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Validation and application of a quantitative LC-MS/MS assay for the analysis of first-line anti-tuberculosis drugs, rifabutin and their metabolites in human breast milk

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ABSTRACT

Breast milk is the preferred method of infant nutrition. Breastfeeding infants born to mothers treated for TB may be at risk of drug toxicity through breast milk exposure, or potentially be vulnerable to select for drug resistance with low level drug exposure. Except for isoniazid, the quantification of first-line TB drugs including rifabutin in breast milk has not been previously described and will provide much-needed insight to TB drug exposure in breastfeeding infants. We developed and validated a novel method to quantify several first-line TB drugs and their major metabolites in breast milk. Accuracy and precision were assessed during three consecutive, independent validation batches over a calibration range of 0.300–30.0 μg/mL for isoniazid and ethambutol, 0.150–15.0 μg/mL for acetyl isoniazid, desacetyl rifampicin, rifampicin, and pyrazinamide, 0.0150–1.50 μg/mL for rifabutin, and 0.00751–0.751 μg/mL for deacetyl rifabutin in breast milk. The method was reproducible for all analytes when using breast milk from six different sources and was not influenced by matrix effects with a mean regression precision (CV(%)) ranging between 1.0 and 2.8. The average recovery of analytes from the matrix was 76.7–99.1%, with a CV(%) between 0.4 and 4.4, while the average process efficiency was between 74.4 and 93.1% with a CV(%) between 1.9 and 8.3. Although only acetyl isoniazid, isoniazid, ethambutol, and pyrazinamide were successfully assayed in breast milk, samples taken from mothers treated for rifampicin-resistant TB and the inclusion of all first-line TB drugs, including rifabutin in the assay development and validation process will allow future quantification of these analytes in breast milk.

1. Introduction

First-line tuberculosis (TB) drugs, including rifabutin, used for the treatment of drug-sensitive TB are considered compatible with breastfeeding by the Centers for Disease Control and Prevention (CDC) [1]. Previous studies indicated that concentrations of first-line TB drugs in breast milk were lower than the recommended therapeutic doses for infants [2–4]. Although several studies report low transfer rates of some TB drugs to breast milk [2–7], there are no published bioanalytical methods for first-line TB drugs, including rifabutin and their metabolites in breast milk.. In a study by Singh *et al.* [7], quantification of isoniazid in breast milk was reported but the method used was validated for the determination of isoniazid in serum, not breast milk [8].

Considering the immaturity of infant drug metabolism, including the cytochrome P450 system where most TB drugs are metabolised [9–11], infants exposed to TB drugs through breastfeeding could be vulnerable to drug toxicity. Low infant drug exposure via breastfeeding could also potentially be prophylactic for infants exposed to TB [12]. In theory, low infant drug exposure via breast milk, could also select for drug resistance in infants should they develop TB. An enhanced understanding of TB drug exposure in breast milk is critical considering breastfeeding is favoured as the optimal source of infant nutrition, particularly in resource-limited settings where TB is an endemic [13].

Rifabutin, a member of the rifamycin-group of TB drugs, is of

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Table 1
Transitions, source, and collision cell settings for the eight analytes and respective internal standards.

Analyte	Ion transitions (*m/z*)	Dwell time (ms)	Declustering potential (V)	Entrance potential (V)	Collision energy (eV)	Collision cell exit potential (V)
Acetyl isoniazid	180.1 → 138.2	85	51	10	21	12
Acetyl isoniazid-d4	184.0 → 142.1	85	36	10	23	8
Isoniazid	138.1 → 121.1	85	51	10	29	10
Isoniazid-d4	142.1 → 83.2	85	61	10	41	6
Ethambutol	205.2 → 55.2	100	46	10	43	4
Ethambutol-d4	209.2 → 120.3	100	51	10	23	8
Pyrazinamide	124.1 → 81.1	65	40	10	23	6
Pyrazinamide-15N-d3	128.1 → 84.0	65	56	10	25	16
Desacetyl rifampicin	781.4 → 749.5	85	46	10	17	42
Desacetyl rifampicin-d3	786.4* →754.5	85	61	10	21	48
Rifampicin	823.5 → 791.4	85	81	10	23	52
Rifampicin-d3	828.5* →796.4	85	86	10	25	22
Rifabutin	847.5 → 815.5	85	101	10	37	52
Rifabutin-d7	855.5 → 823.6	85	106	10	31	52
Deacetyl rifabutin	805.4 → 773.5	85	51	10	33	54
Deacetyl rifabutin-d7	812.4 → 780.3	85	61	10	27	22

*Third isotope was used.

equivalent potency to rifampicin but has several pharmacokinetic and toxicological advantages, particularly when administered to patients co-infected with HIV who are treated with antiretroviral drugs [14]. Unlike rifampicin which requires a dose adjustment, rifabutin may be co-administered with integrase inhibitors, which are now included in first-line ART regimens [15]. Rifabutin may also be used as a substitute drug for treatment-related toxicities related to rifampicin [14]. The exposure of rifabutin in breast milk is unknown.

Breast milk has an overly complex and variable composition containing cells, oligosaccharides, proteins, and lipids. Consequently, accurate quantification of drugs and metabolites in breast milk is challenging [16]. A validated bioanalytical method to measure the concentrations of first-line TB drugs in breast milk is urgently required. We validated a novel quantification method for the analysis of the first-line TB drugs (ethambutol, isoniazid, pyrazinamide, and rifampicin) and their metabolites (acetyl isoniazid and desacetyl rifampicin), including rifabutin and its metabolite deacetyl rifabutin, in human breast milk using high-performance liquid chromatography with tandem mass spectrometry (LC-MS/MS), and explored the application of these assays in a clinical context.

2. Materials and methods

2.1. Chemicals and reagents

Acetyl isoniazid, deacetyl rifabutin, desacetyl rifampicin, ethambutol, isoniazid, pyrazinamide, rifabutin and their deuterated internal standards: acetyl isoniazid-d4 (isotopic purity: 99.4%), deacetyl rifabutin-d7 (isotopic purity: 98.5%), desacetyl rifampicin-d3 (isotopic purity: 99.6%), ethambutol-d4 (isotopic purity: 99.1%), isoniazid-d4 (isotopic purity: 99.3%), pyrazinamide-15 N,d3 (isotopic purity: 99.5%), and rifabutin-d7 (isotopic purity: 97.2%), were purchased from Toronto Research Chemicals (North York, Canada). Rifampicin reference material was purchased from the United States Pharmacopeia (Rockville, United States) and its deuterated internal standard, rifampicin-d3 (isotopic purity: 98.9%), was purchased from Toronto Research Chemicals (North York, Canada). Ammonium acetate, ascorbic acid, and dimethyl sulfoxide were purchased from Sigma-Aldrich (Darmstadt, Germany). Formic acid and ammonium bicarbonate were purchased from Merck (Darmstadt, Germany) and Acros Organics (Fair Lawn, United States), respectively. Acetonitrile and methanol were purchased from Honeywell (Charlotte, United States). LC-MS/MS grade millipore water was produced in-house (Merck-Millipore, Germany).

