in six-fold in accurately verified QCH and QCL samples stored for 340 days at −80 °C. The observed concentrations of the analytes in the test samples were compared to nominal concentrations and expressed as %difference. Stability was considered acceptable if the %difference from the nominal concentrations was ≤ 15 % for both QCs and the CV(%) was ≤ 15 %.

2.5.8.3. Benchtop stability. To assess the stability of the analytes when samples are left and/or processed on-bench, QCH and QCL samples were thawed at room temperature for 2 h before being extracted and analyzed together with freshly prepared calibration standards. The measured concentrations of the analytes in the test samples were compared to nominal concentrations and expressed as %difference. Stability of the analytes under these conditions was considered adequate if the %difference was ≤ 15 % for both QCs and the CV(%) was ≤ 15 %.

2.5.8.4. Freeze-thaw stability. The stability of the analytes during repeated freezing and thawing cycles was assessed at QCH and QCL concentrations. Five freeze-thaw cycles were evaluated which included thawing for 2 h at room temperature and freezing for 12–24 h in-between each cycle. Test samples were analyzed in six-fold and the concentrations of the analytes determined from analysis of freshly prepared calibration standards. The observed concentrations were compared to nominal concentration and expressed as %difference. The stability of the analytes under these conditions was considered acceptable if the %difference was below 15 % for both QCs and the CV(%) was ≤ 15 %.

2.5.8.5. Autosampler stability and reinjection reproducibility. The autosampler stability of the analytes in the final extract was assessed by leaving an extracted validation batch in the autosampler for extended periods (up to 172 hours) and then reinjecting the batch in its entirety. The QCH and QCL peak area ratios (n = 6) were compared to those of the initial values of the batch to calculate %difference. A %difference and CV(%) of ≤ 15 % was needed to confirm adequate stability of the analytes in the autosampler for the period tested. Reinjection reproducibility is assessed according to accuracy and precision criteria, as described in 2.5.1.

2.5.9. Sample filtration/sterilization

The clinical samples used in this study were collected from study participants with active and infective TB. Therefore, all clinical samples were sterilized in a BSL-3 facility before being handled in a BSL-2 laboratory for analysis. Sterilization involved filtration of the samples by centrifugation, using 0.2 μm Costar Spin-x cellulose acetate, Costar Spin-x 0.2 μm nylon, and Nanosep MF 0.2 μm centrifugal filters. To assess any influence of the filtration process on the analytical integrity, QCH and QCL samples were filtered twice by loading 200 μL of the samples on the centrifugal filter devices and centrifuging for 10 minutes at 13,000 *g*. The filtrates were compared to QCH and QCL samples that had not been filtered (reference) in six-fold to assess analyte recovery. Peak area ratios of reference samples were compared to those of filtered samples, and the %difference and CV(%) had to be ≤ 15 % to prove no effect due to filter sterilization. In addition to this test, freeze-thaw stability of filtered samples was assessed for 3 cycles by thawing QCH and QCL filtered samples at room temperature for 2 hours and freezing for 12–24 h in between each freeze-thaw cycle. The observed concentrations of freeze-thaw stability samples were compared to nominal concentration and accuracy was expressed as %difference. Freeze-thaw acceptance criteria applied as per Section 2.5.8.4 above.

2.6. Clinical application

The bioanalytical method was used to analyze sparsely collected CSF samples from the LASER-TBM and TBM-KIDS clinical studies. LASER-TBM was a phase IIa trial aimed at evaluating the pharmacokinetics and safety of intensified therapy comprising high-dose rifampicin plus linezolid, administered together with the standard companion drugs (EMB, INH, PZA) for adults with HIV-associated TB meningitis [20]. TBM-KIDS was a phase I/II randomized, open-label trial aimed at evaluating the pharmacokinetics, safety, and treatment outcomes of high-dose rifampicin with and without levofloxacin, compared to

Table 2
Summary of accuracy and precision data for isoniazid and pyrazinamide, calculated over three independent validation batches.

