Journal of Mass Spectrometry and Advances in the Clinical Lab 32 (2024) 11–17

Contents lists available at ScienceDirect

Journal of Mass Spectrometry and Advances in the Clinical Lab

journal homepage: www.sciencedirect.com/journal/journal-of-mass-spectrometry-and-advances-in-the-clinical-lab

Research Article

A validated liquid chromatography-tandem mass spectrometry assay for the analysis of isoniazid and its metabolite acetyl-isoniazid in urine

Sydwell Poulo Maputla, Willem Van Dalen, Anton Joubert, Jennifer Norman, Sandra Castel, Marthinus van der Merwe, Lubbe Wiesner *

Division of Clinical Pharmacology, Department of Medicine, University of Cape Town, Cape Town, South Africa

ARTICLE INFO

Keywords:
Isoniazid
Acetyl-isoniazid
Solid phase extraction
LC-MS/MS
Urine
Validation

ABSTRACT

Introduction: Isoniazid (INH) is one of the most effective and potent first-line anti-tubercular drug. INH is also effectively administered as a preventative monotherapy and has been shown to significantly reduce TB incidence. INH is primarily metabolised to acetyl-isoniazid (AcINH) in the liver. AcINH is mainly excreted in urine presenting as a target for monitoring adherence to INH therapy.

Objective: The study aimed to develop and fully validate a bioanalytical method using liquid chromatography-tandem mass spectrometry for the quantification of INH and AcINH in human urine.

Methods: The samples were prepared using solid phase extraction, with the internal standards isoniazid-d4 and acetyl-isoniazid-d4 being used. The extracts were chromatographed on an Atlantis T3 analytical column with an isocratic mobile phase. For detection, a AB Sciex™ API 5500 triple quadrupole mass spectrometer was used at unit resolution in the multiple reaction monitoring mode, following positive electrospray ionization.

Results: The analytical method demonstrated sufficient sensitivity, as indicated by average signal-to-noise ratios of 7.07 and 6.23 at the lower limit of quantification for INH and AcINH, respectively. Validation was performed over three consecutive batches, demonstrating accuracy, precision, and overall robustness based on peak area ratios within the analytical range of 0.234–30.0 μg/mL for both INH and AcINH. All required validation experiments were assessed and met the acceptance criteria guidelines of the US Food and Drug Administration and European Medicines Agency. The validated method was utilized to measure concentrations of AcINH in urine as a means of assessing adherence to the intake of isoniazid in order to prevent TB infection during a phase III open-label multicenter trial.

Conclusion: A bioanalytical method was developed and fully validated for quantifying isoniazid (INH) and acetyl-isoniazid (AcINH) in 100 μL of human urine.

Introduction

According to the World Health Organization (WHO), Mycobacterium tuberculosis (Mtb) is one of the leading infectious organisms, estimated to have infected 1.9 billion people worldwide, resulting in latent tuberculosis (TB) [1]. Between 5 and 10 % of infected individuals may progress to the active stage of TB infection during their lifetime [2,3]. In its 2021 global tuberculosis report, the WHO reported an estimated 10 million active TB cases, with 1.3 million related deaths worldwide [4].

Isoniazid (INH) is one of the most effective and potent drugs against

Abbreviations: %Accuracy, The concentration of the analyte found against the nominal concentration expressed as a percentage; %Difference, The difference between the concentrations of the analyte found against a reference found concentration expressed as a percentage; AcINH, Acetyl Isoniazid; CV(%), Percentage Coefficient of Variation; DMSO, Dimethyl sulfoxide; EMA, European Medicines Agency; F/T, Freeze-thaw Stability; FDA, Food and Drug Administration; FIA, Flow injection analysis; HPLC, High Performance Liquid Chromatography; INH, Isoniazid; IPT, Isoniazid preventative therapy; ISS, Internal standard solution; ISTD, Internal standard; LC-MS/MS, Liquid Chromatography with Tandem Mass Spectrometry; LLOQ, lower limit of quantification; MDR-TB, Multi-drug resistant tuberculosis; MeOH, Methanol; MRM, Multiple reaction monitoring; *Mtb*, *Mycobacterium tuberculosis*; n, Number of determinations; NAT2, N-acetyltransferase 2; QC, Quality Control; SPE, Solid phase extraction; SS, Stock Solution; STD, Calibration Standard; STDEV, Standard Deviation; TB, Tuberculosis; TRC, Toronto Research Chemicals; ULOQ, Upper limit of quantification; WHO, World Health Organization.

* Corresponding author.
E-mail address: lubbe.wiesner@uct.ac.za (L. Wiesner).

https://doi.org/10.1016/j.jmsacl.2024.02.001
Received 9 December 2022; Received in revised form 29 January 2024; Accepted 1 February 2024
Available online 6 February 2024

Table 1
MS/MS Settings for Isoniazid, Acetyl-Isoniazid, and their internal standards.