2.2. Stock solutions, calibration standards, and quality control samples

Acetyl isoniazid, desacetyl rifampicin, ethambutol, isoniazid, pyrazinamide, and rifampicin stock solutions were prepared in methanol at 5 mg/mL, while rifabutin and deacetyl rifabutin were prepared in acetonitrile at 1 mg/mL. These stock solutions were used to prepare working solutions on crushed ice for the calibration standards (STD) and quality control (QC) samples in a mixture of methanol and acetonitrile (3:1, v/v). Calibration STDs and QC samples were prepared by spiking working solutions of the analytes into drug free human breast milk on crushed ice to obtain the desired concentrations over a calibration range of 0.300–30.0 μg/mL for isoniazid and ethambutol, 0.150–15.0 μg/mL for acetyl isoniazid, desacetyl rifampicin, rifampicin, and pyrazinamide, 0.0150–1.50 μg/mL for rifabutin and 0.00751–0.751 μg/mL for deacetyl rifabutin.

2.3. Collection of breast milk

We used drug-free human breast milk donated by Milk Matters human milk bank, South Africa (HREC number 639/2019) for the preparation of STDs and QCs for method development and validation. For our exploration of isoniazid, acetyl isoniazid, ethambutol, and pyrazinamide in breast milk, we used breast milk samples from women (≥18 years old) on treatment for rifampicin-resistant (RR-) TB at King Dinuzulu-Hospital (KDH) in Durban, South Africa [17]. KDH is a specialist provincial rifampicin-resistant TB hospital where, until recently, all pregnant women with RR-TB in KwaZulu-Natal province were referred for care. We obtained 1 mL breast milk samples by manual expression from breastfeeding mothers approximately-six weeks post-delivery at the following time-points: immediately prior to maternal dosing and at 2, 4, and 6 h post dose. Patient A and B were treated with a minimum of five anti-TB drugs including isoniazid, ethambutol, and pyrazinamide, dosed at 900 mg, 1200 mg, and 1750 mg, respectively.

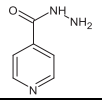
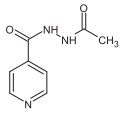
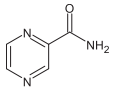
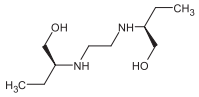
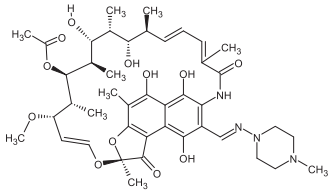
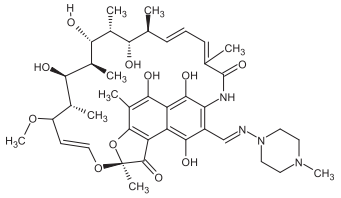
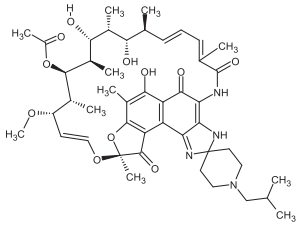
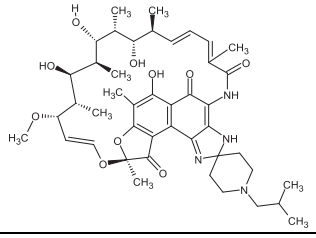
2.4. Calculation of infant daily dosage

To assess the potential effects that the observed concentration may have on the infant, the calculations below were used [18].

Infant daily dosage = F (bioavailability of the drug in exclusively breastfed infants) × milk drug concentration × daily milk volume ingested (150 mL/kg/day).

$$\textbf{Maternal weight} - \textbf{adjusted dose}\ (\textbf{mg/kg/day}) = \frac{\text{Maternal dose (mg)}}{\text{weight (kg)}}$$

Table 2
Chemical structures of the TB drugs with their respective molecular weights and logP values.

Isoniazid Mw = 137.14 logP = −0.69	
Acetyl isoniazid Mw = 179.18 logP = -0.78	
Pyrazinamide Mw = 123.11 logP = −1.20	
Ethambutol Mw = 204.31 logP = −0.06	
Rifampicin Mw = 822.94 logP = 2.9	
Desacetyl rifampicin Mw = 780.90 logP = 1.50	
Rifabutin Mw = 874.00 logP = 3.7	
Desacetyl rifabutin Mw = 804.97 logP = 3.13	

$$\textbf{Relative infant dosage}\ (\%) = \frac{\text{Infant daily dosage (mg/kg/day)}}{\text{Maternal dose (mg/kg/day)}} \text{x}100$$

Infant dosage relative to therapeutic dose (%) = Infant daily dosage × therapeutic infant dosage.
(mg/kg/day).

2.5. Ethics

We obtained ethics approval from the Human Research Ethics Committee (HREC) of the South African Medical Research Council (EC017-6/2016) and from the HREC of the Faculty of Health Sciences, University of Cape Town (711/2019). Informed consent was obtained from participants in a language of choice (English or isiXhosa).

2.6. Sample analysis

Sample analysis involved a combination of protein precipitation (done on crushed ice) and solid phase extraction (SPE). The precipitation solution was a mixture of acetonitrile and methanol (1:1, v/v) containing the internal standards at different concentrations: 0.1 μg/mL for ethambutol-d4, 0.4 μg/mL for deacetyl-rifabutin-d7, 1 μg/mL for acetyl isoniazid-d4, pyrazinamide-^{15}N,d3, and rifabutin-d7, 2 μg/mL for desacetyl-rifampicin-d3 and rifampicin-d3 and 3 μg/mL for isoniazid-d4. Spiked calibration STDs and QC samples were thawed on ice and vortex mixed before 200 μL aliquots were taken and 400 μL precipitation solution, kept on crushed ice, was added. Following another vortex mixing step, precipitated samples were centrifuged for 5 min at ~ 20 238 g. A volume of 125 μL of each supernatant was removed and dried under a stream of nitrogen at room temperature (~22 °C) for ~ 45 min. The samples were reconstituted with 800 μL of 50 mM ammonium bicarbonate solution, followed by vortex mixing before being loaded to Strata-X columns (Phenomenex, 33 μm × 200 mg/3 mL) that were conditioned with methanol and equilibrated by LC-MS/MS grade millipore water followed by 20 mM ammonium acetate solution. The interfering components were washed out with LC-MS/MS grade millipore water, followed by the elution of the more polar analytes (acetyl isoniazid, isoniazid, ethambutol, and pyrazinamide) using 0.1% formic acid in a mixture of acetonitrile and LC-MS/MS grade millipore water (1:4, v/v). The less polar analytes (deacetyl rifabutin, rifabutin, desacetyl rifampicin, and rifampicin) were eluted using 0.1% formic acid in a mixture of acetonitrile and LC-MS/MS grade millipore water (6:4, v/v). A volume of 200 μL of the more polar eluent was transferred to a 96 well plate for analysis by LC-MS/MS. A volume of 150 μL of the less polar eluent was transferred to a different 96 well plate containing 50 μL of a 50 μg/mL ascorbic acid solution in LC-MS/MS grade millipore water and was mixed gently prior to analysis by LC-MS/MS.

