INTRODUCTION

Tuberculosis (TB) is one of the infectious diseases with the highest morbidity and mortality in the world [1]. First-line treatment of TB consists of isoniazid, rifampin, pyrazinamide and ethambutol during the first two months, continuing with isoniazid and rifampin for another four months [1]. Although treatment is often successful, healthcare providers around the world are confronted with treatment failure daily. Recently, Pasi Panodya et al. showed that risk on treatment failure was almost nine fold higher in patients with low drug exposure compared to patients with higher drug exposure [2]. Therefore, monitoring of drug exposure in patients at risk for low drug exposure is worthwhile to explore in a prospective manner [3].

To be able to evaluate drug exposure a fast, accurate and simple method for determination of isoniazid, rifampin, pyrazinamide and ethambutol in serum is valuable. Like for other antimicrobial drugs, it would be ideal to determine all four compounds in the same sample using a single method of analysis [4,5]. Song et al. published a method for the simultaneous determination of all first-line anti-TB drugs using liquid chromatography-tandem mass spectrometry (LC-MS/MS) [6]. Unfortunately, the polar compounds isoniazid, pyrazinamide and ethambutol elute near the void volume of the system, co-eluting with endogenous compounds resulting in substantial ion suppression [6,7]. As matrix effects may vary within and

Quantification of isoniazid, pyrazinamide and ethambutol in serum using liquid chromatography-tandem mass spectrometry

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between subjects, ion suppression at the time of elution of the compounds of interest should be avoided or overcome using a stable isotope-labelled internal standard [8–10].

Zhou et al. used hydrophilic interaction chromatography (HILIC) to quantify isoniazid, rifampin, pyrazinamide, ethambutol and streptomycin [11]. They tested five different HILIC columns and several mobile phase compositions. An initial attempt by the authors to determine isoniazid and ethambutol using HILIC was abandoned, as our method suffered from split peaks and bad repeatability. Recently, Kim et al. developed a LC-MS/MS method for the simultaneous quantification of 20 anti-TB drugs. The method consisted of two different precipitation methods, different solid and mobile phases [12].

We successfully applied ultrafiltration as a means of sample preparation for the polar compound ribavirin [13] and we were interested whether this might also be applied for the first-line anti-TB drugs. However, rifampin shows high protein binding of around 80-90% [14], which makes it a less suitable candidate for ultrafiltration compared to isoniazid, pyrazinamide and ethambutol. Therefore, it was decided to split the patient sample, followed by a subsequent separate analysis of rifampin and a separate one for isoniazid, pyrazinamide and ethambutol together resulting in more reliable results within the same runtime. A previously developed method will be used for the analysis of rifampin [7,15]. The objective of this study was to develop and validate a simple and rapid LC-MS/MS method for the determination of isoniazid, pyrazinamide and ethambutol in human serum.

Materials and methods

Chemicals, reagents and disposables

Isoniazid (purity >99%) was purchased from Bufa (Uitgeest, The Netherlands). Ethambutol dihydrochloride (purity >99%) and pyrazinamide (purity ≥98%) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). The internal standard isoniazid-D4 was produced by CDN Isotopes (Pointe-Claire, Canada). The internal standard ethambutol-D4 was synthesized by Syncom (Groningen, the Netherlands). Acetonitrile (Ultra LC-MS-grade) was obtained from BioSolve (Valkenswaard, The Netherlands). Ammonium acetate and acetic acid (p.a. quality) from Merck were purchased from VWR (Amsterdam, The Netherlands). Water was purified with a Milli-Q system (Millipore Corporation, Billerica, MA, USA). Blank human serum and plasma was obtained from healthy volunteers according to the appropriate guidelines of the University Medical Center Groningen.

Pall Nanosep 30K Omega centrifugal devices, Varian non-deactivated 2 mL borosilicate glass screw top vials and 600 μL polypropylene screw top vials were purchased from VWR (Amsterdam, The Netherlands). The inserts were made from Schott Fiolax®-clear glass and were purchased from Aluglas (Uithoorn, The Netherlands).