	Isoniazid					Pyrazinamide				
Accuracy and precision runs (1–3)	QcDil	QCH	QCM	QCL	LLOQ	QcDil	QCH	QCM	QCL	LLOQ
n	6 of 6	18 of 18	18 of 18	18 of 18	18 of 18	6 of 6	18 of 18	18 of 18	18 of 18	18 of 18
CV (%)	3.7	7.1	7.3	7.8	9.0	2.5	10.1	6.7	6.8	6.4
Accuracy (%)	88.4	106.3	104.5	107.8	102.1	88.5	99.7	97.8	103.9	99.1
r^2 values (n = 3)	0.9993					0.9993				

standard treatment (EMB, INH, PZA, and RIF) in patients with pediatric TB meningitis [21].

3. Results and discussion

3.1. Method development

Method development was initiated by establishing the optimal detection parameters of the analytes on a Sciex API4000 triple quadrupole mass spectrometer using electrospray ionization in the positive mode. Separate reference solutions of INH, PZA, and their respective internal standards were prepared at a concentration of 200 ng/mL each, in a solvent consisting of methanol-acetonitrile-formic acid (50:50:0.1, v/v/v). These solutions were separately infused at 10 μL/min with a syringe pump into the source of the mass spectrometer. Automated compound optimization was performed to establish compound-dependent parameters for the detection of each analyte and internal standard (see Table 1). Fig. 1a and b depict representative fragmentation spectra showing the precursor and fragment ions of INH and PZA, respectively. Flow injection analysis was used to optimize the nebulizer gas (55), turbo gas (60), curtain gas (30), source temperature (550 °C), and ion spray voltage (4500 V).

Using the optimized detection method, the chromatography of INH and PZA was investigated with the aim of establishing a robust chromatographic method with an acceptable retention factor, resolution, and symmetrical peak shapes, suitably compatible with electrospray ionization. This was achieved by reversed-phase chromatography on an Agilent 1260 high performance liquid chromatography system using an Atlantis T3, 2.1 mm × 100 mm, 3 μm analytical column, applying a gradient mobile phase consisting of water, formic acid, methanol, and acetonitrile as indicated in the table imbedded in Fig. 2. Methanol tends to increase back-pressure on the system, whereas acetonitrile has a higher elution strength than methanol, which could result in a lower retention factor and poor resolution for INH and PZA. Mixing the two solvents addressed these concerns. A representative chromatogram displaying the baseline separation of the two analytes is also depicted in Fig. 2.

Following the optimization of the separation and detection methods, the final objective of the method development was to establish a robust, reproducible extraction method and to achieve an extract with minimal interfering matrix components. INH and PZA are basic molecules with pKa values of 3.35 and 3.62 for INH and PZA, respectively [22]. Therefore, adding a basic solution, such as ammonium bicarbonate, promotes the hydrophobicity of both INH and PZA, facilitating the extraction of the analytes by hydrophobic interaction. However, using a mixed-mode extraction medium provides additional functionalities to retain these polar analytes selectively and more efficiently on an extraction medium. We therefore tested and finally selected the Phenomenex Strata-X 33 μm x 30 mg/well 96-well plate solid phase medium, in which π-π interaction (targeting the conjugated bonds of the pyridine and pyrazine rings in INH and PZA, respectively), hydrogen bonding (targeting the carboxy hydrazine and carboxy amide groups on INH and PZA, respectively), and hydrophobic interactions at high pH are suitable extraction mechanisms. This resulted in high recoveries with minimal interference from co-extracted matrix components when eluting the retained analytes with 100 μL 0.1 % formic acid in acetonitrile (to ensure complete elution by disrupting the π-π and hydrophobic interaction), followed by 200 μL methanol-acetonitrile-formic acid (50:50:0.1, v/v/v/) (to match mobile phase B composition in order to prevent peak splitting for INH and PZA during gradient elution). Finally, because of the small volume of CSF available from pediatric subjects, the 96-well plate format was selected based on its suitability for handling small-volume extractions and high sample throughput, as well as its advantages in terms of reduced solvent consumption.

