DEVELOPMENT AND OPTIMIZATION OF AN ECONOMIC METHOD FOR QUANTITATION OF AZITHROMYCIN IN HUMAN PLASMA BY TANDEM MASS SPECTROSCOPY (LCMS/MS) FOR CLINICAL TRIALS

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ABSTRACT

The present study deals with development and optimization of an economic method for quantitation of Azithromycin in human plasma by Tandem Mass Spectroscopy (LCMS/MS) for Clinical studies. Enalapril (stable and economic) was used as an internal standard. Azithromycin was extracted from plasma using an Oasis HLB solid-phase extraction cartridge (Waters Corporation, USA). The elution was carried out with degassed 0.1% formic acid buffer in methanol (5:95, v/v, economic as compare to commonly used acetonitrile). After elution the elute was evaporated to dryness in nitrogen, and was reconstituted in 100 µl mobile phase (same as elution buffer) and 10 µl of the sample was injected into an HPLC system containing Chromolith RP C18, 100*4.6 mm column connected to LCMS/MS system. The lower limit of quantitation (LLOQ) was 0.95ng/ml, without interfering peaks. The calibration curve was linear (R=0.9979) over a concentration range 0.95ng/ml to 951.63 ng/ml. Retention time for Azithromycin and its internal standard was 1.26 minutes and 1.45 minutes. The accuracy (85%-115%) and precision (CV ≤ 15%) were acceptable. The mean recoveries for Azithromycin at 3, 425 and 875 ng/ml were: 75.44±2.86%, 79.23±4.475% and 74.84±2.86% (n=6), respectively. Azithromycin was stable in plasma for at least 6 hours at room temperature and for at least four freeze and thaw cycles (% change = 3.73 at LOQ and 4.46 at ULQ). It was observed that the method is selective for Azithromycin as no peak interference was observed from plasma matrix while blank plasma or plasma with the drugs was run at RT of internal standard and analyte. The developed method has adequate sensitivity, specificity, accuracy and precision to measure Azithromycin in human plasma and, it can be considered as a cost effective, fast and with the acceptable range of recovery (80% - 90%) for BE studies/clinical trials.

Key words: Azithromycin, Bioanalytical method, validation, LCMS/MS.
INTRODUCTION

In almost all countries, a BE studies against the standard formulation is a key feature of ANDA submission to FDA by manufacturer, who wishes to produce a generic drug. The analytical method used in an in-vivo BA/BE study to measure the concentration of the active drug ingredient or therapeutic moiety, or its active metabolite, in body fluids or excretory products, or the method used to measure an acute pharmacological effect. This method needs to be accurate, precise, sensitive and also selective for particular compound. [1] Bioanalytical LC-MC/MS methods are widely used to quantitate drugs and metabolites in physiological matrices. The methods could be applied to human clinical pharmacology/preclinical studies, non-human pharmacology/toxicology studies. Present study was designed to develop and validate analytical method for estimation of Azithromycin in human plasma using LCMS/MS method for clinical trials and BE studies.

Azithromycin, a semi-synthetic macrolide antibiotic structurally related to erythromycin, has been used in the treatment of Mycobacterium avium, toxoplasmosis, and cryptosporidiosis. After oral administration, it is rapidly distributed to peripheral tissues and concentrations in tissues are known to significantly exceed those of plasma or serum. Azithromycin is the first macrolide antibiotic belonging to the azalide group. Azithromycin is derived from erythromycin by adding a nitrogen atom into the lactone ring of erythromycin A, thus making the lactone ring 15-membered.

As per US national library of medicine [2] azithromycin is used to treat certain infections caused by bacteria, such as bronchitis; pneumonia; sexually transmitted diseases (STD); and infections of the ears, lungs, skin, and throat. It works by stopping the growth of bacteria. Azithromycin is also used sometimes to treat H. pylori infection, and other infections. It is also used sometimes to prevent heart infection in patients having dental or other procedures and to prevent STD. [3] Azithromycin has been found to be an effective alternate to penicillin in the treatment of less severe cases of leptospirosis. [4]

Azithromycin does not have a specific UV chromophore and thus, UV detection gives only low sensitivity for the determination of azithromycin in plasma. The methods that have been reported are based on high performance liquid chromatography (HPLC)
Various extraction methods too have been reported for extraction of Azithromycin form plasma are, protein precipitation, pressurized liquid extraction (PLE), liquid–liquid extraction, solid phase extraction. For this project, the method was developed on a sample which is extracted by solid phase extraction (SPE). The objective of the study was to develop simple, fast, sensitive, selective, accurate and economic method for quantitation of Azithromycin in human plasma following SPE by LCMS/MS.