Equipment and conditions

Experiments were performed on an Agilent 6460A (Santa Clara, Ca, USA) triple quadrupole LC-MS/MS system, with an Agilent 1200 series combined LC-system. The mass selective detector operated in heated electrospray positive ionisation mode and performed dynamic multiple reaction monitoring (DMRM) with unit mass resolution. High purity nitrogen was used for both the source and collision gas flows. Precursor ions, product ions, optimum fragmentor voltages and collision energy values are listed in Table 1. For all substances the capillary voltage was set at 4000 V, gas temperature at 320°C, gas flow at 13 L/min, nebulizer gas at 60 psi, sheath gas temperature at 400°C, sheath gas flow at 12 L/min and the nozzle voltage at 0 V. The Agilent 1290 autosampler and the 1260 TCC column oven were set at a temperature of 20°C.

Separation was performed on an Atlantis T3 reversed-phase C18 analytical column (2.0 x 100 mm, 3 μm particle size) from Waters (Etten-Leur, The Netherlands). The mobile phase consisted of acetonitrile, water and a 200 mM ammonium acetate buffer pH 5.0. Chromatographic separation was performed using the mobile

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parent Ion (m/z)</th>
<th>Product Ion (m/z)</th>
<th>Fragmentor Voltage (Volts)</th>
<th>Collision Energy (Volts)</th>
<th>Retention time (Min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethambutol</td>
<td>205.1</td>
<td>116.1</td>
<td>76</td>
<td>13</td>
<td>1.15</td>
</tr>
<tr>
<td>Ethambutol-D4</td>
<td>209.1</td>
<td>120.1</td>
<td>76</td>
<td>13</td>
<td>1.15</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>138.1</td>
<td>121.0</td>
<td>72</td>
<td>11</td>
<td>1.41</td>
</tr>
<tr>
<td>Isoniazid-D4</td>
<td>142.1</td>
<td>125.0</td>
<td>72</td>
<td>11</td>
<td>1.40</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>124.0</td>
<td>81.0</td>
<td>72</td>
<td>20</td>
<td>1.72</td>
</tr>
</tbody>
</table>
phase gradient listed in Table 2 at a flow of 500 μL/min, which resulted in a run time of 2.5 minutes. Peak area ratios of the analytes and their associated internal standards were used to calculate concentrations. Agilent Masshunter software for quantitative analysis (version B.04.00) was used for quantification of the analysis results. Vortexing was performed with a Labtek multi-tube vortexer (Christchurch, New Zealand). Centrifugation was performed using a unthermostated Hettich EBA 21 centrifuge (Tuttlingen, Germany) with a 45º -fixed angle rotor. Temperature was not controlled because no effect of increasing temperature was observed during earlier experiments (data not shown).

**Method development**

The analytical range was based on clinical pharmacokinetic data and set at 0.2–8 mg/L for isoniazid and ethambutol and 2–80 mg/L for pyrazinamide [3,16]. As stable isotope-labelled internal standards are known to compensate for matrix effects [9,10], isoniazid-D4 and ethambutol-D4 were used as such. Since a stable isotope-labelled internal standard for pyrazinamide was unavailable, no internal standard was used for the quantification of this compound.

**I. Protein binding and ultrafiltration**

Reported protein binding levels vary considerably for the three compounds: ranging from 0 to 74% for isoniazid [17,18] from 5 to 50% for pyrazinamide [17,19] and from 10 to 30% for ethambutol [17,20]. However, one expects protein binding to be limited as isoniazid, pyrazinamide and ethambutol are very polar compounds. If protein binding proves to be low, ultrafiltration as a means of deproteinization of the serum samples can be applied. Although ultrafiltration is commonly used for determining free unbound drug levels in serum [21], it has previously been applied as a means of sample preparation for other polar compounds such as acyclovir, ganciclovir, ribavirin and zidovudine [13,22–25]. The obtained ultra filtrate is purely aqueous which is particularly suitable for injecting onto LC-systems that are equilibrated under the same conditions. To investigate the applicability of ultrafiltration for the assay, it was decided to determine protein binding of the three compounds using the 30 kDa centrifuge filters applied for the sample preparation. The Pall Nanosep 30 K Omega centrifugal device was chosen because it removes over 99.5% of all serum proteins, shows low protein binding, allows for fast processing times and fits standard micro-centrifuges [13].

Protein binding was determined by measuring a calibration curve with calibration standards at seven levels prepared in human serum and compare it to an equivalent calibration curve prepared in water with added blank ultra filtrate to compensate for matrix effects. The ratio of the slopes of these curves shows the extent of protein binding. In this experiment, calibration curve samples were freshly prepared and processed within 30 minutes to minimise alterations to isoniazid other than the irreversible protein binding under investigation. This period is comparable to other studies on protein binding [26,27]. Inert type I borosilicate glass inserts were used to minimise any adsorption of ethambutol to glass inserts.