3.2. Method validation

The accuracy and precision of this assay was assessed across 3 validation batches and the summary of the accuracy and precision QCs is presented in Table 2. The assay proved to be accurate and precise, demonstrating reproducibility when a different analyst performed the extraction and when a different analytical column of the same specification was used for one of the batches. In addition, the assay demonstrated good linearity with r^2 values of > 0.999 for both INH and PZA when a quadratic fit weighted by 1/x was used over the ranges 0.0568–15.0 μg/mL and 0.234–60.0 μg/mL for INH and PZA, respectively. These ranges were determined using the C_{max} values of 12.0 μg/mL and 37.7 μg/mL reported for INH and PZA, respectively [14,15], extended over 8 half-lives to capture concentration levels anticipated to result from the doses given in the LASER-TBM (5 mg/kg and 25 mg/kg for INH and PZA respectively) and TBM-KIDS (10 mg/kg and 25 mg/kg for INH and PZA respectively) trials.

Dilution integrity was proven to be accurate and precise, indicating that samples with concentrations of up to 24.0 μg/mL and 96.0 μg/mL for INH and PZA, respectively, can be diluted to within the calibration ranges for reliable and accurate quantification. Partial volume dilution was also shown to be accurate and precise with accuracy and precision values of 96.4 % (CV(%) = 6.6 %) and 103.3 % (CV(%) = 4.8 %) for INH and PZA, respectively.

Sensitivity and specificity met the acceptance criteria for both INH and PZA. Fig. 3 shows the unprocessed chromatograms of LLOQ samples in six different CSF sources for INH and PZA, respectively. The signal-to-noise ratios of both INH and PZA in all six CSF sources were above 5 with the average signal-to-noise ratios of 13.6 and 45.3 for INH and PZA, respectively. The chromatograms of the six blank matrices showed no interfering peaks at the retention times of the two analyte peaks. No carryover was observed in the double-blank samples for all the validation runs.

Matrix effects assessment showed slope variabilities of 1.3 % and 4.5 % for INH and PZA, respectively, indicating that co-extracted matrix components do not influence the assay. Matrix effects were also evaluated quantitatively by comparing the analyte/ISTD responses of INH and PZA at QCH, QCM, and QCL concentrations in pure injection solution without matrix to those in post-extracted blank matrix, expressed as a percentage. A percentage of less than 100 % would indicate ion suppression, with a value greater than 100 % indicating ion enhancement. The values were 95.9 % (CV(%) = 3.3 %) and 95.3 % (CV(%) = 4.7 %) for INH and PZA, respectively, indicating slight ion suppression, well within the 15 % acceptance limit for both %difference and CV(%). In addition, recovery and process efficiency were assessed and recoveries were found to be 83.9 % (CV(%) = 2.2 %) and 71.3 % (CV(%)