**MATERIALS AND METHODS**

Azithromycin and Enalapril were purchased from Cadila Pharmaceuticals Limited, Ankleshwar. Methanol, Acetonitrile, and formic acid were of HPLC grade and de-ionized water was obtained from a Milli-Q de-ionized (DI) water system (Millipore, Bedford, MA). Drug free plasma was obtained from CRO, Cadila Pharmaceuticals Limited, Dholka.

**Equipment and chromatographic conditions**

The HPLC system with solvent delivery mode (LC-20AD, Shimadzu Corporation, North America) and auto sample injector (SIL-HTC, Shimadzu Corporation, North America). MS detector API 4000 (MDS SCIEX, Ontario, Canada), was coupled with LC system. Data acquisition was performed using Analyst version 1.4.2 (Applied Biosystem) system software. The analytical column decided, was a Chromolith RP-18, 100 X 4.6mm with 5 µm packed particle size. The mobile phase decided after several trails, was a methanol: 0.1% formic acid, pH 5.5 (95:5 v/v) with a flow rate of 1.0 ml/minute. The HPLC solvent mobile phase was filtered through a 0.2 µm nylon membrane, degassed by sonication, (Cleaner, Life care, Mumbai). Injection volume was kept at 10µl/min. Acetonitrile and water (70:30v/v) was used for column or needle was solution. The SPE cartridge used was a hydrophilic-lipophilic balanced copolymer extraction column (1 ml, 30 mg, Waters Oasis HLB, Waters, Milford, MA).

**MS tuning**

Tuning of mass spectrometer involves optimizing voltages, currents, flows, and the like for the ion source parameters to achieve the maximum mass spectral sensitivity and proper resolution. The MRM experiment is accomplished by specifying the parent
mass of the compound for MS/MS fragmentation and then specifically monitoring for a single fragment ion. In this method, only data on analytes of interest were collected. All other compounds were ignored. It is more sensitive than full scan mode.

100 ng/ml of azithromycin solution injected via FIA (Flow Injection Analysis) into detector via Harvard Apparatus at flow rate 10 μl/min. The molecular weight of compound Q1 mass was determined by Scan. Then daughter ion was scanned and Q3 was determined.

**Preparation of Calibration standard**

Stock solutions of azithromycin (1000 μg/ml) and Enalapril (1000 μg/ml) were prepared in methanol-water (70:30, v/v). Azithromycin stock solution was diluted with a mobile phase to make a working solution at a concentration of 1 μg/ml. Calibration standards were prepared by spiking azithromycin working solution into drug free human plasma (total volume 500 ml) to obtain concentrations of 1, 2, 5, 25, 125, 400, 650, 850 and 1000 ng/ml. 475 μL of plasma was transferred to a micro centrifuge tube and 25 μL of working calibration standard of azithromycin was added for preparation of calibration standard and vortexed for 15 seconds. 50 μL of IS working solution was then added and vortexed for 15 seconds. Calibration curves were obtained using nine calibration standards and calculated using peak area ratios for Azithromycin for the corresponding concentration. Linear calibration curves were generated by a linear regression equation with R² not less than 0.9900.

**Sample preparation**

For sample extraction, three methods used mainly are, protein precipitation (PPT), liquid –liquid extraction (LLE) and solid phase extraction (SPE). After not getting satisfactory results in terms of peak interferences, resolution and recovery, PPT and LLE was not optimized further. SPE was performed. The processes of sample extraction were: the cartridge was conditioned with 1 ml of methanol, allowed the cartridge to dry, equilibrated with 1 ml of water, loaded 1 ml of plasma sample and IS 20 μl onto the Oasis HLB SPE columns. SPE cartridges were washed with 2 ml of Milli-Q water for twice, eluted with 1 ml of methanol, evaporated the elute to dryness with nitrogen gas at a temperature of 25-30°C for 40 minutes and reconstituted in 100 μl of mobile phase, vortexed for 15 seconds and transferred the contents into appropriate pre-labeled vials.
Method validation [18]

The processes of method validation and their criteria followed the guidelines for Bioanalytical Method Validation of the US FDA (2001). Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine, is reliable and reproducible for the intended use. The fundamental parameters for this validation include (1) accuracy, (2) selectivity, (4) sensitivity, and (5) stability.

Preparation of quality control samples

Quality control (QC) samples were prepared in plasma using a stock solution separated from that used to prepare the calibration curve, at the three different concentrations of the spiked plasma (3, 425 and 875 ng/ml). The result of the QC samples gave the basis for accepting or rejecting the run. At least four of the six QC samples had to be within ± 15% of their nominal concentration.

Sensitivity

Sensitivity was measured in terms of lower limit of quantification (LLOQ). LLOQ was decided such that, $C_{max}$ for Azithromycin equals to 20 times the LLOQ. Six matrices spiked LLOQ were prepared, using the same spiking dilution used to prepare the LLOQ standard of the calibration curve. Six LLOQ samples were processed and injected along with calibration curve standards in the same range used for calculation of precision and accuracy. Back-calculated the concentration of the LLOQ samples against calibration curve standards where the %CV was within 20% and accuracy was within the range 80-120%.

Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Blank plasma samples of the appropriate were obtained from at least six sources (six different donors). One sample, each of hemolyzed plasma and a sample having high lipid content were also analyzed. Each blank plasma sample was tested for interference, selectivity was ensured at the lower limit of quantification (LLOQ) and each sample from at least six different donors were analyzed individually for interference. Selectivity was assessed by comparing the
chromatograms of the drug-free plasma from the six different donors, and the plasma spiked with Azithromycin and IS.

Accuracy

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy was determined by replicate analysis of samples containing known amounts of the analyte. Accuracy was measured using a minimum of five determinations per concentration. The %CV for each concentration level did not exceed 20% and accuracy was within the range of 85-115%.

Recovery

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. The recovery of analyte(s) and internal standard was evaluated by comparing the bioanalytical results for extracted QC samples with aqueous solutions equivalent to 100% recovery of LQC, MQC and HQC. The recoveries of azithromycin extraction at three different concentrations of the spiked pooled plasma (3, 425 and 875 ng/ml) were evaluated and comparing the peak areas of azithromycin with the IS, and then comparing with those obtained from direct injection of the azithromycin dissolved in methanol at the same concentrations. The mean recoveries were calculated for each concentration. The % CV of the mean analyte(s) and internal standard(s) recoveries must be ≤ 15% at low middle and high quality control concentration level

Stability

The short-term stock solution and working solution stability were examined by keeping the standard stock solution and working solution at room temperature and analyzed at 6 hours 30 minutes. Results were compared with those obtained after analyzing freshly prepared samples at similar concentration. The long-term stability of Azithromycin in plasma was tested after storage of the spiked pooled plasma at LQC and HQC concentration, after four freeze and thaw cycles

RESULTS

MS tuning
Analytes were analyzed by mass spectrometry by multiple reaction monitoring mode using the respective [M + H] + ions. For Azithromycin, parent ion molecular weight was 749amu, while product ion molecular weight was 591amu. For Enalapril, parent ion molecular weight was 377amu, while product ion molecular weight was 234amu.

**Method of SPE extraction**

The main aim of the extraction process is to get maximum recovery of analyte and metabolite from the biological samples without interferences during HPLC determination. SPE based on the principles of separation due to adsorption and/or absorption. The SPE column, Oasis HLB, is of polymeric construction designed to have a hydrophilic-lipophilic balance (HLB) that gives a high recovery rate and good reproducibility for acidic, basic, and neutral compounds. Azithromycin was extracted from the spiked plasma and purified using Oasis HLB. Washing with 2 ml of Milli-Q water provided acceptable recoveries and no peaks interfering with determination of azithromycin or IS.

**Linearity of calibration curves**

Calibration curve was found linear, accurate and precise over the range 0.95 ng/mL to 951.63 ng/mL for azithromycin. The regression coefficient (r) is 0.9979 which is greater than 0.9900 for azithromycin and the equation of the standard curve was y = 0.00745 x + 0.00211.

**Selectivity**

No significant interference (in terms of retention time or resolution) was observed at the retention times for analyte in eight different lots of blank human plasma samples including one haemolysed and one lipemic plasma. The retention times for IS and azithromycin in spiked plasma are shown in Fig 1. The retention times for IS and azithromycin were about 1.45 and 1.26 minutes respectively.

**Accuracy**

For lowest calibration standard, % accuracy was 96.67, 101.04, and 101.97%, with an average of 99.89%, which is in between 80-120%. For other calibration standards, % accuracy was in the range of 85-115%. Any two consecutive samples were not outside the acceptance criteria range. First and last calibration standard complied with
the acceptance criteria (Table 1). The results demonstrate the values were within the acceptable range and the method is accurate.

![Representative chromatogram of blank human plasma sample](image)

**Figure 1**
Representative chromatogram of blank human plasma sample

**TABLE 1: %ACCURACY FOR THE DETERMINATION OF AZITHROMYCIN IN PLASMA**

<table>
<thead>
<tr>
<th>Sample No</th>
<th>Initial concentration (ng/ml)</th>
<th>Calculated concentration (ng/ml)</th>
<th>% Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>951.63</td>
<td>910.21</td>
<td>95.65</td>
</tr>
<tr>
<td>2</td>
<td>808.89</td>
<td>749.34</td>
<td>92.64</td>
</tr>
<tr>
<td>3</td>
<td>618.56</td>
<td>632.34</td>
<td>102.23</td>
</tr>
<tr>
<td>4</td>
<td>380.64</td>
<td>367.70</td>
<td>96.60</td>
</tr>
<tr>
<td>5</td>
<td>118.95</td>
<td>130.00</td>
<td>109.29</td>
</tr>
<tr>
<td>6</td>
<td>23.80</td>
<td>25.84</td>
<td>108.56</td>
</tr>
<tr>
<td>7</td>
<td>4.76</td>
<td>4.51</td>
<td>94.79</td>
</tr>
<tr>
<td>8</td>
<td>1.90</td>
<td>1.88</td>
<td>99.19</td>
</tr>
<tr>
<td>9</td>
<td>0.95</td>
<td>0.96</td>
<td>101.04</td>
</tr>
</tbody>
</table>
Lower limits of quantification (LLOQ)