**II. Adsorption**

During early method development, a significant decline in signal for ethambutol was observed when the aqueous test mixtures were injected from the standard non-deactivated glass vials compared to plastic vials. To investigate this observation, an adsorption test was performed. A test solution with isoniazid, pyrazinamide and ethambutol was prepared and was divided over a polypropylene vial, a glass vial and a glass insert made out of type I borosilicate glass. The samples were shaken vigorously for one minute and five repetitive injections were made from each type of vial.

**III. Carry-over**

Carry-over from high isoniazid and ethambutol standards was observed during method development. Therefore, an experiment in fivefold was performed to evaluate the magnitude of the carry-over by injecting five LLOQ (Lower Limit of Quantification) samples after a HLQ.
(Higher Limit of Quantification with 8.0 mg/L isoniazid and ethambutol and 80 mg/L pyrazinamide) sample.

Sample preparation
For the calibration (reference) standards and Quality Control (QC) samples, separate stock solutions of 200 mg/L isoniazid and ethambutol and 2000 mg/L pyrazinamide in water were prepared. Calibration standards and QC samples were freshly prepared immediately before use by diluting appropriate amounts of stock solutions with blank human serum or plasma. QC samples were freshly prepared as stability of isoniazid is known to be limited [28,29]. The total amount of stock solutions added to the serum was less than 5% of the final volume. Calibration standards at eight levels ranged from 0.2-8.0 mg/L for isoniazid and ethambutol and from 2.0-80 mg/L for pyrazinamide. QC samples consisted of LLOQ, Low, Medium, High and over-the-curve (OTC) levels for each compound (Table 3). The internal standard solution was prepared with 0.25 mg/L isoniazid-D4 and 0.25 mg/L ethambutol-D4 in water.

QC samples for the freeze/thaw stability were prepared beforehand. Separate Low and High level QC samples in human serum were thawed, frozen and analysed on three consecutive days. The samples for determining the bench top stability were freshly prepared and analysed immediately and after being kept for 24 h at ambient temperature.

Calibration standards, QC or human serum samples were homogenised and aliquots of 10 µL were directly transferred onto the upper reservoir of the centrifuge filters and 250 µL of internal standard solution was added. The centrifuge filters were closed and the samples were briefly homogenised using a vortex mixer. Filtration was done by centrifugation for 10 minutes at 14000 g at an angle of 45°. A 5 µL volume of the ultra filtrate was injected onto the LC-MS/MS system. Samples for the stability test were processed in the same way as the samples for the validation and routine analysis.

Analytical validation
In accordance with the ‘Guidance for Industry – Bioanalytical Method Validation’ of the FDA [30], method validation included selectivity, linearity, accuracy, precision and stability. The linearity was assessed by using eight point calibration curves on three days for the three compounds. To determine accuracy and precision, LLOQ, Low, Medium and High QC samples were analysed. All QC samples were analysed in fivefold in three separate runs on separate days.

The calibration model was chosen by evaluating the accuracy results at LLOQ, Low, Medium and High concentration level of the first precision and accuracy run. The simplest linear model at three weighting factors (x, 1/x and 1/x²) at which all accuracies were within the specifications of the FDA was chosen and set for the entire validation and ensuing bioanalysis.

Automatic immuno-assays do not exist for the quantification of anti-TB drugs. Therefore, all assays for anti-TB drugs are ‘in-house’ developed and validated chromatographic methods [31]. To be able to compare obtained results between laboratories, it is important to participate in an external quality control programme. Recently, such a proficiency testing programme has been set up [31] and we participated in the programme with the developed method.

Matrix effect
The matrix effect was assessed using six different human sera and six different human edetate plasmas adding up to 12 different matrices. The first set (A) was prepared of QC stock in water to evaluate the neat MS response of the compounds and the internal standards. The second set (B), 12 different calibration sets were prepared with internal standard solution and after ultrafiltration QC stock was added.

The matrix factor was calculated as the ratio of the peak area of the spiked standards after sample preparation (A) to the corresponding peak area of the neat solution standards (A) [9]. The internal standard-normalised matrix factor was calculated by dividing matrix factor of isoniazid and ethambutol by the matrix factor of their internal standard [9]. As pyrazinamide was determined without internal standard, the matrix factor was calculated without normalisation.