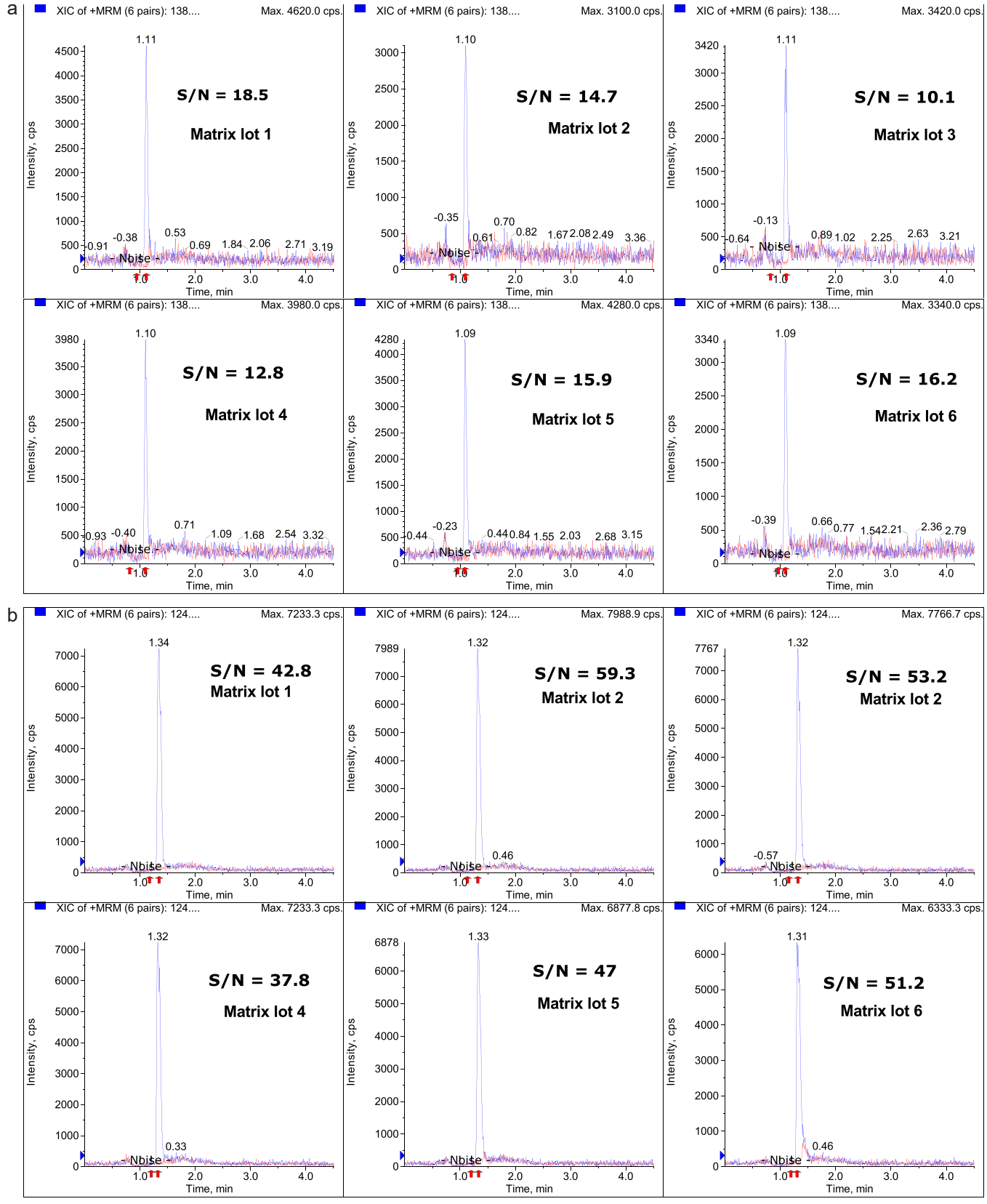


Fig. 3. a: Unprocessed chromatograms of INH at LLOQ concentration, in six different cerebrospinal fluid matrix lots (in blue), overlaid with the chromatograms of blank matrixes (red). b:Unprocessed chromatograms of PZA at LLOQ concentration, in six different cerebrospinal fluid matrix lots (in blue), overlaid with the chromatograms of blank matrixes (red).

Table 3
Summary of results of stability tests performed as part of the validation.

				Isoniazid		Pyrazinamide	
Stability test	Test sample/condition	Duration	n	CV(%)	%difference	CV(%)	%difference
Stock solution	at −80 °C	108 days (INH) 334 days (PZA)	3	0.9	2.8	0.2	−4.9
	On crushed ice	3 hours (INH) 24 hours (PZA)	3	1.3	−2.1	0.5	−3.7
	At 4 °C	3 hours (INH) 24 hours (PZA)	3	2.2	−1.4	0.7	−4.7
	At room temperature	3 hours (INH) 24 hours (PZA)	3	1.2	0.0	2.2	−6.3
Working solution (−80 °C)	Highest WS	26 Days	6	3.8	−6.5	4.1	2.4
	Lowest WS		6	4.9	5.5	3.7	−1.2
Working solution (RT)	Highest WS	6 Hours	6	9.6	−6.7	2.4	−7.0
	Lowest WS		6	2.9	−2.6	1.5	−5.1
Working solution (4 °C)	Highest WS		6	9.2	−6.6	2.3	−7.5
	Lowest WS		6	3.1	−1.9	1.5	−6.4
Working solution (20 °C)	Highest WS		6	6.2	−9.3	3.3	−3.2
	Lowest WS		6	3.2	−1.1	2.9	−4.6
Matrix stability (−80 °C)	QCH	340 Days	6	2.0	−12.2	5.3	−12.3
	QCL		6	3.2	12.3	3.0	−9.6
Concomitant medication	QCH	N/A	6	4.7	−1.5	4.4	9.9
	QCL		6	5.1	−6.6	2.2	−9.2
Freeze-thaw	QCH at RT for 2hrs	3 cycles (INH)	6	2.7	−0.3	3.9	−2.6
	QCL at RT for 2hrs	5 cycles (PZA)	6	5.5	14.3	3.0	0.5
Filtrate freeze-thaw	QCH at RT for 2hrs	3 cycles	6	2.3	−14.3	3.1	−13.3
	QCL at RT for 2hrs		6	5.1	−10.3	2.8	−8.9
Benchtop	QCH at RT for 2hrs	2 Hours (INH)	6	4.7	4.9	1.8	1.5
	QCL at RT for 2hrs	4 Hours (PZA)	6	6.4	12.7	3.4	−1.9
Autosampler stability	QCH extract at 8 °C	172 Hours	6	3.1	−0.2	2.2	−4.2
	QCL extract at 8 °C		6	4.9	9.2	2.1	8.7