	Isoniazid	Isoniazid-d4	Acetyl-Isoniazid	Acetyl-Isoniazid-d4
Protonated molecular ion mass (*m/z*) $[M+H]^+$	138.0	142.0	180.0	184.1
Product ion mass (*m/z*) Quantifier	121.0	125.1	138.1	125.0
Product ion mass (*m/z*) Qualifier	66.1		121.0	
Dwell time (ms)	150	150	150	150
Declustering potential (V)	91	31	81	56
Entrance potential (V)	10	10	10	10
Collision energy (eV)	19	21	31	31
Collision cell exit potential (V)	12	16	12	12

Mtb and is considered one of the four first-line drugs used in the treatment of TB [5,6]. INH is also effectively used as a preventative therapy [7] and has been shown to significantly reduce TB incidence in household contacts of patients with drug-resistant TB [7,8].

Challenges associated with INH preventative therapy (IPT) include the lengthy treatment duration, ranging from 6 to 12 months, and the potential development of INH liver toxicity [7,9]. However, IPT has been shown to have a success rate of up to 90 % when completed successfully [10–12]. The participation and adherence of study subjects play a crucial role in studying any preventative therapy. Therefore, monitoring adherence during preventative therapy studies is essential [13].

INH is primarily metabolized in the liver by the enzyme N-acetyltransferase 2 (NAT2) to the non-toxic inactive metabolite acetyl-isoniazid (AcINH) [14]. AcINH accounts for approximately 75 % of the administered INH dose, while the remaining INH is primarily eliminated through the kidneys and excreted in urine [15]. Analyzing urine for the presence of INH and AcINH provides a convenient and non-invasive method for assessing adherence.

Amlabu et al. utilized both colorimetric (Arkansas test) and tandem mass spectrometry (LC-MS/MS) assays to detect INH and its metabolite AcINH in urine for adherence monitoring [16]. However, the Arkansas test showed a decrease in sensitivity when measuring the analytes in urine 4 h after dosage, rendering it unsuitable for monitoring adherence at that time point. In contrast, the LC-MS/MS method exhibited no sensitivity limitations and could successfully monitor adherence up to 24 h after dosage. It is worth noting that the study did not provide details regarding the validation of the LC-MS/MS assay according to the guidelines of the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA).

Here, the development of a robust bioanalytical method for quantifying INH and AcINH in 100 μL of human urine using solid phase extraction and LC-MS/MS analysis is described. The method was successfully validated following guidelines set by the US FDA [17] and EMA [18].