Stability
The stability of the compounds was extensively evaluated as a part of a larger investigation on the stability of many anti-TB drugs. Human serum standards were prepared by adding appropriate amounts of stock solution to blank matrices. Analyses were performed in duplicate and time point 0 served as the reference. The final serum concentrations for the Low concentration samples were 0.51 mg/L isoniazid, 20.2 mg/L pyrazinamide and 1.00 mg/L ethambutol. For the High concentration samples, the values were 6.89 mg/L, 62.4 mg/L and 5.09 mg/L for isoniazid, pyrazinamide and ethambutol respectively. The prepared human serum standards were divided into 0.5 mL aliquots and stored in sealed plastic tubes at four different conditions: ambient temperature (18-24°C), re-
Although isoniazid and ethambutol elute within a short period, no interference was observed. However, some tailing of ethambutol was observed. We were unable to further diminish this tailing.

The results of the protein binding tests are presented in Table 4. As protein binding indeed proved to be low, ultrafiltration as a means of sample preparation could be applied.

In the adsorption test, ethambutol showed a significant loss of approximately 35% in peak area when injected from the standard glass vial (Fig. 2). The isoniazid response showed a small but significant decrease when injected from the polypropylene and the glass vial. Pyrazinamide was unaffected by the different materials. The type I borosilicate glass insert showed the least absorption for most compounds and was chosen as the standard vial. The test for carry-over showed that it was caused by a memory effect of the column. This memory effect was proven by repeating the gradient three times during an analytical run.

To determine freeze/thaw stability, separate Low and High QC samples were thawed, frozen at -80°C and analysed on three consecutive days.

One portion of human serum was divided into 1 mL aliquots in 10 mL glass containers and freeze-dried using a Leybold Lyovac GT4 lyophilizer according to local standard operating procedure by authorized personnel. Then they were sealed and refrigerated at 4-8°C. Freeze-dried samples were reconstituted by adding 1 mL of water, immediately before use.

Clinical application

After validation the analytical procedure was used in routine patient care and a clinical study evaluating the influence of food and fasting on the pharmacokinetics of first-line anti-TB drugs. Twenty patients included in this study were diagnosed with drug susceptible TB and provided written informed consent. The study protocol was approved by the Medical and Health Research Ethics Committee of the Gadjah Mada University in Yogyakarta, Indonesia.

Results

Method development

Using the Waters Atlantis T3 column, best peak shapes and repeatable retention times were obtained by starting and ending the mobile phase gradient in 100% aqueous conditions. Mean retention times were 1.15 min for ethambutol, 1.41 min for isoniazid and 1.72 min for pyrazinamide, respectively (Table 1). The hold-up time of the system is approximately 0.9 min. To optimize the chromatography of the compounds a mobile phase buffer pH 5 was used. This resulted in acceptable peak shapes (Fig. 1). Although isoniazid and ethambutol elute within a short period, no interference was observed. However, some tailing of ethambutol was observed. We were unable to further diminish this tailing.

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The test for carry-over showed that it was caused by a memory effect of the column. This memory effect was proven by repeating the gradient three times during an analytical run. During this experiment, isoniazid and ethambutol peaks were observed at the interval of the orig-

<table>
<thead>
<tr>
<th>Compound</th>
<th>Stock (mg/L)</th>
<th>Calibration Standards (mg/L)</th>
<th>QC samples (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LLOQ</td>
<td>Low</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>200</td>
<td>0.2, 0.3, 0.5, 0.8, 1.5, 3.0, 5.0, 8.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>2000</td>
<td>2.0, 3.0, 5.0, 8.0, 15, 30, 50, 80</td>
<td>2.0</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>200</td>
<td>0.2, 0.3, 0.5, 0.8, 1.5, 3.0, 5.0, 8.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Table 3. Concentration of stock solutions, calibration standards and QC samples.

QC: Quality Control, LLOQ: Lower Limit of Quantification, OTC: over-the-curve

Figure 1. Chromatograms of blank (dotted line) and LLOQ concentrations (solid line) of ethambutol (0.2 mg/L), isoniazid (0.2 mg/L) and pyrazinamide (2.0 mg/L).
and <15% CV for the other QC samples. The maximum overall bias was 10.7% and the maximum overall CV was 8.1%, which is well within the limits set by the FDA.

We participated with this method in the second round of an interlaboratory quality control program for the measurement of anti-TB drugs [31]. All values were within the limits of 20% set by the organisation of the programme.