Table 4
Concomitant medications and the concentrations at which their interference on the analysis of INH and PZA was investigated.

Analyte	Final concentrations of ConMed mix in CSF (μg/mL)
Ethambutol (EMB)	2.32
Levofloxacin (LVF)	4.86
Delamanid (DLM)	0.825
Delamanid metabolite (DM6705)	0.825
Rifampicin (RIF)	2.52
Clofazimine (CLF)	2.08
Linezolid (LZD)	30.0
Ethionamide (ETA)	23.2
Kanamycin (KAN)	11.6
Teridazone (TZD)	42.3
Bedaquiline (BDQ)	0.0540
N-desmethyl metabolite (M2)	0.0520
Dolutegravir (DLTG)	3.69

= 11.9 %), for INH and PZA, respectively, whereas process efficiencies were found to be 80.4 % (CV(%) = 3.6 %) and 67.9 % (CV(%) = 12.6 %), respectively.

Table 3 shows the results of the various stability tests performed as part of the validation process. The analytes were found to be stable under most of the tested conditions, with some stability concerns for INH upon repeated freeze-thaw cycles and when exposed to room temperature during on-bench activities. When test samples were taken through five freeze-thaw cycles, only PZA met acceptance criteria. Re-assessment over three freeze-thaw cycles confirmed stability for both PZA and INH. Therefore, when repeat analysis is required, samples can only be frozen and thawed three times for INH analysis, whereas samples for PZA analysis can be frozen and thawed five times. Both analytes are only stable for two hours when exposed to room temperature and all on-bench work at room temperature must therefore be performed within two hours.

Concomitant medications tested at the concentrations listed in Table 4 had no influence on the specificity of the assay, with accuracy

Table 5
Precision and %difference of observed peak area ratios of INH and PZA in sterilized and unsterilized samples.

			Isoniazid		Pyrazinamide	
Filter type	Test sample	n	CV (%)	% difference	CV (%)	% difference
Costar spin-x cellulose acetate 0.2 μm	QCH	6	2.5	4.6	1.1	3.0
	QCL		1.4	−1.2	2.5	0.2
Costar spin-x Nylon 0.2 μm	QCH	6	2.8	2.1	1.1	2.6
	QCL		2.5	−0.6	2.4	−0.4
Nanosep MF 0.2 μm	QCH	6	2.4	−0.7	1.8	1.7
	QCL		6.6	−0.3	3.8	0.5

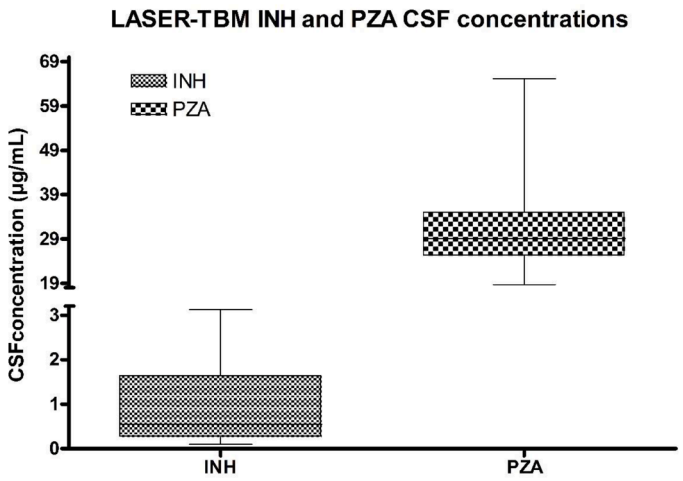


Fig. 4. Box and whisker plot of INH and PZA concentrations (LASER-TBM study).