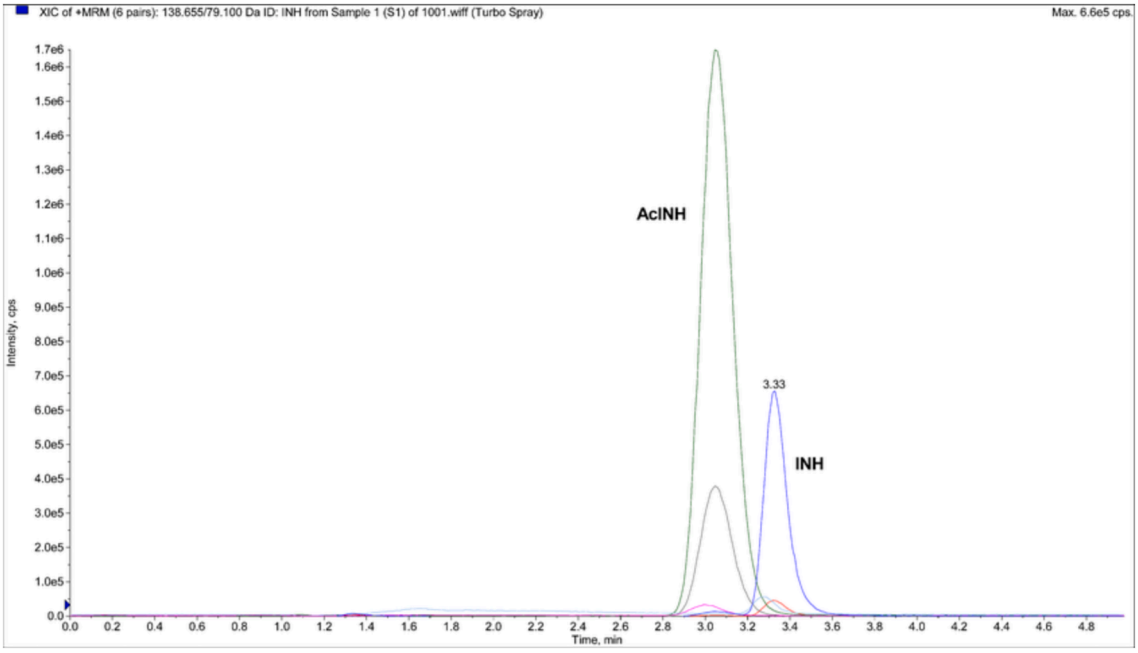


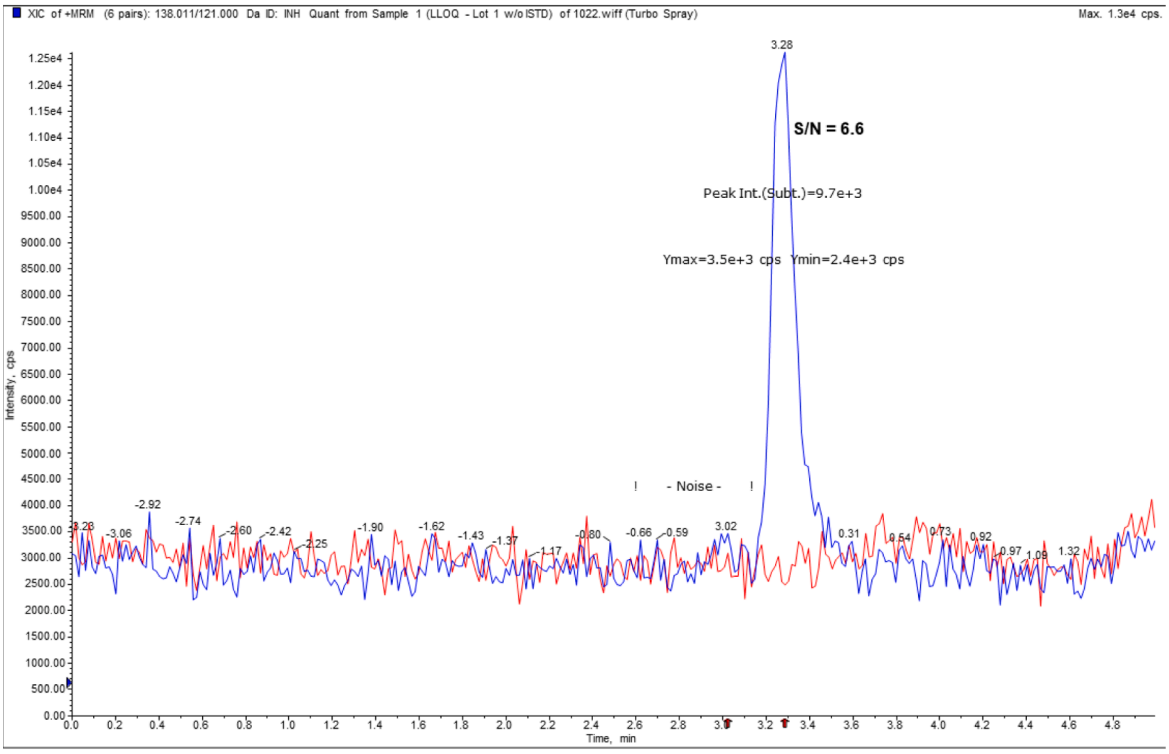
Fig. 1. Representative chromatogram indicating separation of INH and AcINH on an Atlantis T3 3 μm, 2.1 mm × 100 mm analytical column using an isocratic mobile phase which consisted of 5 mM ammonium acetate and acetonitrile (98:2, v/v) at a flow rate of 0.250 mL/min.

Table 2
Accuracy and precision of quality controls indicated in a combined summary of the three validation batches for isoniazid and acetyl-isoniazid.

	Isoniazid					Acetyl-Isoniazid				
Validation 1–3	QC-Dil	QCH	QCM	QCL	LLOQ	QC-Dil	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
Precision CV (%)	4.4	6.0	5.2	7.3	7.6	2.8	5.2	3.3	6.3	10.5
Accuracy (%)	102.6	102.3	102.7	101.4	95.2	99.5	100.6	99.7	98.3	102.5
r^2 values (n = 3)	0.9993					0.9983				

**QC-Dil = 48.0 μg/mL, QCH = 24.0 μg/mL, QCM = 12.0 μg/mL, QCL = 0.586 μg/mL, LLOQ = 0.234 μg/mL.*

2 (A)



2 (B)

