A simple, high-throughput and validated LC–MS/MS method for the determination of azithromycin in human plasma and its application to a clinical pharmacokinetic study

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Abstract
A sensitive, specific and rapid liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method was developed and validated to quantify azithromycin concentrations in human plasma. Azithromycin (AZI) is the most common outpatient prescribed antibiotic in the US and clinical studies have demonstrated the efficacy and safety of AZI in many bacterial infections. To support a clinical study, we developed a high-throughput LC–MS/MS method to process up to 250 samples per day to quantify AZI in human plasma. Samples were prepared by solid-phase extraction. Separation was achieved with an ACE C18 column (2.1 x 100 mm, 1.7 μm) equipped with a C18 guard column. The mobile phase consisted of 0.1% formic acid and methanol–acetonitrile (1:1, v/v) at a flow rate of 0.25 ml/min. The ionization was optimized with positive electrospray source using multiple reaction monitoring transition, m/z 749.50 > 591.45 for AZI and m/z 754.50 > 596.45 for AZI-d5. Extraction recoveries were approximately 90% for AZI. The assay was linear from 0.5 to 2,000 ng/ml and required only 100 μl of plasma with a total analysis time of 4.5 min. The method was

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Azithromycin (AZI), a macrolide antibiotic, is the most commonly prescribed outpatient antibiotic in the USA (Durkin et al., 2018). AZI not only has a broad spectrum antimicrobial activity against Gram-positive, Gram-negative and atypical pathogens, but also produces immunomodulatory effects for patients with inflammatory disorders (Amsden, 2005). It is widely used for the treatment of upper and lower respiratory tract infections and skin and soft tissue infections as well as the treatment of sexually transmitted diseases (Peters et al., 1992). Alongside its clinical efficacy, AZI has a number of advantages including tolerability, simple dosage regimens and minimal drug–drug interactions (Larson et al., 2010).

Azithromycin is also widely used in global health. In particular mass drug administration (MDA) with azithromycin is a cornerstone of the World Health Organization strategy for the elimination of trachoma as a public health problem and for the eradication of yaws (Emerson et al., 2006; World Health Organization, 2012). Through these programs several hundred million doses of AZI are administered in low- and middle-income country settings. There is also increasing interest in expanding these programs owing to the potential for MDA with AZI to reduce childhood mortality (Keenan et al., 2018). Considering the central role of AZI in several public health programs it is imperative to develop and validate a sensitive bioanalytical method for AZI quantification in order to support the development of efficacious drug treatment regimens.

Current analytical methods developed for quantification of AZI in human plasma or serum include high-performance liquid chromatography with electrochemical detection (HPLC–ECD) (Kees et al., 1998; Raines et al., 1998; Supattanapong & Konsil, 2008), HPLC with fluorescence detection (HPLC–FD) (Bahrami et al., 2005; Sastre Toraño & Guchelaar, 1998; Wilms et al., 2005) and HPLC with ultraviolet detection (HPLC–UV). HPLC–UV is rarely used today owing to the lower sensitivity (LLOQ = 30 ng/ml) associated with the absence of an UV chromophore of AZI (Ebrahimzadeh et al., 2010). The HPLC–ECD and HPLC–FD methods have been also largely replaced by liquid chromatography–mass spectrometry (LC–MS) owing to their limited sensitivity with the lowest limit of quantification of 10 ng/ml (Bahrami et al., 2005) and their large biological sample requirement (500–1,000 μl; Supattanapong & Konsil, 2008; Wilms et al., 2005). Liquid chromatography tandem mass spectrometry (LC–MS/MS; Ahmed et al., 2012; Barrett et al., 2005; Chen et al., 2007; Filist et al., 2014; Jiang et al., 2012; Nirogi et al., 2005; Ren et al., 2008; Yüzüük et al., 2007; Zhou et al., 2007) has become the most frequently used method for AZI quantification in human plasma or serum during the last decade. However, the reported methods require a large sample volume (200–500 μl) and a relatively long total run time (up to 14 min), as well as complex sample pretreatment procedures.