2.7. Instrumentation

2.7.1. Chromatographic separation

Chromatographic separation of the analytes was achieved using three different chromatography methods and two different analytical columns. All three methods used mobile phases consisting of 0.1% formic acid in LC-MS/MS grade millipore water (solvent A) and 0.1% formic acid in a mixture of acetonitrile and methanol (1:1, v/v) (solvent B) on an Agilent 1200 HPLC (Agilent, United States). Chromatographic separation of the more polar analytes (acetyl isoniazid, isoniazid, ethambutol, and pyrazinamide) was achieved using gradient elution with an Atlantis 100 Å T3 column, 2.1 mm × 100 mm, 3 μm (Waters, United States). The total run time was 5 min applying the following gradient: 0.00–0.50-min, 100% solvent A, 2.00–2.15-min, 10% solvent A, 3.50–5.00-min, 100% solvent A, at a flow rate of 0.150 mL/min. The chromatographic separation of the less polar analytes was achieved using two different isocratic elution methods for rifampicin and rifabutin along with their metabolites. Both these isocratic methods included a 3-min run time at 0.4 mL/min, using a Poroshell 120 EC-C18 column, 4.6 mm × 50 mm, 2.7 μm (Agilent, United States), with the separation of rifampicin and desacetyl rifampicin employing a mobile phase consisting of 20% solvent A and 80% solvent B, while the mobile phase for rifabutin and deacetyl rifabutin separation consisted of 30% solvent A and 70% solvent B.

2.7.1.1. Mass spectrometry conditions. An AB Sciex API4000 (Sciex, Germany) mass spectrometer at unit resolution in the multiple reaction monitoring mode was used to monitor the transition of the protonated precursor ions to product ions as shown in Table 1. Electrospray ionisation was used for ion production in the positive mode.

The instrument gas settings for the more polar analytes were set at 40.0, 60.0, 40.0, and 6.00 (arbitrary units) for the nebuliser gas, turbo

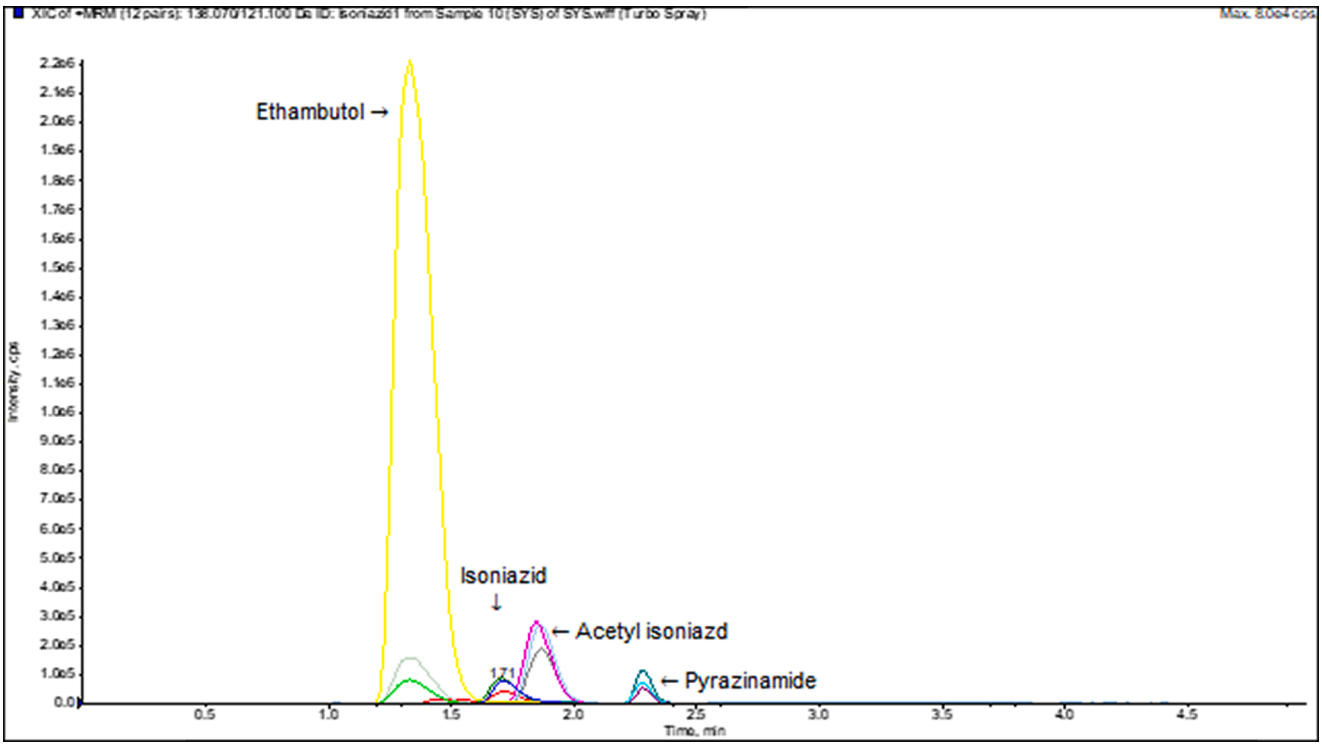


Fig. 1. Chromatographic representation of the small more polar analytes showing two transitions for each analyte and their respective internal standards at concentrations of 6.01 μg/mL, 3.00 μg/mL, 6.01 μg/mL, and 3.00 μg/mL for isoniazid, acetyl isoniazid, ethambutol, and pyrazinamide, respectively.