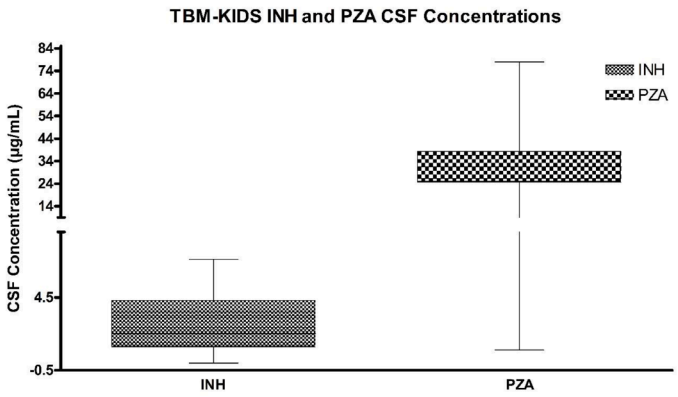


Fig. 5. Box and whisker plot of INH and PZA concentrations (TBM-KIDS study).

and precision criteria met for both INH and PZA (Table 3).

Assessment of the influence of sterilization of subject samples on the measured analyte concentrations produced accuracy and precision data within criteria, indicating that all three of the 0.2 µm filter types tested are suitable for sample sterilization (Table 5). In addition, three cycles of freezing and thawing of the filtered samples did not have a notable effect on the measured concentrations of the two analytes. This indicates that the sterilized CSF samples of trial subjects can be subjected to repeat analysis if required.

The validation met FDA and EMA criteria, demonstrating that the analytical method is robust, accurate, repeatable, and can be used for the quantification of INH and PZA in CSF samples collected from trial participants.

3.3. Application to clinical samples

The validated method proved to be suitable when used to analyze clinical sparce samples collected during the LASER-TBM and TBM-KIDS studies. The calibration ranges of both INH and PZA were appropriate for determining INH and PZA concentrations in CSF. Figs. 4 and 5 show the INH and PZA concentrations measured in CSF samples of the LASER-TBM and TBM-KIDS study participants, respectively. The mean concentrations measured for INH were 0.886 µg/mL and 2.74 µg/mL, respectively, for the LASER-TBM and TBM-KIDS studies, whereas the mean concentrations measured for PZA were 31.1 µg/mL and 31.4 µg/mL, respectively. Fig. 6 shows a representative unprocessed extracted ion chromatogram from a patient sample showing INH and PZA peaks at retention times of approximately 1.1 and 1.3 respectively.

The INH dose in the LASER-TBM study was 5 mg/kg (flat dose 300 mg daily), whereas the participants of the TBM_KIDS study received 10 mg/kg. This explains why the concentrations of INH were lower in the LASER-TBM study than in the TBM-KIDS study. For PZA, 25 mg/kg was used in both studies, explaining the comparable average concentrations measured.

The statistical analysis and study outcomes of the LASER-TBM [20] and the TBM-KIDS [21] studies are both published. We report the INH and PZA concentrations measured in CSF as part of the bioanalytical method application.

4. Conclusion

A high-throughput, rapid LC-MS/MS bioanalytical assay has been developed to simultaneously measure INH and PZA in CSF. The method validation per the FDA and EMA guidelines proved the assay to be reproducible, repeatable, and robust. The method determined INH and PZA concentrations accurately and precisely in CSF samples from the LASER-TBM and TBM-KIDS studies, demonstrating the suitability of the concentration ranges of 0.0568–15.0 µg/mL and 0.234–60.0 µg/mL for INH and PZA, respectively, at administered doses of 5–10 mg/kg INH and 25 mg/kg PZA.