Our goal was to develop an LC–MS/MS method for the quantitation of AZI that could detect concentrations <1 ng/mL with a simple extraction procedure and less total analysis time that could be used to support clinical studies on the use of AZI in MDA regimens.

2 | EXPERIMENTAL

2.1 | Chemicals and materials

AZI and AZI-d5 (internal standard) were purchased from Toronto Research Chemicals (North York, Canada). Sodium bicarbonate was purchased from Sigma Aldrich (St Louis, MO, USA). Solvents including methanol (MeOH), acetonitrile (MeCN) and formic acid (FA) were HPLC grade or better and were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Waters Oasis HLB solid-phase extraction (SPE) cartridges (1 ml:30 mg) were purchased from Waters Inc. (Massachusetts, USA). Blank human plasma was purchased from Equitech-Bio Inc. (Kerrville, Texas, USA). Ultrapure water was generated using a Barnstead Genpure water purification system (ThermoFisher Scientific).

2.2 | Standard solutions preparation

A stock solution of AZI was prepared in a 2 ml volumetric flask by dissolving 4.0 mg AZI in methanol and made up to 2 ml with a final concentration of 2 mg/ml. The stock solution was further diluted with methanol to prepare mixed calibration control standards (CCs) and quality control samples (QCs). The internal standard stock solution was prepared by dissolving 1 mg of AZI-d5 in 1 ml of methanol, and further diluted with methanol to prepare working IS stock solutions of 1 μg/ml.

The further 10 calibration standards (CSs) were prepared by diluting stock solutions with methanol to 20, 17.5, 2, 0.5, 0.2, 0.1, 0.2, 0.1 and 0.005 μg/ml. A total of 10 calibration points with a concentration range of 0.5–2,000 ng/ml for AZI were prepared by spiking 10 μl of working standard solution into 90 μl of human plasma. Four different concentrations in four replicates were prepared as QCs, comprising a lower limit of quantification QC (LLOQ, 0.5 ng/ml), low QC (LQC, 1.5 ng/ml), medium QC (MQC, 500 ng/ml) and high QC (HQC, 1,500 ng/ml). All stock solutions, mixed working solutions, CCs, QCs and stock solutions were stored at –20°C.
2.3 | Plasma sample preparation

All CCs, QCs and patient samples were prepared via SPE utilizing Waters Oasis HLB cartridges (30 mg/1 ml; Waters Inc., Milford, MA, USA). All CCs and QCs were prepared by the addition of working standard solution into 100 µl of blank human plasma. For study samples, 100 µl of human plasma was added to a 2 ml polypropylene tube. The IS (10 µl) and 60 mM sodium bicarbonate (600 µl, pH 11) were added to all CCs, QCs and study samples prior to SPE. The sample was vortexed thoroughly for 30 s before SPE. The SPE cartridges were conditioned using 1 ml of MeOH followed by 1 ml of water before loading the samples. A two-step washing procedure was utilized and included washing with water (1 ml) followed by 15% MeOH (1 ml) after loading the samples. Finally, the samples were eluted into glass tubes (13 × 100 mm) using MeOH (2 ml). The eluate was dried under nitrogen at 37°C, and the dried residue was reconstituted with 200 µl of 0.1% aqueous formic acid–MeOH/MeCN (65:35, v:v), followed by vortexing for 30 s and centrifugation at 2,054 g for 5 min. Finally, the supernatant (165 µl) was transferred into autosampler vials and a volume of 2 µl was injected onto the column.

2.4 | Instrumentation

The Shimadzu Nexera UPLC system consisted of two LC-30 AD pumps, a CTO-30AS column oven and a SIL-30 AC autosampler, together with an LC–MS/MS 8060 system (Shimadzu Scientific Instruments, Columbia, MD, USA). An electrospray ionization source in positive ionization mode was used to ionize samples. LabSolutions Instruments, Columbia, MD, USA). An electrospray ionization source was used for data acquisition.