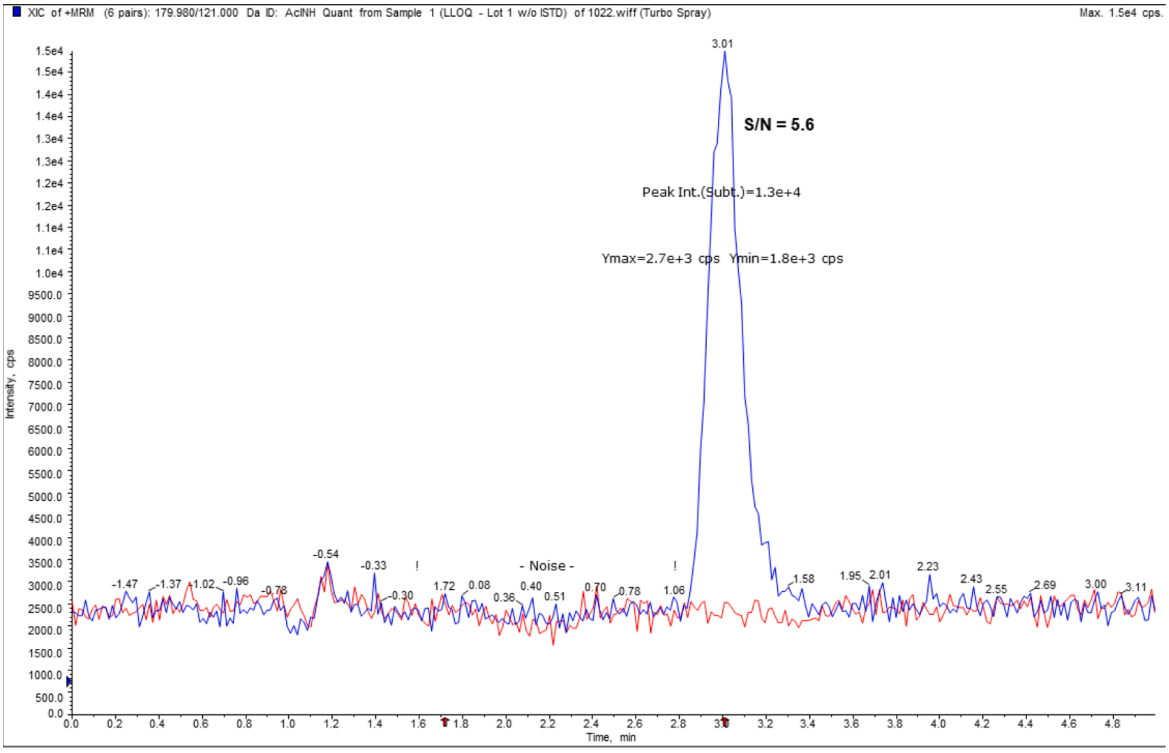


Fig. 2. Chromatograms of the LLOQ samples of isoniazid (A) and acetyl-isoniazid (B) overlayed with a double blank sample.

Table 3
Summary of the results of stability experiments for isoniazid.

Stability test	Test sample and duration		n	Precision CV (%)	% Difference
Stock solution stability	108 days at −80 °C		3	0.9	2.8
	3 h at room temperature		3	1.2	0.0
	3 h on ice		3	1.3	2.1
	3 h at 4 °C		3	2.2	1.4
Working solution stability	88 days at −80 °C	High WS	6	2.9	5.0
		Low WS	6	4.3	4.4
	4 h on ice	High WS	6	5.1	2.2
		Low WS	6	2.2	5.0
Matrix stability	10 days at −80 °C	High QC	6	2.2	4.5
		Low QC	6	1.3	0.4
	24 h at −20 °C	High QC	6	2.0	4.0
		Low QC	6	3.5	6.6
Freeze-thaw stability	3 cycles	High QC	6	3.2	3.9
		Low QL	6	7.0	0.6
Benchtop stability	4 h at room temperature	High QC	6	6.4	0.8
		Low QL	6	7.5	4.5
Autosampler stability	100 h at 8 °C (autosampler)	High QC	6	5.6	4.9
		Low QL	6	4.2	0.4
Concomitant medication	High QC		6	1.8	12.4
	Low QL		6	9.1	0.3

**High QC = 24 μg/ml, Low QC = 0.586 μg/ml, High WS = 30 μg/ml, Low WS = 0.586 μg/ml.*

Table 4
Summary of the results of stability experiments for acetyl-isoniazid.

Stability test	Test sample and duration		n	Precision CV (%)	% Difference
Stock solution stability	105 days at −80 °C		3	1.6	0.9
	3 h at room temperature		3	1.6	0.9
	3 h on ice		3	0.0	0.0
	3 h at 4 °C		3	0.0	0.0
Working solution stability	88 days at −80 °C	High WS	6	2.2	0.5
		Low WS	6	1.7	9.9
	4 h on ice	High WS	6	1.3	1.4
		Low WS	6	1.6	0.5
Matrix stability	233 days at −80 °C	High QC	6	2.3	2.0
		Low QC	6	4.6	11.7
	24 h at −20 °C	High QC	6	2.5	7.3
		Low QC	6	3.5	9.4
Freeze-thaw stability	3 cycles	High QC	6	3.5	1.3
		Low QL	6	3.3	1.9
Benchtop stability	4 h at room temperature	High QC	6	3.1	3.1
		Low QL	6	3.5	0.1
Autosampler stability	100 h at 8 °C (autosampler)	High QC	6	1.6	5.5
		Low QL	6	7.5	1.0
Concomitant medication	High QC		6	4.7	3.7
	Low QL		6	8.8	8.9

**High QC = 24 μg/ml, Low QC = 0.586 μg/ml, High WS = 30 μg/ml, Low WS = 0.586 μg/ml.*

Materials and methods

Chemicals and consumables

Reference standards for isoniazid (97.1 % purity), acetyl-isoniazid (98.0 % purity), isoniazid-d4 (INH-d4; used as internal standard 98.0 % purity), and acetyl-isoniazid-d4 (AcINH-d4; used as internal standard 98.0 % purity) were purchased from Toronto Research Chemicals (TRC).

Ammonium acetate and dimethyl sulfoxide (DMSO) (high purity grade) were purchased from Sigma-Aldrich (Darmstadt, Germany). Ammonium bicarbonate (high purity grade) was purchased from Acros organics (Geel, Belgium). Acetonitrile and methanol (LC-MS grade) were purchased from Honeywell (Burdick and Jackson, Muskegon, Michigan), and LC-MS grade water was produced in-house using the Milli-Q water purification system (Merck Millipore, Darmstadt, Germany). Strata X 33 μm Polymeric Reversed Phase 200 mg/3mL solid phase extraction (SPE) columns were purchased from Phenomenex (California, United States).