We have demonstrated the ability of our method to quantify INH and PZA concentrations in CSF within the validated ranges and have shown its potential as an analytical tool for PK and drug monitoring studies.

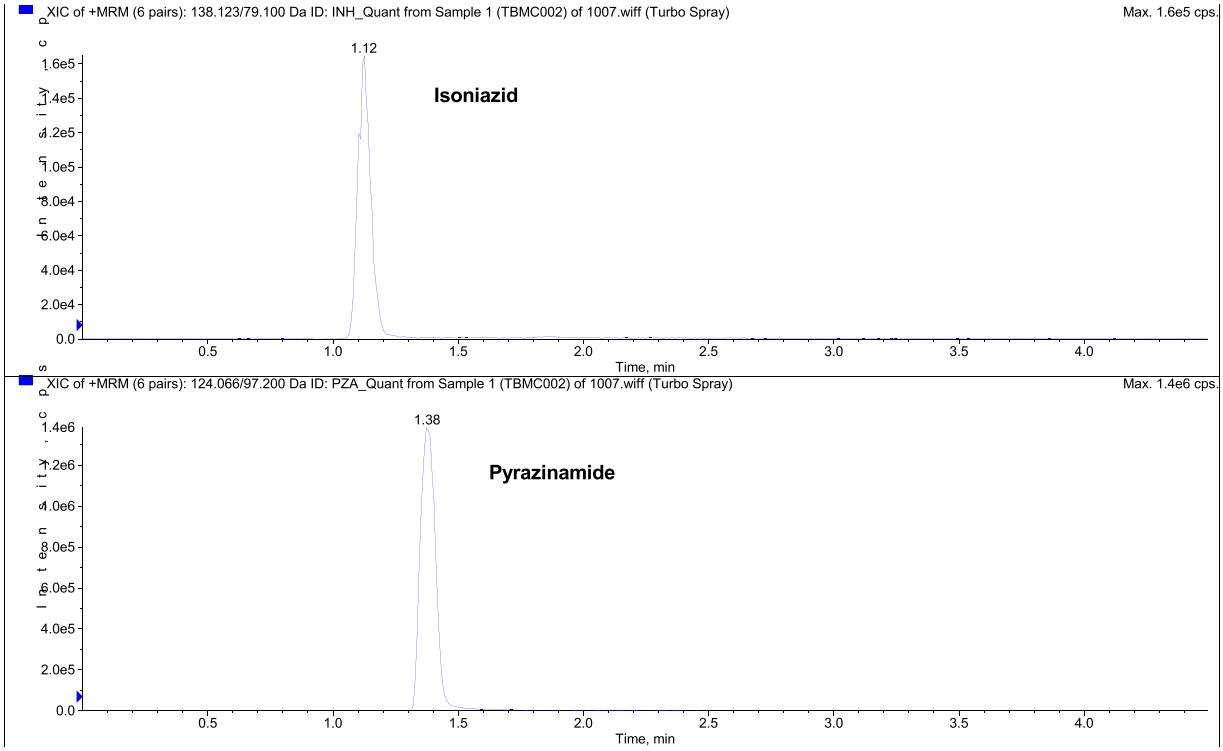


Fig. 6. A representative unprocessed (raw) chromatogram from a patient sample showing INH and PZA chromatographic peaks.

Ethics statement

Informed consent was obtained from all matrix donors and trial participants, and ethics approval of this study was granted by the University of Cape Town Human Research Ethics Committee (HREC:293/2018 and HREC:478/2019) for LASER-TBM and TBM-KIDS, respectively.

CRediT authorship contribution statement

Kelly E Dooley: Writing – review & editing, Resources, Funding acquisition, Conceptualization. **LUBBE WIESNER:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Sean Wasserman:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Marthinus Marthinus van der Merwe:** Writing – review & editing, Supervision, Methodology. **Edda Zangenberg:** Writing – review & editing. **Anton Joubert:** Validation, Supervision, Methodology. **Sandra Castel:** Writing – review & editing, Supervision, Project administration, Methodology. **Sydwell Poulo Maputla:** Writing – original draft, Validation, Methodology, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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