2.5 | Liquid chromatographic and mass spectrometric conditions

Sample separations were completed using an ACE C18 column (2.1 × 100 mm, 1.7 µm, Advance Chromatography Technologies Ltd, UK) equipped with a C18 guard column (Waters, Milford, MA, USA). The mobile phase consisted of 0.1% aqueous formic acid (solvent A) and MeOH–MeCN (1:1; v/v; solvent B). The chromatographic separation was achieved using 5.1 min gradient elution. The initial mobile phase composition was 35% B, increasing to 75% B over 3.0 min, then held constant for 1 min, and finally brought back to the initial conditions of 35% B in 0.10 min followed by 1 min of re-equilibration. The constant flow rate was maintained at 0.25 ml/min throughout the analysis.

The MS–MS conditions, including desolvation line temperature (250°C), interface temperature (300°C), heat block temperature (400°C), nebulizer gas (2.0 L/min) and drying gas flow (10 L/min) were optimized accordingly. Multiple reaction monitoring (MRM) was used to detect and quantitate AZI. The MRM transitions, voltage potential (Q1, Q3) and collision energy (CE) parameters are shown in Table 1.

2.6 | Method validation

The developed UPLC–MS/MS method for AZI quantification in human plasma was validated according to the guidance for Bioanalytical Method Validation of the US Food and Drug Administration (FDA, May, 2018). Fundamental parameters including selectivity, specificity, sensitivity, accuracy, precision, linearity, carryover, recovery, matrix effects and stability were assessed to ensure the acceptability of the method performance.

Linearity was determined by plotting the calibration curve with concentrations ranging from 0.5 to 2,000 ng/ml. A blank sample and 10 different non-zero concentrations were used as calibration points. The determination coefficient ($r^2$) was calculated for each calibration curve using linear regression and was required to be 0.998 or better. Intra- and inter-assay accuracy and precision were assessed for the method by spiking QC samples at four known AZI concentrations (LLOQ, LQC, MQC and HQC) with five replicates. The accuracy and precision were expressed as the percentage deviation from the nominal concentration (% bias) and percentage relative standard deviation (% RSD), respectively. The criteria for acceptability of the data included accuracy within ±15% (bias) from the nominal values and a precision of within ±15% relative standard deviation (%RSD), except for the LLOQ, for which it should not exceed ±20% for accuracy as well as precision:

$$\text{Bias} = \frac{\text{observed concentration} - \text{nominal concentration}}{\text{nominal concentration}} \times 100$$

$$\text{RSD} = \frac{\text{relative standard deviation (SD/mean} \times 100\text{)}}{100}$$

The recoveries of AZI and IS were determined by comparing the peak area of an analyte spiked before extraction with that post extraction, and then multiplying by 100. The recoveries were evaluated at LQC, MQC, and HQC concentrations for total of three replicates (Matuszewski et al., 2003). Matrix effects were studied by comparing

| TABLE 1 | Summary of optimized MS/MS parameters: precursor ions, fragment ions, voltage potential (Q1), collision energy (CE), voltage potential (Q3), and retention time for analyte and internal standard |
|----------|-------------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Analytes** | **Multiple reaction monitoring transition m/z (Q1 > Q3)** | **Q1 (V)** | **CE (V)** | **Q3 (V)** | **Retention time (min)** |
| Azithromycin | 749.50 > 591.45 | −40 | −29 | −22 | 2.5 |
| Azithromycin-d5(IS) | 754.50 > 596.45 | −40 | −31 | −22 | 2.5 |
the peak areas of QCs in post-extraction spiked human plasma with the peak area in methanol.

Stability tests, including bench-top storage stability (20°C for 4 h), long-term stability (−80°C for 30 days), freeze–thaw stability (room temperature to −80°C to room temperature for three cycles) and autosampler stability (4°C for 36 h) parameters were validated by comparing the mean peak area of AZI with the corresponding mean peak area in a fresh solution under the respective conditions.

Dilution integrity was determined by spiking the human plasma with AZI at 3,000 and 7,500 ng/ml, which is above the upper limit of quantification (2,000 ng/ml). Then concentrations were diluted with pooled blank human plasma at dilution factors of 2 and 5 in five replicates and analyzed. The accuracy and precision should be within the set criteria (±15%), similar to the QC samples, to ensure dilution integrity.