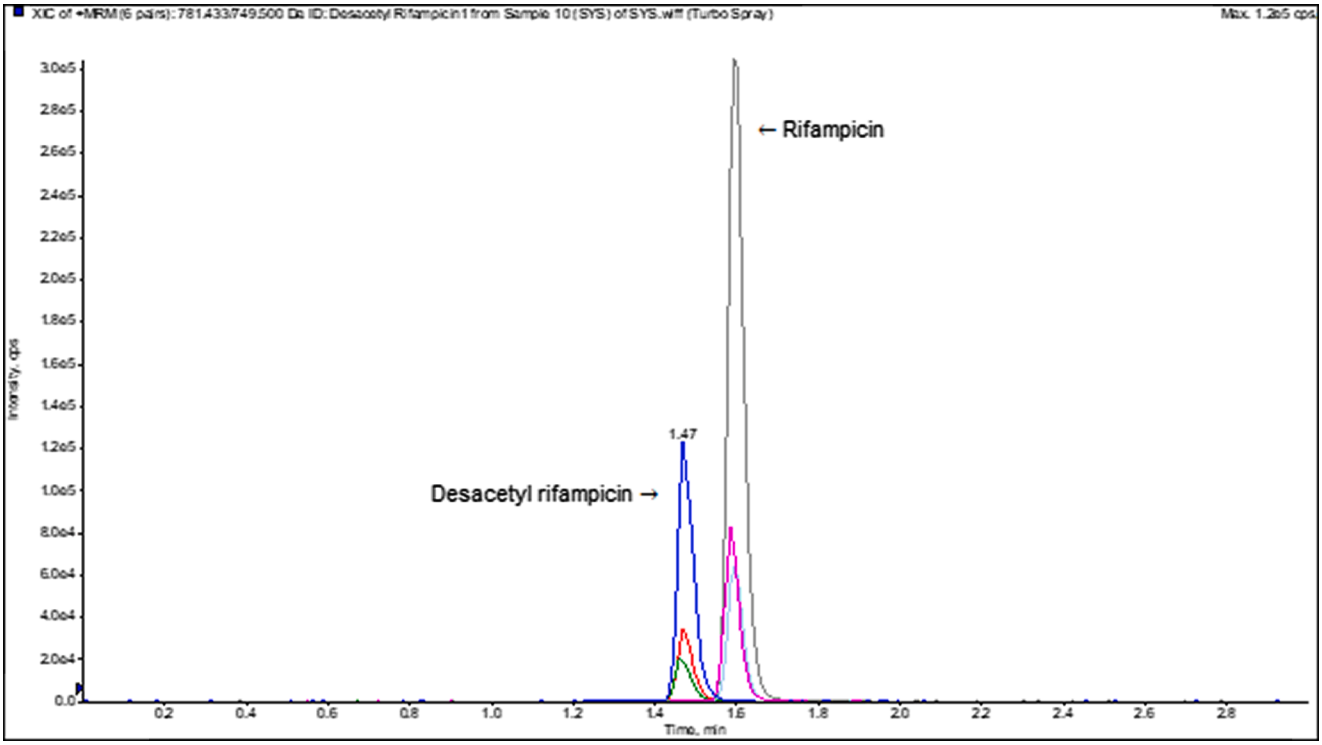


Fig. 2. Chromatographic representation of rifampicin and desacetyl rifampicin showing two transitions for each analyte and their respective internal standards at a concentration of 3.00 μg/mL for both rifampicin and desacetyl rifampicin.

gas, curtain gas, and collision gas, respectively. The ion source temperature was set at 550 °C and the ion spray voltage at 5000 V. For the less polar analytes, these settings were at 40.0, 40.0, 30.0, and 8.00 (arbitrary units) for the nebuliser gas, turbo gas, curtain gas, and collision gas, respectively, the ion source temperature was set at 300 °C and ion spray voltage at 5000 V. The instrument was interfaced with a computer running Analyst® version 1.6.2 software (AB Sciex™, Germany) for data processing.

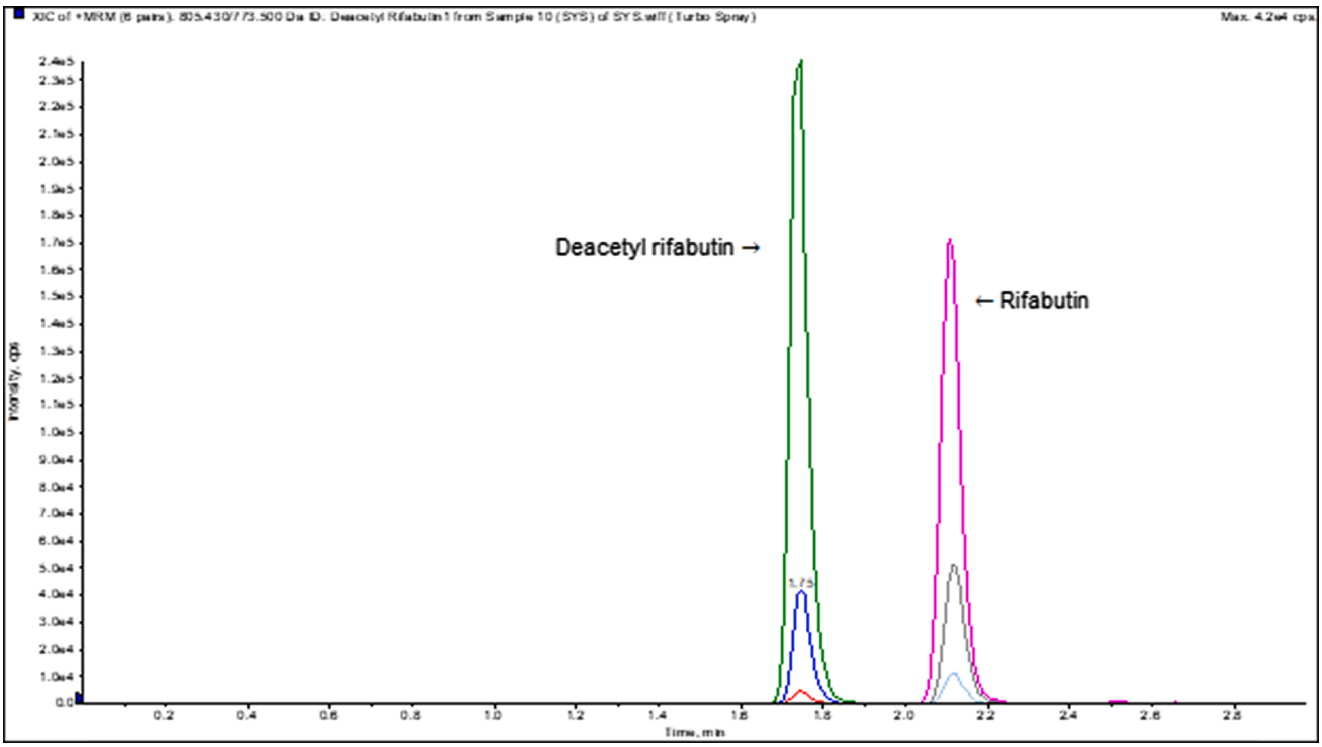


Fig. 3. Chromatographic representation of rifabutin and deacetyl rifabutin showing two transitions for each analyte and their respective internal standards at a concentration of 0.302 μg/mL and 0.151 μg/mL for rifabutin and deacetyl rifabutin, respectively.

2.8. Method validation

Validation of the method was done according to the USA Food and Drug Administration (FDA) [19] and European Medicines Agency (EMA) [20] bioanalytical method validation guidelines.

Calibration curves were each based on seven calibration STDs in duplicate and inter-day and intra-day accuracy and precision were proven with QCs in sixfold at four different concentration levels (low, medium, and high), including the lower limit of quantification (LLOQ), assessed in three validation batches. These batches also included the assessment for carry-over by injecting a double blank sample (without analyte and internal standard) following a high concentration standard. In one of the three batches, dilution integrity of the samples was assessed using a QC sample twice the high QC concentration of each analyte in sixfold. LLOQ QCs were used to assess both sensitivity and selectivity in six different matrix sources, investigating signal to noise ratios at this level and interference from endogenous compounds, internal standards, and different analytes included in the method.

Matrix effects and recovery were assessed using six different sources of biological matrix at three concentration levels. Moreover, matrix effects were assessed using a method described by Matuszewski *et al.* [21,22]. Process efficiency was assessed using an injection solution, prepared at three concentration levels to assess the effect of both extraction recovery and matrix presence on analyte response/quantitation. Stability tests included stock and working solution stability (short and long term), a minimum of two freeze–thaw cycles, benchtop stability for five h on ice and at room temperature, and long-term matrix stability. Concomitant medication was evaluated to assess for potential interference from commonly co-administered HIV drugs (abacavir, atazanavir, bictegravir, darunavir, dolutegravir, efavirenz, emtricitabine, lamivudine, lopinavir, moxifloxacin, nevirapine, raltegravir, ritonavir, tenofovir, tenofovir alafenamide, and zidovudine).