Collection and storage of urine samples

Drug-free human urine was donated by healthy volunteers not taking INH and screened for the presence of INH and AcINH before use. The urine was used to prepare calibration standards (STDs), quality control samples (QCs), and validation experiments. Clinical samples were collected and stored at approximately −80 °C until analysis. The necessary ethics approval from the University of Cape Town Human Research Ethics Committee (HREC: 089/2020) and informed consent were obtained.

Sample preparation and extraction procedure

Patient urine samples, STDs, and QC samples were thawed unassisted at room temperature. The samples were mixed by vortex for approximately 30 s, thereafter, 100 μL of each sample was transferred to a 1.5 mL microcentrifuge tube. An aliquot of 500 μL of the internal standard solution (ISS) (50 mM ammonium bicarbonate containing 0.050 μg/mL INH-d4 and AcINH-d4) was added to each sample aliquot except for the double blank sample in which an ISS free 50 mM ammonium bicarbonate solution was added. Samples were vortexed for approximately 30 s and sonicated on ice for approximately 5 min.

SPE was performed in batches of 48 samples simultaneously, using a positive pressure Biofuge system and Phenomenex Strata X SPE columns. The SPE columns were conditioned with 2 mL acetonitrile followed by 2 mL of HPLC grade water and 2 mL of a 10 mM ammonium acetate solution. The sample mixture was loaded onto the SPE cartridges and washed twice with 2 mL HPLC grade water, and all excess water on the cartridge was removed under maximum flow for 10 s. The analytes were eluted using a 2-step process with 100 μL acetonitrile followed by 750 μL of a 5 mM ammonium acetate and acetonitrile solution (98:2, v/v) (also used as the mobile phase). The eluant was briefly mixed by vortex, and 200 μL was transferred to a 96-well plate for analysis.

Instrumentation

Chromatography was performed on an Agilent 1260 series High-Performance Liquid Chromatography system (Agilent, California, USA). Separation of INH, AcINH and their internal standards, INH-d4 and AcINH-d4, was achieved on a reversed-phase Atlantis T3, 3 μm, 2.1 mm × 100 mm analytical column (Waters, California, USA). The

column oven temperature was set to 30 °C. A solution of 5 mM ammonium acetate and acetonitrile (98:2, v/v) was used as a mobile phase for isocratic elution at a flow rate of 0.250 mL/min for a total runtime of 5 min. The mobile phase was degassed with helium and sonicated prior to use. A sample volume of 1 μL was injected, and the temperature of the autosampler was set to ~8 °C.

Detection was performed by tandem mass spectrometry on an AB Sciex™ API 5500 Q trap mass spectrometer (AB Sciex™, Germany). Electrospray ionisation in the positive mode was used for ion production. The source conditions were optimised by flow injection analysis (FIA) with the following parameters: 50 psi, 55 psi, 40 psi, medium, 550 °C and 5000 V for Nebuliser gas, Turbo gas, Curtain gas, Collision gas, Source Temperature and Ion Spray Voltage, respectively. Multiple reaction monitoring (MRM) at unit resolution was used to monitor the transition of the protonated precursor ions to the product ions. Analyst® software version 1.7 using Analyst classic algorithm (AB Sciex™, Germany) was used for data processing.

Method validation

Accuracy and precision determination

STDs and QCs were prepared by spiking working solutions prepared in methanol into blank urine at room temperature. Stock solutions prepared in methanol for INH at 2 mg/mL and in DMSO for AcINH at 2 mg/mL were used to prepare working solutions. Eight STDs were prepared in blank urine at concentrations of 30.0, 15.0, 7.50, 3.75, 1.88, 0.938, 0.469, and 0.234 μg/mL for both INH and AcINH. The LLOQ was selected at the lowest concentration expected in clinical samples, based on signal-to-noise ratio (≥5) and reliable quantification (accuracy and precision of ±20 %) as per FDA and EMA guidelines. QCs were prepared at concentrations of 48.0, 24.0, 12.0, 0.586, and 0.234 μg/mL for QC-dilution, QC-high, QC-medium, QC-low, and QC-LLOQ, respectively.

The assay's accuracy and precision were assessed by analysing three independent runs of freshly prepared STDs in duplicate and six replicates of each QC. Validation experiment and stability QCs were also assessed in these runs in six replicates. The calibration curves of both analytes were validated over the concentration range of 0.234–30.0 μg/mL using a quadratic regression with a weighting of 1/x and a linear regression with a weighting of $1/x^2$ using area ratios of analytes/internal standards for INH and AcINH, respectively. The QC dilution sample was used to validate the analysis of the dilution of samples above the upper level of quantitation (30.0 μg/mL) using a 5-fold (1:4) dilution with a blank matrix to a concentration of 48.0 μg/mL.

Stock solution stability

Short-term stock solution stability for INH at 2 mg/mL in methanol and AcINH at 2 mg/mL in DMSO was assessed for 3 h at room temperature, on ice, and at approximately 4 °C. Long-term stability was assessed at ~ -80 °C for 108 days for INH and 105 days for AcINH. Test samples were assessed against freshly prepared reference samples at the same concentration. Triplicate dilutions (10 μL stock solution + 990 μL solvent) using 100 % methanol for each test and reference sample were performed before analysis. The absorbance was measured using a spectrophotometer at an absorbance wavelength of 262 nm. Test samples' mean absorbance was compared to that of the reference samples to determine stability.