Inoculated samples reanalysis was performed to confirm the reliability of the bioanalytical methods during analysis of study samples (Bland & Altman, 1999; Fluhler et al., 2014). Thirty-three clinical samples (from three volunteers, approximately ~10% of total study samples) were reanalyzed and the reproducibility assessed against their original concentrations. The acceptance criteria for IRS were that 67% of the repeated sample results should be within ±20% of the mean. The percentage difference of the results is determined with the following equation:

\[
\text{Percentage difference} = \left( \frac{\text{repeat concentration} - \text{original concentration}}{\text{mean}} \right) \times 100
\]

### 2.6.1 Calculations and statistical evaluation

The mean value, SD, bias and RSD were calculated by using Microsoft Excel 2016. LabSolutions LCMS software version 5.80 (Shimadzu Scientific Inc., Columbia, MD, USA) was used for linear regression analysis.

### 2.7 Clinical application

The aim of this method development was to develop a sensitive bioanalytical method utilizing a simple SPE process and requiring a total run time of <5 min while requiring 100 μl of plasma for the determination of AZI concentrations in a clinical study. The study protocol was approved by the Papua New Guinea medical research advisory committee. The trial was registered at ClinicalTrials.gov (NCT03664063). Written consent forms were obtained from each participant. Equal numbers of men and women were included in the study arms. The AZI used was generic AZI (500 mg tablet) produced by Kern Pharmaceuticals for the study. Volunteers were instructed to fast overnight and were given by direct observation AZI 30 mg/kg (maximum 2 g/dose) in three treatment regimens (John et al., 2020). Serial blood samples were collected at baseline, 1, 2, 3, 4, 6, 8, 12, 24, 48 and 72 h following the dose. Plasma samples were stored at −80°C until analysis. The plasma concentration–time data were analyzed utilizing noncompartmental methods utilizing Phoenix WinNonlin version 6.3 (Pharsight Corporation, CA, USA). The maximal concentration (Cmax) and the time to reach Cmax (Tmax) were determined by direct visual inspection of the data. The half-life (t1/2) was calculated using the following equation: t1/2 = 0.693/Kel. The area under the plasma concentration–time curve from zero time to the last time point (AUC0–t) was calculated using the linear trapezoidal rule. The area under the concentration–time curve from zero time to infinity (AUC0–∞), was calculated using the following equation: AUC0–∞ = Cmax/Kel + Clast/Kel, where Cmax is the last measurable concentration. Apparent volume of distribution (Vz/F) and apparent clearance were calculated using the formulas dose/(F × AUC0–∞) and dose/AUC0–∞, respectively.

**FIGURE 1** Mass spectra of azithromycin (AZI) and AZI-d5 spectra. (a) Q1 spectra of AZI in positive electrospray ionization mode, showing a prominent precursor ion at m/z 749.50. (b) MS/MS spectra of AZI in positive electrospray ionization mode, showing prominent product ion at m/z 591.45. (c) Q1 spectra of AZI-d5 in positive electrospray ionization mode, showing prominent precursor ion at m/z 754.55. (d) MS/MS spectra of AZI-d5 in positive electrospray ionization mode, showing prominent product ion at m/z 596.45.
3 | RESULTS

3.1 Method development and optimization

The precursor and product ions for analytes of interest were determined by the direct infusion of 1 μg/ml stock solutions. During method development, we compared electrospray ionization source and an atmospheric pressure chemical ionization source to optimize the signal intensity. The electrospray ionization source in positive mode provided the best signal intensity of AZI and the IS (Figure 1). The major MRM transitions for AZI m/z 749.50 > 591.45 and IS were 754.50 > 596.45, respectively. Optimized parameters including voltage potential Q1 and Q3 and collision energy are shown in Table 1.

In order to achieve better sensitivity of this method, we further optimized the chromatographic conditions by evaluating various analytical columns, reducing the injection volume and modifying the mobile phase. We found that AZI and IS were resolved from extraneous peaks within 2.5 min by an ACE C18 column (2.1 × 100 mm, 1.7 μm) equipped with a C18 guard column (Waters, Milford, MA, USA). By comparing various injection volumes, we found that injecting 2 μl of sample resulted in the best peak shape and improved the sensitivity of the method compared with a larger injection volume of 5 μl. Various mobile phase components, including MeOH, water, MeCN and FA, were evaluated by comparing the peak intensity and peak shape. Finally, a gradient elution profile consisting of 0.1% FA (solvent A) and methanol–acetonitrile (1:1, v/v, solvent B) at a flow rate of 0.25 ml/min was used in our method to achieve rapid chromatographic resolution and protect the column from accumulated endogenous compounds. The resolution of AZI and IS was achieved in 2.5 min.