3. Results and discussion

3.1. Method development

3.1.1. Chromatography development

The initial strategy was to develop a method that would allow for the analysis of all eight analytes in breast milk in a single chromatographic method. This proved to be challenging due to the differences in analyte polarities (Table 2). Different gradient mobile phase compositions consisting of formic acid in LC-MS/MS grade millipore water and in a mixture of acetonitrile and methanol were investigated in combination with two different C18 analytical columns: an Agilent Poroshell (2.7 μm, 4.6 mm × 50 mm) and an Atlantis 100 Å T3 (3 μm, 2.1 mm × 100 mm). Acceptable peak resolution and separation were obtained on the Agilent Poroshell column for six of the analytes (rifampicin, desacetyl rifampicin, rifabutin, deacetyl rifabutin, pyrazinamide, and ethambutol), but isoniazid and acetyl isoniazid co-eluted, each as a double peak. Chromatographic separation and peak resolution were acceptable on the Atlantis T3 column for seven of the eight analytes, but not for pyrazinamide. Single peaks were observed for isoniazid and acetyl isoniazid with improved resolution. Despite the advantages a multiplexed assay would offer, it was more important to have a robust method that ensures the quality of the results. Therefore, chromatography was redeveloped, with the analytes divided into three groups. The small more polar analytes (isoniazid, acetyl isoniazid, pyrazinamide, and ethambutol) were grouped together. The rifamycin-type analytes (rifampicin, desacetyl rifampicin, rifabutin, and deacetyl rifabutin) were separated into two methods for each of the parent compounds and the respective metabolites. Chromatographic separation of the three systems is shown in Figs. 1 - 3.

3.1.2. Extraction method development

Due to the complexity of breast milk as a biological matrix, a combination of protein precipitation and SPE was used. Different organic solvents as precipitation solvents (acetonitrile, methanol, and a 1:1

Table 3
Validation summary over three days for all analytes in breast milk.

Analytes	Sample tested		Intra-day (n = 6)		Inter-day (n = 18)	
	QC type	Nominal Conc. (μg/mL)	%Accuracy	Precision CV (%)	%Accuracy	Precision CV (%)
Isoniazid	QC LLOQ	0.300	85.8	3.6	87.2	4.2
	QCL	0.751	89.6	3.9	94.1	9.9
	QCM	12.0	89.2	1.5	94.6	8.1
	QCH	24.0	90.0	1.8	94.5	6.8
	QC DIL	48.1	107.9	2.1		
Acetyl isoniazid	QC LLOQ	0.150	84.4	2.6	87.3	3.6
	QCL	0.375	88.9	1.8	89.9	2.0
	QCM	6.01	91.7	2.4	92.3	2.0
	QCH	12.0	92.7	2.0	93.9	3.2
	QC DIL	24.0	90.1	3.1		
Pyrazinamide	QC LLOQ	0.150	91.6	3.1	90.9	4.2
	QCL	0.375	92.2	2.5	92.8	2.2
	QCM	6.01	91.6	2.8	93.7	2.8
	QCH	12.0	95.2	4.0	96.2	3.9
	QC DIL	24.0	91.1	1.9		
Ethambutol	QC LLOQ	0.300	86.4	1.5	87.2	2.9
	QCL	0.751	91.7	1.9	91.9	2.3
	QCM	12.0	95.1	2.6	95.2	2.6
	QCH	24.0	96.5	1.5	96.0	3.4
	QC DIL	48.1	92.2	1.6		
Rifampicin	QC LLOQ	0.150	89.7	3.3	87.1	3.5
	QCL	0.375	90.6	3.0	89.3	3.4
	QCM	6.01	91.3	2.0	91.4	2.1
	QCH	12.0	93.0	1.5	92.5	3.4
	QC DIL	24.0	88.5	1.6		
Desacetyl rifampicin	QC LLOQ	0.150	85.7	3.6	90.7	6.7
	QCL	0.375	88.4	4.1	87.9	3.2
	QCM	6.01	96.8	6.9	94.1	6.0
	QCH	12.0	95.8	5.5	94.1	5.1
	QC DIL	24.0	88.6	3.7		
Rifabutin	QC LLOQ	0.0150	91.1	2.9	89.7	7.6
	QCL	0.0377	94.4	4.3	92.5	4.3
	QCM	0.603	94.0	2.3	95.7	2.5
	QCH	1.21	94.7	3.5	97.0	4.4
	QC DIL	2.41	95.7	2.7		
Deacetyl rifabutin	QC LLOQ	0.00751	87.1	3.6	86.5	5.1
	QCL	0.0188	89.4	1.8	90.7	2.3
	QCM	0.302	91.1	2.7	93.5	4.4
	QCH	0.603	92.3	3.8	93.6	3.1
	QC DIL	1.21	90.9	4.6		

QCL- Quality control low.
QCH- Quality control high.
QCM- Quality control medium.
QC DIL- Quality control dilution.
QC LLOQ- Quality control lowest level of quantification.

mixture of both), breast milk sample volumes (50 μL, 100 μL, 150 μL, and 200 μL) and precipitation solvent volumes (100 μL, 200 μL, 300 μL, and 400 μL) were investigated. The optimal combination for protein precipitation was found to be 200 μL of breast milk sample with 400 μL of a precipitation solvent consisting of a mixture of acetonitrile and methanol (1:1 (v/v)).

The overly complex nature of breast milk necessitated further clean-up of matrix components and therefore following protein precipitation, SPE on mixed mode extraction media was investigated. Furthermore, the use of SPE allowed for optimal extraction of the analytes by selectively eluting them due to differences in their polarity. Strata-X extraction cartridges were chosen for the combined retention mechanisms of hydrophobic interaction and hydrogen bonding. Even so, it was found that retention of the more polar analytes could only be accomplished in the complete absence of any organic solvent. Therefore, following the protein precipitation step, the supernatants were evaporated under nitrogen gas and the residues reconstituted with a 50 mM ammonium bicarbonate solution before application to the SPE column. Following complete retention of all analytes and rinsing to get rid of more polar background components, stepwise elution with increasing organic content allowed further sample clean-up which resulted in two selective sample fractions. The sample preparation method is described in Section 2.6.

3.2. Method validation

3.2.1. Calibration Curve, intra and inter-day accuracy and precision

Accuracy and precision were assessed over three consecutive, independent runs. A summary of the results for the three validation runs is shown in Table 3. The calibration curves fitted quadratic regressions (weighted by 1/x for six out of the eight analytes; rifampicin and its metabolite, desacetyl rifampicin, were weighted by $1/x^2$) over the range of 0.300–30.0 μg/mL for isoniazid and ethambutol, 0.150–15.0 μg/mL for acetyl isoniazid, pyrazinamide, desacetyl rifampicin, and rifampicin, 0.0150–1.50 μg/mL for rifabutin, and 0.00751–0.751 μg/mL for deacetyl rifabutin in breast milk.

3.2.2. Matrix effects, recovery and process efficiency

The method was shown to be reproducible for all analytes when using six different lots of breast milk. The method was not influenced by matrix effects with a mean regression precision (CV(%)) less than 5 and ranging between 1.0 and 2.8. The average recovery for the analytes was consistent throughout all three QC levels (low, medium, and high) and ranged between 85.5 and 99.1% with a CV(%) ranging between 0.4 and

Table 4
Summary of the recovery, process efficiency, and matrix effects results based on the average of the high, medium, and low-quality control samples, n = 6.