Working solution stability

Short-term working solution stability was assessed for 4 h on ice, in methanol at the highest (1000 μg/mL) and lowest (4.88 μg/mL) working solution concentrations for both INH and AcINH. Long-term working solution stability was assessed for 88 days in methanol at ~ -80 °C. The working solutions were diluted in triplicate to 30 μg/mL for the high concentration and to 0.234 μg/mL for the low concentration in 5 mM ammonium acetate and acetonitrile (98:2, v/v) containing 0.500 μg/mL of the internal standards, before analysis by LC-MS/MS.

Reinjection reproducibility and autosampler stability

Reinjection reproducibility was assessed to determine the possibility of reanalysis of an analytical batch by reinjection in the case of instrument interruptions. Autosampler stability was assessed to determine the stability of analytes at the autosampler temperature. Following the injection of the first validation batch, the extracted samples (96-well plate) remained in the autosampler at a temperature of ~8 °C, and the batch was reinjected after 100 h. The peak area ratios (n = 6) of the initial injections were compared to that of the reinjected batch to assess autosampler stability and reinjection reproducibility.

Stability in matrix

QC high and QC low samples were prepared, verified, and stored at the intended study sample storage temperature (~−80 °C). An additional storage condition (~−20 °C) was tested to provide flexibility at sites that do not have access to −80 °C freezers. Each QC level was analysed in six replicates against a freshly prepared calibration curve, and observed concentrations were compared to the nominal concentrations to determine the stability of INH and AcINH in urine.

Freeze-thaw stability

QC high and QC low samples were frozen at ~ -80 °C and put through three freeze–thaw cycles, consisting of 2 h of thawing time at room temperature followed by 12–24 h of freezing between each cycle. These samples were analysed against a freshly prepared valid calibration curve and assessed for accuracy against the nominal QC concentrations.

Benchtop stability

QC high and QC low samples were left on bench at room temperature and on ice for 4 h and then analysed against a freshly prepared calibration curve. The observed concentrations were compared to the nominal QC concentrations to determine stability.

Recovery

Recovery, the extraction efficiency of the analytical procedure, was determined by extracting six different blank matrix pools and preparing each with the analyte at QC high, QC medium, and QC low concentrations. The analytical responses of these QCs were compared to QCs spiked in the same six blank matrix sources before extraction. The internal standards were included to normalise the extraction recovery and to express the results as comparative peak area ratios. However, recoveries of the internal standards were not assessed.

Process efficiency

Process efficiency compares the response of the mass spectrometer observed for extracted samples to those observed for neat unextracted samples spiked into injection solvent where the matrix is not present. The effects of both the extraction recovery and the presence of matrix on analyte response were included in this assessment. QCs prepared at high, medium, and low QC concentrations from six different matrix lots were extracted according to the analytical method and compared to QCs spiked at high, medium, and low QC concentration in triplicate in a 5 mM ammonium acetate and acetonitrile solution (98:2, v/v). The internal standards were used to normalise for the extraction, and the peak area ratios observed after extraction were compared to the peak area ratios of the neat samples and expressed as percentage process efficiency.

Matrix effects

Matrix effects refer to interference(s) of the analytical process by components in a sample other than the analyte being studied. Such interferences mainly influence the ionisation of the analytes of interest. For this assessment, the method described by Matuszewski [19] was used to quantify the effect of the matrix across the calibration range of the assay using different matrix sources. A minimum of six blank sources of urine were extracted without internal standard and spiked at high,

medium, and low QC concentrations and one internal standard concentration. The peak area ratios for each concentration level in each matrix source were used to generate simple linear regressions for each matrix lot. The variability observed between the slopes of the different regressions provides an estimate of the variability attributed to the presence of different matrix components (matrix effects).

Concomitant medication effects

Concomitant medications were tested for potential interference, especially those administered as part of the study and commonly prescribed to the study population. Concomitant medications (ethambutol, pyrazinamide, delamanid, DM6705, rifampicin, and 25-deacetyl-rifampicin) were tested at approximately 15.0 μg/mL, which is the mid-level concentration of this assay as no precise levels of the tested concomitant medications in urine are reported. High and low QCs were prepared in urine containing the concomitant medications and extracted in six replicates together with reference high and low QCs without concomitant medications. The mean peak area ratios observed in the test samples were compared to those observed in the reference samples. Selectivity in the presence of concomitant medication was also assessed by evaluating a blank and a double-blank sample in the presence of concomitant medications.

Specificity/selectivity and carryover

Selectivity or specificity relates to the ability of the analytical method to distinguish and quantify the analyte of interest in the presence of other compounds involved in the analytical process. To assess carryover, a double blank sample was injected following the injection of the highest calibration standard (30.0 μg/mL) and monitored for any response of the analyte and internal standard. Six different lots of urine were extracted as double blank samples, without analyte and internal standard. Aliquots from the duplicate six lots of urine were extracted as blank samples, with only the internal standard added. Finally, aliquots from the duplicate six lots of urine were spiked with the analytes at the LLOQ concentration without internal standards and extracted. This experiment was done to monitor and ensure that the measured transitions are due to the presence of the analytes and not the background components of the matrix.