In order to accomplish this, an efficient extraction method had to be developed. We tested many extraction methods including protein precipitation, liquid–liquid extraction and SPE. The Oasis® HLB cartridge was found to have a high extraction efficiency for the analytes of interest and with a resulting clean baseline. Conversely, the SPE method provided an efficient sample cleanup with improved reproducibility, high recovery and negligible matrix effect in comparison with protein precipitation and liquid–liquid extraction (data not shown). The mean recoveries were >90% utilizing the final SPE method.

3.2 Assay validation

3.2.1 Selectivity and specificity

Comparison of the chromatogram of blank human plasma samples from six different sources with those of analytes spiked samples was used to determine the selectivity and specificity of this method. With the final method, there were no co-eluting peaks interfering with the quantification near the retention times of AZI and the IS (Figure 2). The retention times were 2.5 min for both AZI and the IS.

3.2.2 Sensitivity

The signal-to-noise ratio was required to be >3 at limit of detection and >10 at the LLOQ to determine the assay limits for quantitation. The lowest concentration for quantitation in assay with RSD < 20% was taken as the LLOQ and was found to be 0.5 ng/ml for AZI.
3.2.3 | Intra- and inter-assay variation

The intra-day and inter-day accuracy (expressed as bias) and precision (expressed as RSD) results for AZI for the LLOQ, LQC, MQC and HQC samples are presented in Table 2. All of the QCs were deemed accurate and precise, with values within ±15% (bias) and ±15% (RSD), except for the LLOQ, where it did not exceed ±20% for the RSD and bias.

3.2.4 | Linearity

The assay was linear from 0.5 to 2,000 ng/ml. Weighted linear regression analysis ($1/x^2$ weighting) was used while performing data analysis. Each calibration curve achieved a determination coefficient of 0.998 or better. The lowest concentration on the calibration curve was 0.5 ng/ml, which was defined as the LLOQ.

3.2.5 | Carryover

No significant carryover effect (<20%) was found in the zero samples after the HQC samples injection.

3.2.6 | Recovery and matrix effects

The extraction recoveries of AZI and IS are shown in Table 3. Calculated matrix factor were in the range of 90–110%, did not exceed ±15%. Therefore, the interference of ion suppression or enhancement was negligible.

3.2.7 | Stability

The effect of long-term storage and analysis stability was performed and consisted of bench-top stability, long-term stability (30 days), freeze–thaw stability and autosampler stability. All QCs were found to be within the range of 85–115% of the actual concentration for the stability studies Table 4.

3.2.8 | Dilution integrity

The precision for the dilution integrity of 1:2 and 1:5 dilutions was within the acceptance limit of ±15% for precision (CV) and 85.0–115.0% for accuracy.

3.2.9 | Incurred samples reanalysis

The percentage difference in concentration at all time points (1–72 h) were within ±20% of the average value for 90% of the samples, except for three time points out of 33 samples (Figure 3a; Bland & Altman, 1999; Fluhler et al., 2014). In addition, there was good linear correlation ($r^2 = 0.9953$, with a slope of 0.6824) obtained between concentrations of reanalyzed and original (Figure 3b). Thus, no significant differences were found between reanalyzed and original concentrations, which were within the acceptance limits of ±20% of the mean value.