Analytes	%Recovery						%Process efficiency						Matrix effects			
	QCH		QCM		QCL		QCH		QCM		QCL		QCH	QCM	QCL	Area Ratio vs concentration regression slope CV (%)
	Average % Recovery	Average precision CV (%)	Average % Recovery	Average precision CV (%)	Average % Recovery	Average precision CV (%)	Average % Process efficiency	Average precision CV (%)	Average % Process efficiency	Average precision CV (%)	Average % Process efficiency	Average precision CV (%)	Average precision CV (%)			
Isoniazid	**89.6**	**2.8**	**84.4**	**4.3**	**82.4**	4.3	86.9	2.8	82.9	4.3	73.7	4.3	1.0	1.3	1.8	1.0
Acetyl isoniazid	**94.2**	**3.5**	**90.0**	**2.3**	**88.5**	2.0	88.2	3.5	86.6	1.9	79.2	2.0	1.5	2.3	1.0	1.5
Pyrazinamide	**98.1**	**4.9**	**90.8**	**4.4**	**92.6**	5.6	90.0	4.9	85.3	4.4	80.7	2.9	2.2	2.0	5.6	2.3
Ethambutol	**79.1**	**2.4**	**76.2**	**1.5**	**74.9**	1.8	75.6	2.4	76.7	1.5	71.1	1.8	1.2	1.3	1.6	1.3
Rifampicin	**94.2**	**3.6**	**91.3**	**4.4**	**90.2**	4.5	76.7	3.6	77.9	4.4	73.8	4.5	2.6	2.4	1.7	2.8
Desacetyl rifampicin	**95.1**	**6.2**	**87.9**	**4.4**	**90.9**	3.4	81.4	6.4	82.6	4.6	79.6	3.4	2.4	4.4	3.3	2.6
Rifabutin	**101**	**2.7**	**97.7**	**3.6**	**98.2**	5.6	94.7	3.9	94.4	3.6	88.6	4.3	1.9	2.8	5.6	1.9
Deacetyl rifabutin	**93.3**	**4.7**	**92.6**	**2.9**	**92.9**	6.3	93.5	4.7	96.8	4.0	88.9	5.1	2.7	2.9	6.3	2.8

4.4 while the average process efficiency ranged between 74.4 and 93.1% with a CV(%) ranging between 1.9 and 8.3. Results for recovery, process efficiency, and matrix effects for each analyte are presented in Table 4.

3.2.3. *Carry over, sensitivity, specificity, and crosstalk*

No significant analyte peaks were observed for all analytes in the double blank samples. The method further showed sufficient selectivity and specificity. The average signal to noise ratio for each of the analytes was greater than five, indicating acceptable sensitivity of the assay. Representative chromatograms of an LLOQ sample overlayed with a blank sample for assessing sensitivity for each analyte are presented in Figs. 4 and 5. No crosstalk was observed between any of the analytes and metabolites within and between the three-chromatography system.

3.2.4. *Stability*

Stock solutions were shown to be stable at ~ −80 °C for up to 73 days for acetyl isoniazid and isoniazid, 334 days for ethambutol and pyrazinamide, 190 days for desacetyl rifampicin, 405 days for rifampicin and 405 days for rifabutin and deacetyl rifabutin. Long-term matrix stability for isoniazid, acetyl isoniazid, ethambutol, pyrazinamide, rifampicin, and desacetyl rifampicin in breast milk were demonstrated up to 127 days at ~ −80 °C and up to 43 days for rifabutin and deacetyl rifabutin. Long-term matrix stability was demonstrated up to 127 days at ~ −20 °C for isoniazid, ethambutol, and pyrazinamide, while it was shown to be stable for at least five days for acetyl isoniazid, rifampicin, desacetyl rifampicin, rifabutin, and deacetyl rifabutin. This stability data covers the duration these samples were prepared, verified, and stored until they were analysed.

All eight analytes were stable in breast milk at room temperature and on ice, for a minimum of five h. Sample freezing (fresh vs frozen) did not influence the accuracy and precision of the assay, and seven of the eight analytes were stable for at least three freeze–thaw cycles, the exception being isoniazid, which was stable for two freeze–thaw cycles. Autosampler stability and reinjection reproducibility was shown for up to 48 h for all analytes.

3.2.5. *Effect of concomitant medication and dilution integrity*

The presence of concomitant medications was assessed and shown to have no influence on the analyte assays. The dilution assessment showed that concentrations reported above the upper limit of the validated calibration curves may be diluted fivefold and be re-analysed for all analytes. Should the concentrations obtained for pyrazinamide be over the fivefold validated dilution, the samples can be diluted 10-fold and re-analysed.

3.3. *Clinical application*

Understanding the exposure of TB drugs in breast milk is critical to inform policies on breastfeeding in infants born to mothers treated for TB. We therefore used the validated assays to determine the concentrations of isoniazid, acetyl isoniazid, ethambutol, and pyrazinamide in breast milk samples obtained from patients on treatment for RR-TB; the concentration vs time profiles for two patients are presented in Fig. 6. As the complete 24-h profiles for the patients were not available, we refer to the peak concentration as the highest concentration obtained within the sampling interval. We found the exposures of isoniazid, ethambutol, and pyrazinamide in human breast milk to be higher than previously reported [7,23,24] but lower than the therapeutic range when calculating the infant dose received via breastfeeding – see Table 5.

The peak acetyl isoniazid concentrations in breast milk were 3.89 μg/mL and 8.45 μg/mL, for patient A and B, respectively. A recent study by Singh *et al.*, 2008 reported a lower isoniazid concentration range in breast milk (2.0–6.7 μg/mL), which peaked after 1 h post dose with sampling time points of 0, 1, 2, 3, and 4 h in a small series of patients treated for drug-sensitive TB, including daily dose isoniazid 300 mg, rifampicin 450 mg and ethambutol 800 mg [7]. The participants in our