Crosstalk/contribution

This experiment was conducted to test for the presence or lack of contribution between the analytes and their deuterated internal standards. A blank matrix was extracted and then individually spiked with each of the internal standards at 0.050 μg/mL and with each analyte separately at the ULOQ (30.0 μg/mL) and LLOQ (0.234 μg/mL) concentrations. The contribution of the analytes to the internal standards was assessed using the ULOQ samples in the internal standards channels at the retention time of the internal standards for any signal response. Similarly, the blank samples (containing the deuterated internal standards) were used to assess the contribution of internal standards to each analyte channel at the retention time of the analytes. The LLOQ samples were used as reference to determine crosstalk/contribution.

Results and discussion

The assay development began by infusing INH, AcINH, and their internal standards (INH-d4 and AcINH-d4) directly into the ion source using a 1 mL Hamilton syringe connected to a syringe pump operating at a flow rate of 10 μL/min. The analytes and internal standards were prepared at a concentration of 500 ng/mL in a mixture of methanol and water (9:1, v/v) containing 0.1 % formic acid. The most suitable multiple reaction monitoring (MRM) transitions and compound-specific parameters were chosen and summarized in Table 1. The source conditions were determined using flow injection analysis (FIA) and are described in the instrumentation section. Reverse-phase chromatography was employed to separate INH and AcINH, utilizing an isocratic mobile phase consisting of 5 mM ammonium acetate with 2 % acetonitrile (v/v) on an Atlantis T3 column (3 μm, 2.1 mm × 100 mm), as shown in Fig. 1. The retention times for INH, AcINH, and their respective deuterated internal standards were observed to be 3.33 and 3.04 min, respectively.

The next objective was to develop a robust and reproducible extraction method for isolating INH and AcINH from human urine. The composition of urine exhibits high variability compared to other matrices, like plasma and serum, due to factors such as fluid intake, pathological conditions, and diet, resulting in varying levels of urine constituents that can potentially cause matrix effects, such as ion suppression or enhancement [20]. While Amlabu et al. [16] reported a sample dilution LC-MS/MS method for analyzing INH and AcINH in urine, we chose to employ a specific SPE method in order to produce cleaner extracts and minimize excessive matrix components. Strata X 33 μm Polymeric Reversed Phase 200 mg/3 mL SPE cartridges were utilized to load and retain INH and AcINH on the solid phase at pH 8.5 (using 50 mM ammonium bicarbonate).

The validation process demonstrated that the analytical method was robust, reproducible, accurate, and precise. Calibration curves were generated for both analytes, covering a range of 0.234 to 30.0 μg/mL. A quadratic regression weighted by 1/x was employed for INH, while a linear regression weighted by $1/x^2$ was used for AcINH. Accuracy (% Accuracy) and precision (% CV) were evaluated by analyzing QC samples across the concentration range. The regression statistics and values for accuracy and precision for all three runs are summarized in Table 2.

Specificity and sensitivity assessments are illustrated in Fig. 2A and B, showcasing the overlaid chromatograms of the LLOQ samples and double-blank samples. A double-blank sample (without analyte and ISTD), analyzed after the highest calibration standard did not exhibit any chromatographic peaks at the retention times of the analytes, indicating the absence of carryover.

The results of the stability assessment are presented in Tables 3 and 4. The stock solutions of INH and Ac-INH were found to be stable at approximately −80 °C for 108 and 105 days, respectively. Both analytes remained stable for 3 h at room temperature, on ice, and at approximately 4 °C, which corresponds to the maximum anticipated time that stock solutions will be used while preparing working solutions. Working solutions of both INH and AcINH were stable for 88 days at approximately −80 °C and 4 h on ice. In sample extracts, both analytes were stable when left on the instrument at approximately 8 °C for at least 100 h. Reinjection reproducibility experiments demonstrated that extracted samples could be reinjected within 100 h. INH and AcINH were shown to be stable in urine for at least three freeze–thaw cycles and on the bench for 4 h at room temperature and on ice. Short-term and long-term stability of INH in urine was verified for ten days at approximately −80 °C and 24 h at approximately −20 °C. AcINH exhibited short-term and long-term stability in urine for 233 days at approximately −80 °C and 40 days at approximately −20 °C. The average percentage recoveries for high, medium, and low concentrations of INH and AcINH were 92.3 % and 99.8 %, respectively. The average process efficiency was determined to be 90.7 % for INH and 97.2 % for AcINH. No significant matrix effects were observed, with precision estimates across the average regression slopes of 3.0 % for INH and 2.8 % for AcINH.

The inclusion of concomitant medications did not have a significant impact on the assay, as the deuterated internal standards adequately compensated for any potential interference, ensuring the accuracy of the measurements for both analytes.

Application to a clinical study

The validated analytical method was employed to measure the concentration of AcINH in urine samples obtained from participants of the PHOENIX MDR-TB study. In the interim analysis, a total of 733 samples were assayed for AcINH. The clinical study team intends to publish the clinical data once the analysis of the study results has been

completed.