3.3 | Clinical application

The developed and validated UPLC–MS/MS method was successfully applied to a clinical trial (NCT03490123) to study the pharmacokinetics of AZI. This method was able to quantify AZI concentrations up to 72 h after drug administration following oral doses of 30 mg/kg. The plasma concentration time profiles of AZI in 13 subjects are shown in Figure 4. The main pharmacokinetic parameters (mean ± SD) are shown in Table 5. The $C_{\text{max}}$ was $1,480 \pm 798$ ng/ml, occurring at the corresponding $T_{\text{max}}$ of 4.23 ± 2.01 h. The $t_{1/2}$ for AZI was

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**Table 2** Intra and inter-day precision (RSD) and accuracy (bias) for azithromycin in human plasma

| Nominal (ng/ml) | Precision intra-assay (%) | Precision inter-assay (%) | Accuracy intra-assay (%) | Accuracy inter-assay (%) |
|----------------|---------------------------|---------------------------|--------------------------|--------------------------|
| 0.5 (LLOQ)     | 10.6                      | 12.5                      | 3.5                      | 13.7                     |
| 1.5 (LQC)      | 2.0                       | 13.3                      | 7.0                      | 8.3                      |
| 500 (MQC)      | 0.6                       | 4.5                       | 0.9                      | 3.7                      |
| 1,500 (HQC)    | 0.7                       | 6.5                       | -11.9                    | -7.0                     |

LLOQ, Lower limit of quantitation; LQC, low quality control; MQC, medium quality control; HQC, high quality control.

**Table 3** Mean extraction recoveries of the azithromycin from human plasma

| Concentration (ng/ml) | Percentage extraction recovery (mean ± SD, n = 3) |
|-----------------------|-----------------------------------------------|
| LQC                   | 82.2 ± 0.5                                   |
| MQC                   | 93.0 ± 4.5                                   |
| HQC                   | 94.7 ± 6.7                                   |
33.7 ± 8.22 h, which was similar to the reported values in the literature (Ahmed et al., 2012; Liu et al., 2007). The mean AUC₀–ₜ and AUC₀–∞ for AZI were 12,300 and 12,300 h ng/ml, respectively. The mean apparent clearance and volume of distribution of AZI were 126.0 L/h and 6,030 L, respectively.

**DISCUSSION AND CONCLUSION**

LC–MS/MS is the most common bioanalytical method used for measuring AZI in human plasma. Compared with previously reported quantification methods, this method has significant advantages including the requirement of a small plasma volume, a simple SPE method with analyte recovery >90%, increased sensitivity, reduced injection volume, large dynamic range and a short total run time. To the best of our knowledge, this is the first description of a bioanalytical method for the quantitation of AZI following weight-based dosing regimens (30 mg/kg) for the treatment of yaws. The linearity range from 0.5 to 2000 ng/ml was suitable to quantitate the AZI plasma concentration for up to 72 h, allowing for the determination of pharmacokinetic parameters to describe AZI bio-distribution. Regarding to sample preparation procedure, only 100 μl human plasma was used in our method, which was the lowest requirement for bio-matrices compared with published articles, which utilized 200–500 μl (Ben-Eltriki et al., 2013; Chen et al., 2007; Choemunng & Na-Bangchang, 2010; Filist et al., 2014; Zhou et al., 2007). Moreover, the SPE method provided excellent extraction recovery of AZI without a matrix effect, while avoiding methyl tertiary-butyl ether, which is not an environmentally friendly solvent for sample extraction. Finally, we were able to further reduce the total analysis time from 8 min (Filist et al., 2014) to 4.5 min per sample, which enabled us to measure more than 200 samples per day with acceptable accuracy and precision. The study subjects were given about 2–4 times higher doses of AZI compared to the standard dosing regimen.
(1.250–2000 mg) than in previous published pharmacokinetic studies (250–500 mg). We found that the $C_{\text{max}}$ and AUC values were significantly higher, as expected. The $C_{\text{max}}$ and AUC values were around 3 times higher than the reported values when the traditional 250–500 mg dose of AZI was given (Ahmed et al., 2012; Bahrami et al., 2005; Barrett et al., 2005; Ben-Eltriki et al., 2013; Chen et al., 2007; Liu et al., 2007; Zhou et al., 2007). This highly sensitive, rapid, high-throughput bioanalytical method will provide a valuable tool to determine the pharmacokinetic profile of AZI following weight-based dosing regimens.

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CONFLICTS OF INTEREST
There is no conflict of interest to disclose.

DATA AVAILABILITY STATEMENT
Data available on request from the authors.

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