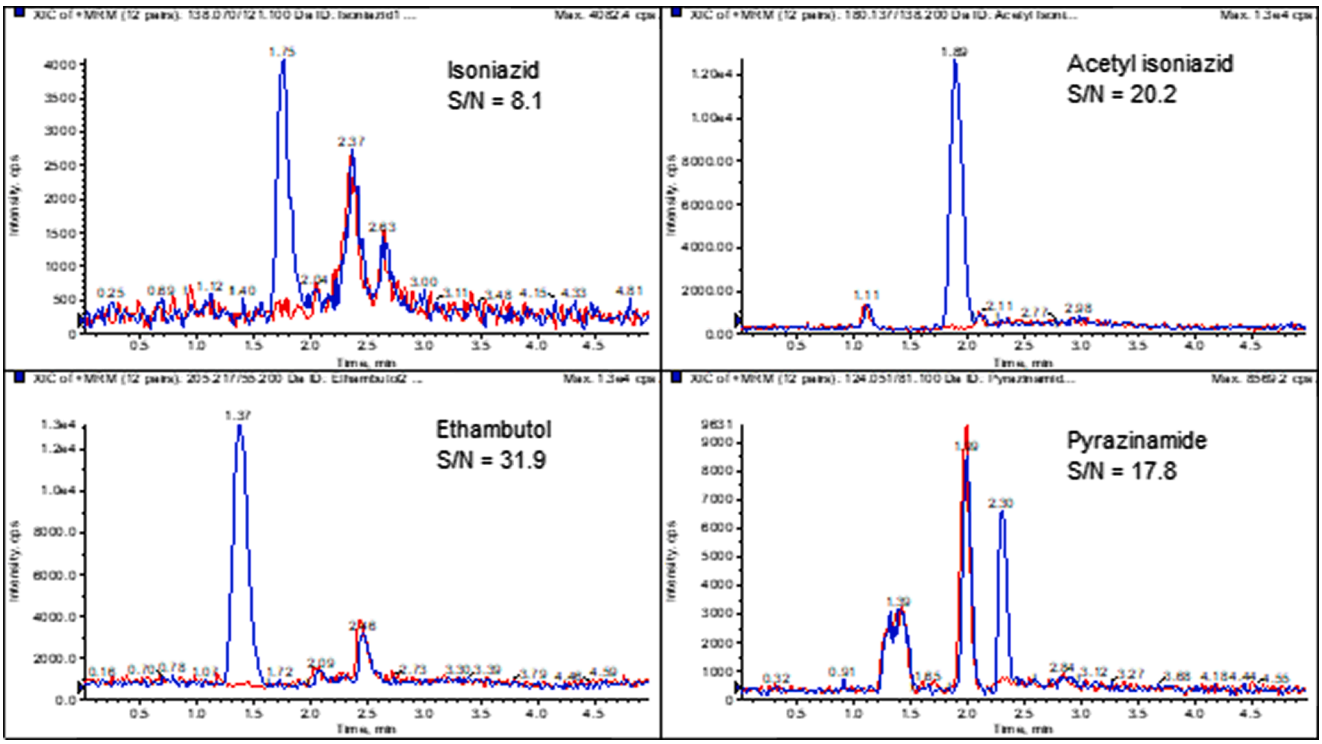


Fig. 4. Representative lower limit of quantification chromatograms of isoniazid, acetyl isoniazid, ethambutol, and pyrazinamide extracted with internal standard and overlaid with a blank sample at concentrations of 0.300 μg/mL, 0.150 μg/mL, 0.300 μg/mL, and 0.150 μg/mL, respectively. The lower limit of quantification is shown in blue and the blank in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

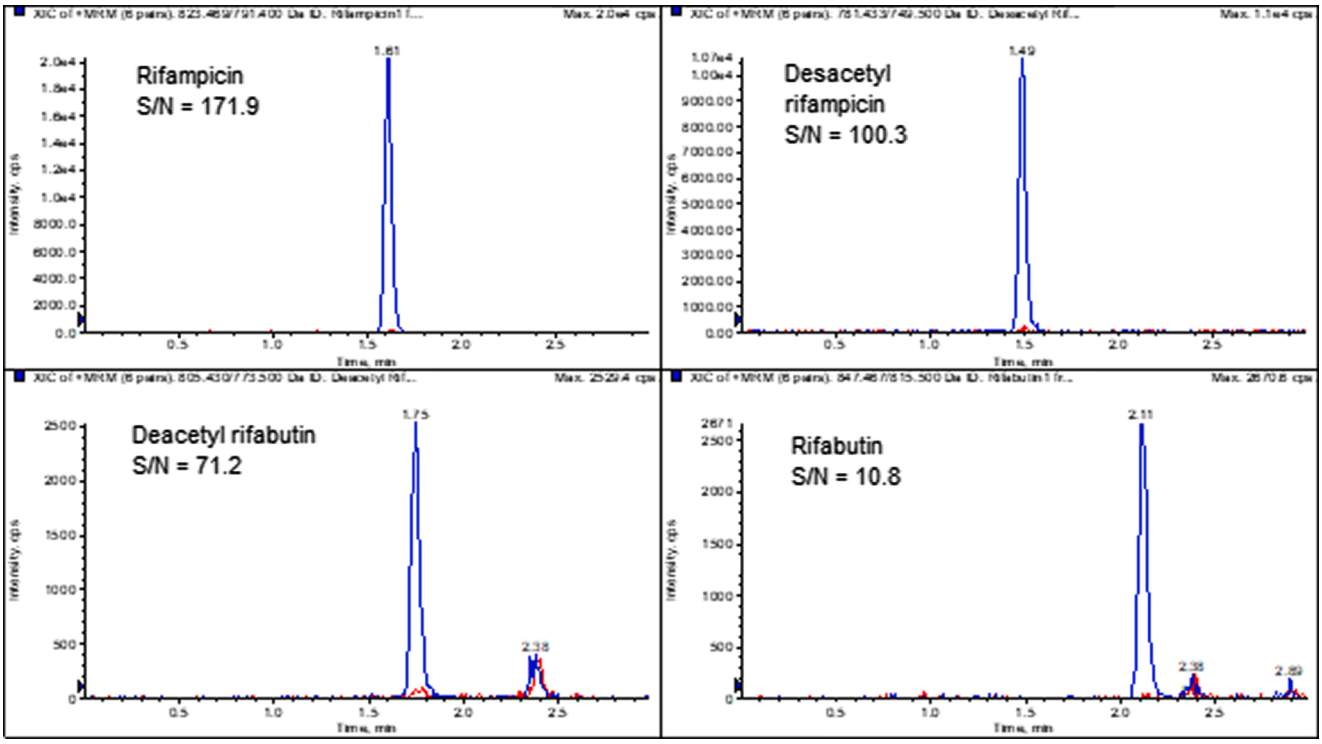


Fig. 5. Representative lower limit of quantification chromatogram of rifampicin, desacetyl rifampicin, rifabutin, and deacetyl rifabutin extracted with internal standard and overlaid with a blank sample at concentrations of 0.150 μg/mL, 0.150 μg/mL, 0.0150 μg/mL, and 0.00751 μg/mL, respectively. The lower limit of quantification is shown in blue and the blank in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