Matrix effect
The results for the matrix effect tests are listed in Table 6. Data for both serum and plasma samples were pooled, as no significant difference was observed between them. For all compounds, a small positive (internal standard-normalised) matrix factor was determined for low and high concentrations. Maximum CV was 6.0% for the Low level of ethambutol, this is within the limit of 15% set by the EMA [9].

Analytical validation
Regarding selectivity, examination of the six independent blank human serum or plasma samples revealed no interfering components at the mass transitions monitored (Fig. 1).

The validation results regarding accuracy and precision are listed in Table 5. Calibration curves for isoniazid and ethambutol were obtained by plotting the ratio of peak area to that of the internal standard against the concentration. Pyrazinamide was not analysed using an internal standard because no stable isotope-labelled pyrazinamide was available. The calibration curves were fitted using least squares linear regression with a weighting factor of 1/x. None of the calibrators was discarded in the validation procedure. The calibration curves were linear in the range of 0.2-8 mg/L for isoniazid and ethambutol and 2-80 mg/L for pyrazinamide. The average correlation coefficients were 0.9987 for isoniazid, 0.9992 for pyrazinamide and 0.9984 for ethambutol. All the calibration curves showed a significant goodness of fit. All obtained results met the acceptance criteria set by the FDA [27]. These criteria were <20% bias and <20% coefficient of variation (CV) for LLOQ QC samples and <15% bias and <15% CV for the other QC samples.
Stability
The results of the stability tests showed that isoniazid is not stable at ambient temperature in human serum (Table 7). However, if it is present in ultra filtrate as in the autosampler test, isoniazid proves to be stable for at least 24 h. Isoniazid serum standards are only stable at -80°C or in freeze-dried conditions for longer periods. From the data in Table 7, it is calculated that average in vitro half-life values for isoniazid were 6 days at ambient temperature, 15 days at refrigerating temperatures and 115 days at -20°C.

None of the three compounds in human serum showed degradation beyond criteria after three freezing and thawing cycles, although the concentrations of the isoniazid and pyrazinamide samples at low concentration level dropped considerably after the third cycle. Pyrazinamide in human serum is stable at all temperatures except at ambient temperature. Measured pyrazinamide concentrations at ambient temperature started to decline significantly after five days. Since the human matrices stored at ambient temperature showed signs of decomposition over time, degradation products may have affected these measurements. None of the measured ethambutol concentrations fell beyond acceptance limits at any of the time points for all storage conditions. A limited concentration drop for all long term stability samples was observed however.

Clinical application
To illustrate the clinical application a concentration-time curve over a 24 h time period of a TB patient recently started on TB treatment is shown in Fig. 3. She received her medication orally on an empty stomach. Including the preparation of calibration standards and QC samples, it takes approximately 1.5 h to analyse the three drugs in these 12 samples. The maximum capacity is almost 200 samples per 24 h. In the same time, the samples can be analysed for rifampin on a separate LC-MS/MS result in a processing time similar to the method of Song [6].

Discussion
The most important aspect from these results presented here is that ethambutol did not show any significant in vitro protein binding in serum using this method, which clearly contradicts earlier results [17,20]. This may partly be caused by the difference in temperature, as this factor is known to influence protein binding [26]. In our experiments we used an unthermostated centrifuge. However it can be observed from the accuracy and precision results in Table 5 that temperature had limited influence on the validation. In the adsorption test, ethambutol showed a significant loss of approximately 35% in peak area when injected from the standard glass vial. Earlier reported protein binding results [17,20] vary and may be obscured by adsorption of ethambutol to non deactivated glass.

Table 6. Internal standard-normalised matrix factor [9].

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (mg/L)</th>
<th>Matrix factor</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid</td>
<td>Low (0.5)</td>
<td>1.128</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>High (6.5)</td>
<td>1.157</td>
<td>5.0</td>
</tr>
<tr>
<td>Pyrazinamide*</td>
<td>Low (5.0)</td>
<td>1.184</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>High (6.5)</td>
<td>1.142</td>
<td>3.3</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>Low (0.5)</td>
<td>1.079</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>Low (6.5)</td>
<td>1.065</td>
<td>5.5</td>
</tr>
</tbody>
</table>
*matrix factor of pyrazinamide is not internal standard-normalised, due to absence of stable isotope-labelled internal standard for pyrazinamide. CV: coefficient of variation

Table 7. Stability testing results for isoniazid, pyrazinamide and ethambutol.