The LLOQ was 0.95 ng/ml for which an acceptable accuracy was in the range 80-120% (99.89%) (Table 2). One such representative chromatogram of LLOQ is shown in Figure 2.

**TABLE 2: SENSITIVITY OF AZITHROMYCIN IN HUMAN PLASMA**

<table>
<thead>
<tr>
<th>Initial concentration (ng/ml)</th>
<th>Calculated concentration * (ng/ml) (n= 6)</th>
<th>% Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.95</td>
<td>1.00 ± 0.093</td>
<td>105.36%</td>
</tr>
</tbody>
</table>

* Mean ± standard deviation

Figure 2

Representative Chromatogram of the Lower Limit of Quantitation Sample-LQC

Recovery

The mean recoveries determined for each of the three concentrations (3, 425 and 875 ng/ml) of azithromycin were 75.44±2.86%, 79.23±4.475% and 74.84±2.86% (n=6), respectively (Table 3).

**TABLE 3: RECOVERY OF AZITHROMYCIN FROM HUMAN PLASMA**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Mean Recovery *</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>LQC</td>
<td>75.44 ±2.86</td>
<td>3.79%</td>
</tr>
<tr>
<td>MQC</td>
<td>79.23 ±4.475</td>
<td>5.65%</td>
</tr>
<tr>
<td>HQC</td>
<td>74.83 ±2.86</td>
<td>1.82</td>
</tr>
</tbody>
</table>

* Mean ± standard deviation
Stability

No significant degradation occurred under any of the experimental conditions. The stability data are shown in Table 4. Azithromycin and IS were found stable in methanol (stock solution) and in diluents (working solution) on bench at room temperature for 06 Hrs. 30 mins. %change should be less than 10% from initial value of analyte as specified in US FDA criterion. Azithromycin was found stable after four freeze and thaw cycles at -30°C ±5°C after reconstituting with mobile phase; it was stable for at least 48 hours for auto-sampler injection.

**TABLE 4: STABILITY OF AZITHROMYCIN IN PLASMA SAMPLES**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Initial concentration* (ng/ml)</th>
<th>Calculated concentration* (ng/ml)</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short term working solution</td>
<td>2.85</td>
<td>2.63±0.63</td>
<td>2.23</td>
</tr>
<tr>
<td>Long term stability</td>
<td>2.85 ±0.175</td>
<td>3.06±0.389</td>
<td>3.73</td>
</tr>
<tr>
<td></td>
<td>832.68±21.311</td>
<td>801.12±37.64</td>
<td>4.46</td>
</tr>
<tr>
<td>Auto sampler stability</td>
<td>2.85 ±0.132</td>
<td>2.66±0.141</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>832.68±8.92</td>
<td>831.08±14.25</td>
<td>2.3</td>
</tr>
</tbody>
</table>

* Mean ± standard deviation

**DISCUSSION**

The LC in combination with MS/MS for the determination of azithromycin in plasma is simple, selective, sensitive, rapid, stable and reproducible. In order to determine the accurate concentration of azithromycin in biological matrix, there were many challenges. The major challenges were establishment of suitable sample preparation technique, setting Instrumental parameters, mobile phase determination etc. Reverse phase chromatography and SPE extraction was developed for sample preparation. Substituting acetonitrile with methanol as one of the constituent of mobile phase and extraction process lowers the cost of the method. SPE is a choice of extraction method as LLE due to, almost complete extraction, less interference, less consumption of organic solvents and easy procedure [19]. Washing the sample with 2 ml of water, gave an acceptable chromatogram without interfering peaks near the retention times for
azithromycin and IS, with almost 80% recovery. The total run time of method was three minutes with 1.26 and 1.45 minutes of retention time of azithromycin and IS respectively. Thus, method is rapid in its performance. The method was fully validated according to USFDA guidelines for Bioanalytical Method Validation and found method is accurate, precise, selective, linear, and stable.

CONCLUSION

SPE was selected to provide simple and clean sample preparation. The LLOQ of the LCMS/MS was 1 ng/ml. This method is found specific, rapid and cost effective and can be applicable for quantitative analysis of Azithromycin during bioequivalence / bioavailability study and clinical trial.

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