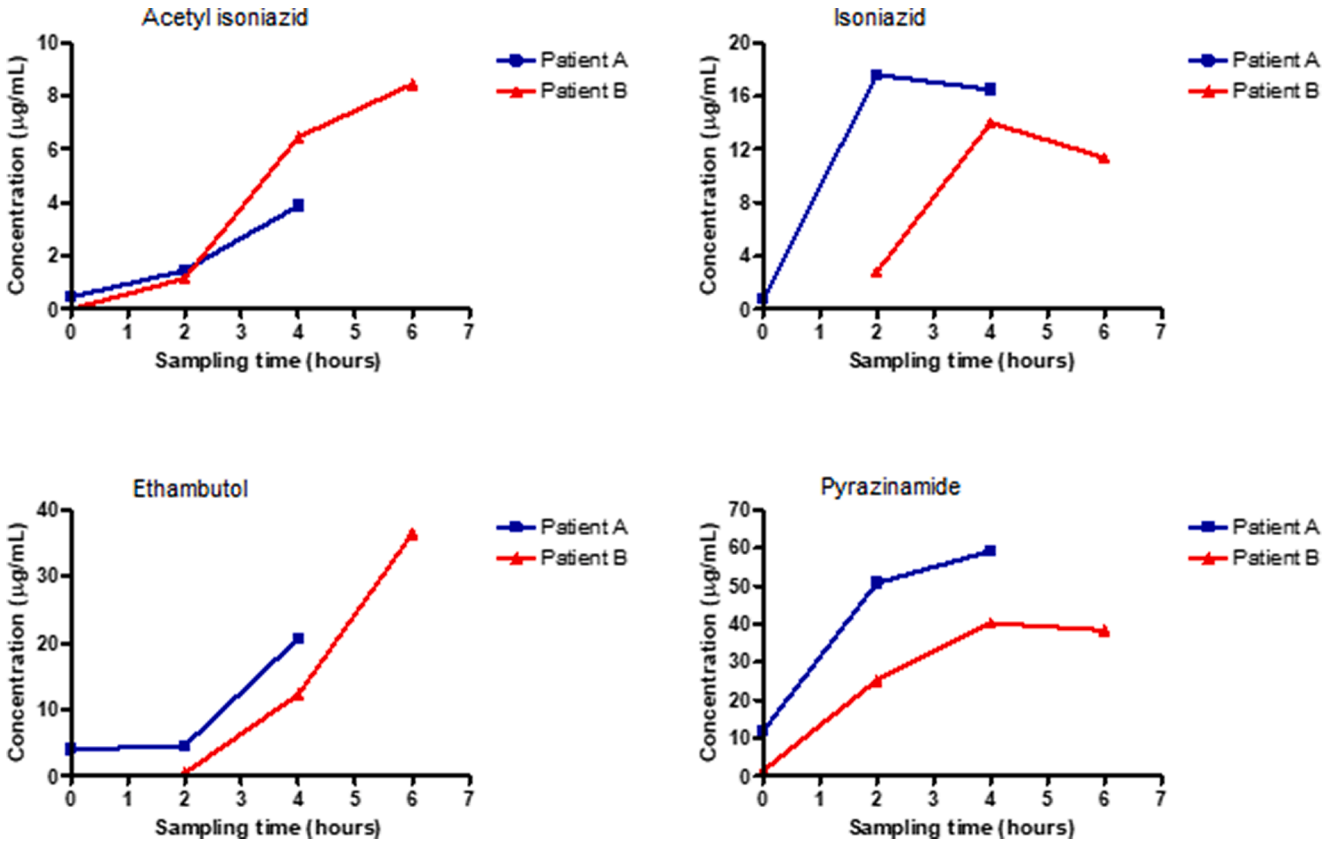


Fig. 6. Concentration vs time profiles of the two patients for acetyl isoniazid, isoniazid, ethambutol, and pyrazinamide. Time zero represents the time before the first dose of the day was given.

Table 5
Infant exposure to drugs in breast milk.

	Breast milk peak concentration µg/mL)	Infant daily dosage (mg/kg/day)	Relative Infant dosage (%)	Therapeutic infant dosage (mg/kg/day) [30]	Infant dosage relative to therapeutic dose (%)
Isoniazid					
Patient A	17.6	2.64	16.4	10–15	17–26
Patient B	14.0	2.10	12.1		14–21
Ethambutol					
Patient A	20.7	3.11	14.5	15–25	12–21
Patient B	36.4	5.46	23.6		21–36
Pyrazinamide					
Patient A	59.2	8.88	28.4	30–40	22–30
Patient B	38.4	5.76	17.1		14–19

[30]: reported therapeutic infant dosage (mg/kg/day).

study were treated with a higher dose of isoniazid (900 mg daily; 15 mg/kg) for rifampicin-resistant TB, which may explain the higher peak isoniazid concentrations we observed (Table 5), compared with the reported concentrations by Singh *et al.* where patients were treated with a lower dose of isoniazid (300 mg daily; 5 mg/kg) for rifampicin-sensitive TB. However, similar breast milk isoniazid concentrations to our study were reported by Berlin and Lee in an older report published in 1979, where patients treated with 300 mg isoniazid daily for drug susceptible TB were found to have breast milk isoniazid and acetyl isoniazid concentrations of 16.6 µg/mL after 3 h and 3.76 µg/mL after 5 h, respectively [25]. In an earlier study in 1954 by Ricci and Copaitich, breast milk isoniazid concentrations in three patients given two doses of isoniazid 5–10 mg/kg (300 mg) for drug-sensitive TB were reported. The observed concentrations were 6 µg/mL for one of the patients, while the other two were in a range of 9–11 µg/mL (no time points given) [26].

Breast milk composition fluctuates after birth and may explain the varying drug concentrations reported. Immediately post-delivery, breast milk is comprised of a high protein, low-fat colostrum. Colostrum is a milk-like fluid produced during the first few days of lactation, containing 2–4 times more protein than mature milk, mostly in the form of immunoglobulins [27]. By day six of lactation, colostrum becomes transitional milk, and finally mature milk by day 14 [28]. During the

colostrum phase, large gaps exist between the alveolar cells of the breast, which enhances the transfer of drugs and maternal proteins into breast milk. Beyond the colostrum phase, these intracellular gaps close, reducing the transfer of drugs and maternal proteins into breast milk [29]. Unfortunately, these two studies [7,25] do not specify the post-delivery period at which the breast milk samples were taken, which may explain the inter-study variability in reported breast milk drug concentrations.

We observed higher ethambutol peak concentrations (Table 5) in our study after a dosage of 1200 mg compared to a 1984 report by Snider *et al.* on two patients with breast milk concentrations of ethambutol of 1.4 μg/mL at 2 h after an oral dose of 15 mg/kg (800 mg) and 4.6 μg/mL (dosage information not provided), respectively [23]. Higher breast milk pyrazinamide peak concentrations were also observed in our study after a dosage of 1750 mg (Table 5) compared with a report by Holdiness in 1984 of one patient with a pyrazinamide breast milk concentration of 1.5 μg/mL at 3 h post dose (1000 mg). For both ethambutol and pyrazinamide, the higher breast milk concentrations reported in our study are likely explained by the higher treatment dose. Other factors affecting breast milk drug concentrations include maternal factors, which influence plasma drug concentrations such as liver/renal impairment or drug-drug interactions. While the concentrations of the measured drugs in breast milk we report are higher than previously described, inter-study variability of sampling time points limits comparison.

Although the use of the first-line TB drugs is not contraindicated during breast feeding, the relative infant dosage (RID) we observed is higher than what is considered safe [18] (Table 5), the significance of which is currently unclear. Infant factors including prematurity of the infant metabolic system, particularly in preterm neonates, could further increase TB drug exposure [31] and consequently, the risk of drug-related toxicity [32]. Whether low-level infant drug exposure through breastfeeding could potentially be protective or alternatively, select for resistance in infants who become infected with TB, requires exploration.

4. Conclusion

We developed and successfully validated a novel, robust quantification method for the analysis of the first-line TB drugs, including rifabutin in breast milk; and applied this method in breast milk samples from patients treated for rifampicin-resistant TB. Breast milk exposures of isoniazid, pyrazinamide, ethambutol, and their related metabolites are higher than previously reported, and requires further study.

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.

Data availability

Data will be made available on request.

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