<table>
<thead>
<tr>
<th>Component</th>
<th>Isoniazid</th>
<th>Pyrazinamide</th>
<th>Ethambutol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Low (%)</td>
<td>High (%)</td>
<td>Low (%)</td>
</tr>
<tr>
<td>Autosampler (ultra filtrate), 24h</td>
<td>96.7</td>
<td>97.4</td>
<td>97.1</td>
</tr>
<tr>
<td>Ambient temperature, 24 h</td>
<td>75.7</td>
<td>78.5</td>
<td>106.1</td>
</tr>
<tr>
<td>Ambient temperature, 51 d</td>
<td>n.d.</td>
<td>28.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>Refrigerator, 51 d</td>
<td>31.3</td>
<td>34.0</td>
<td>106.8</td>
</tr>
<tr>
<td>Freezer (-20°C), 51 d</td>
<td>74.2</td>
<td>67.8</td>
<td>99.7</td>
</tr>
<tr>
<td>Freezer (-80°C), 51 d</td>
<td>101.6</td>
<td>102.5</td>
<td>108.1</td>
</tr>
<tr>
<td>Freeze-dried, 51 d</td>
<td>101.6</td>
<td>95.6</td>
<td>104.9</td>
</tr>
<tr>
<td>Freeze-thaw (3 cycles)</td>
<td>87.0</td>
<td>94.4</td>
<td>86.6</td>
</tr>
</tbody>
</table>

n.d.: not detectable. Low sample: 0.51 mg/L isoniazid, 20.2 mg/L pyrazinamide and 1.00 mg/L ethambutol, High sample: 6.89 mg/L isoniazid, 62.4 mg/L pyrazinamide and 5.09 mg/L ethambutol.
vials. Furthermore, any possible protein binding would be reduced by the extensive dilution of the samples with internal standard solution during sample preparation before the ultrafiltration [32]. By then, variation of any possible protein binding of ethambutol in the diluted samples is compensated by the stable isotope-labelled internal standard.

Protein binding of isoniazid and pyrazinamide is also lower than reported values. Reported (reversible) protein binding values of isoniazid might have been obscured by the instability of isoniazid in serum. The instability of isoniazid is attributed to irreversible protein binding [33]. As protein binding of isoniazid, pyrazinamide and ethambutol proved low, ultrafiltration as a means of sample preparation could be applied resulting in a fast and simple method of analysis.

Significant carry-over and memory effects were observed for isoniazid and ethambutol, during method development. This can be explained by absorption to silica and metal surfaces, due to their polar characteristics. Carry-over was overcome by rinsing the sample needle and needle seat with eluent B in-between sample injections. Memory effects were overcome by adding two flushing and conditioning steps to the original gradient (Table 2).

Bench-top stability testing and the more extensive stability assessment showed that isoniazid is unstable in human serum unless it is freeze-dried or stored at -80°C. Instability of isoniazid in plasma at ambient temperature and -20°C has been reported earlier [28,29]. This instability calls for adequate standard operating procedures and rapid handling of samples. The autosampler stability testing show that isoniazid is stable in ultra filtrate for at least 24 hours proving that serum proteins are involved in the instability of isoniazid. Pyrazinamide serum samples are more stable and can be kept for at least 51 days in a refrigerator. Ethambutol proved to be stable at ambient temperature.

Using this method, almost 200 patient samples can be analysed for isoniazid, pyrazinamide and ethambutol in 24 h. If one makes use of an optimal sampling strategy consisting of three samples to calculate the area under the concentration-time curve, information on drug exposure of 65 patients can be obtained in one day. The presented analytical method has shown to be robust for therapeutic drug monitoring and pharmacokinetic studies of isoniazid, pyrazinamide and ethambutol. It may be of clinical help and importance as the current opinion is moving from standard dosing towards a more drug exposure based evaluation of TB treatment [34].

Conclusion
A fast, simple and reliable LC-MS/MS method has been developed for the determination of isoniazid, pyrazinamide and ethambutol in human serum. Isoniazid, pyrazinamide and especially ethambutol have proven to be low protein bound. Therefore, ultrafiltration as a means of sample preparation could be applied. The method adheres to all the validation criteria and is suitable for the simultaneous determination of isoniazid, pyrazinamide and ethambutol in human serum for therapeutic drug monitoring and pharmacokinetic studies.

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28. Huang L, Marzan F, Jayewardene AL, Lizak PS, Li


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