Conclusion

A selective and robust SPE LC-MS/MS assay was developed and validated following guidelines from the US FDA and EMA for the quantification of INH and AcINH in human urine. In a study involving participants from the PHOENIX MDR-TB study, this assay was employed to measure AcINH concentrations in urine samples as an indicator of adherence to treatment. The assay was implemented without adjustments for fluid intake to compensate for the measured AcINH concentrations. However, for adherence monitoring purposes, the presence of a positive AcINH concentration would be sufficient to determine adherence qualitatively. With the incorporation of fluid intake corrections, the assay has the potential to serve as a reliable quantitative method for pharmacokinetic studies of INH and AcINH in urine. Additionally, the assay demonstrates sensitivity, robustness, and the advantage of being a non-invasive procedure.

Ethics statement

Informed consent was obtained by all matrix donors and trial participant and the ethics approval for this study was approved by the University of Cape Town Human Research Ethics Committee (HREC: 089/2020).

CRediT authorship contribution statement

Sydwell Poulo Maputla: Methodology, Validation, Investigation, Writing – original draft. **Willem Van Dalen:** Validation, Investigation. **Anton Joubert:** Supervision, Investigation. **Jennifer Norman:** Conceptualization. **Sandra Castel:** Conceptualization, Project administration. **Marthinus van der Merwe:** Writing – review & editing. **Lubbe Wiesner:** Conceptualization, Supervision, Resources.

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.

Acknowledgements

The University of Cape Town Clinical PK Laboratory is supported in part via the Adult Clinical Trial Group (ACTG), by the National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health under award numbers UM1 AI068634, UM1 AI068636, and UM1 AI106701; as well as the Infant Maternal Paediatric Adolescent AIDS Clinical Trials Group (IMPAACT), funding provided by National Institute of Allergy and Infectious Diseases (U01 AI068632), the Eunice Kennedy Shriver National Institute of Child Health and Human Development, and the National Institute of Mental Health grant AI068632. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

References

[1] W.H. Organization, Global tuberculosis report 2021: supplementary material. 2022.
[2] W.H. Organization, Guidelines on the management of latent tuberculosis infection, World Health Organization, 2015.
[3] S. Gandra, et al., Trends in antibiotic resistance among major bacterial pathogens isolated from blood cultures tested at a large private laboratory network in India, 2008–2014, Int. J. Infect. Dis. 50 (2016) 75–82.
[4] W.H. Organization, Global Tuberculosis Report 2021, Switzerland, Geneva, 2021.
[5] D. Garg, V. Goyal, Spinal tuberculosis treatment: An enduring bone of contention, Ann. Indian Acad. Neurol. 23 (4) (2020) 441.
[6] D.A. Mitchison, Role of individual drugs in the chemotherapy of tuberculosis, Int. J. Tuberc. Lung Dis. 4 (9) (2000) 796–806.
[7] T. Mudzviti, et al., Tolerability of isoniazid preventive therapy in an HIV-infected cohort of paediatric and adolescent patients on antiretroviral therapy from a resource-limited setting: a retrospective cohort study, Drugs-Real World Outcomes 6 (1) (2019) 37–42.
[8] C.-C. Huang, et al., The impact of isoniazid preventive therapy on tuberculosis among household contacts of isoniazid-resistant patients, bioRxiv (2019) 479865.
[9] B. Durovni, et al., The implementation of isoniazid preventive therapy in HIV clinics: the experience from the TB/HIV in Rio (THRio) study, AIDS (London, England) 24 (Suppl 5) (2010) S49.
[10] G. Fox, et al., Preventive therapy for latent tuberculosis infection—the promise and the challenges, Int. J. Infect. Dis. 56 (2017) 68–76.
[11] A. Uppal, et al., Economic and modeling evidence for tuberculosis preventive therapy among people living with HIV: A systematic review and meta-analysis, PLoS Med. 18 (9) (2021) e1003712.
[12] WHO, WHO operational handbook on tuberculosis. Module 1: prevention-tuberculosis preventive treatment, World Health Organization Geneva, Switzerland, 2020.
[13] W.H. Organization, Guidelines for treatment of drug-susceptible tuberculosis and patient care. 2017.
[14] E.R. Erwin, et al., Pharmacokinetics of isoniazid: the good, the bad, and the alternatives, Tuberculosis 116 (2019) S66–S70.
[15] P. Wang, et al., NAT2 polymorphisms and susceptibility to anti-tuberculosis drug-induced liver injury: a meta-analysis, Int. J. Tuberc. Lung Dis. 16 (5) (2012) 589–595.
[16] V. Amlabu, et al., Isoniazid/acetylisoniazid urine concentrations: markers of adherence to isoniazid preventive therapy in children, Int. J. Tuberc. Lung Dis. 18 (5) (2014) 528–530.
[17] Food and D. Administration, Food and Drug Administration, Bioanalytical Method Validation. 2018.
[18] G. Smith, European medicines agency guideline on bioanalytical method validation: what more is there to say? Bioanalysis 4 (8) (2012) 865–868.
[19] B. Matuszewski, M. Constanzer, C. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC– MS/MS, Anal. Chem. 75 (13) (2003) 3019–3030.
[20] D. Sviridov, G.L. Hortin, Urine albumin measurement: effects of urine matrix constituents, Clin. Chim. Acta 404 (2) (2009) 140–143.