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A new validated bio-analytical liquid chromatographic-tandem mass spectrometric (LC-MS/MS) method for the quantification of Azithromycin in human plasma

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ABSTRACT

A simple, rapid and sensitive LC-MS/MS method was developed and validated for the quantification of azithromycin (AZI) in human plasma. Sildenafil citrate was used as an internal standard. The analytes were extracted from human plasma samples by liquid-liquid extraction technique. The reconstituted samples were chromatographed on a reversed - phase column C18, 50 × 2.1 mm, 5 µm from Waters, by using a 80:20 (*v*:*v*) mixture of acetonitrile and 0.1% formic acid in water as the mobile phase at a flow rate of 0.5 mL/min. The calibration curve was linear (r^2 > 0.99) over the concentration range of 5.0-1500.0 ng/mL. The results of the intra- and inter-day precision and accuracy studies were within the acceptable limits. A run time of 0.5 min for each sample made it possible to analyze more than 400 plasma samples per day.

1. Introduction

Azithromycin designated chemically as 2*R*,3*S*,4*R*,5*R*,8*R*,10*R*,11*R*,12*S*,13*S*,14*R*)-2-ethyl-3,4,10-trihydroxy 3,5,6,8,10,12,14-heptamethyl-15-oxo-11-{[3,4,6-trideoxy-3-(dimethylamino)-β-*D*-xylo-]oxy}-1-oxa-6-azacyclopentadec-13-yl2,6-dideoxy-3-C methyl-3-O-methyl-α-L-ribo-hexopyranoside [1] (Figure 1) is a semi-synthetic macrolide antibiotic of the azalide class. Azithromycin inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit of the bacterial 70S ribosome. It inhibits peptidyl transferase activity and interferes with amino acid translocation during the process of translation. Its effect may be bacteriostatic or bactericidal depending on the organism and the drug concentration. Its long half-life, which enables once daily dosing and shorter administration durations, is a property distinct from other macrolides. It is derived from erythromycin; however, it differs chemically from erythromycin in that a methyl-substituted nitrogen atom is incorporated into the lactone ring as shown in Figure 1.

Like erythromycin, it appears to bind to the same receptor, 50S ribosomal subunits of susceptible bacteria and suppresses protein synthesis. It is used primarily to treat various bacterial infections, such as aerobic gram-positive microorganisms and aerobic gram-negative microorganisms. The incorporation of the nitrogen into the ring significantly alters the chemical, microbiologic and pharmacokinetic properties of AZI. It exhibits a more extensive spectrum of activity, greater acid stability and more favorable pharmacokinetic parameters than erythromycin [1].

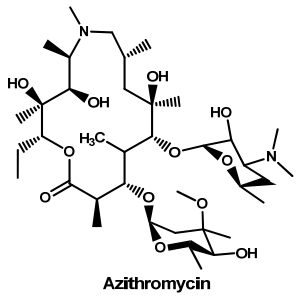

Figure 1. Chemical structure of azithromycin.

Several methods have been developed for determination of AZI in pharmaceutical dosage forms. These methods include

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high-performance liquid chromatography (HPLC) and micro-biological methods.

Chromatographic separation is one of the essential and powerful components of the most quantitative analyses and HPLC is currently the most versatile tool which satisfies the needs for an optimum separation [2]. AZI has been analyzed by spectrophotometric methods [3-5], high-perfor-mance liquid chromatography using fluorescence [6-8], electro-chemical using amperometric detection [9-10] and coulometric detection [11-13] and mass spectrometry detector [14-17] for quantification in bulk material and pharmaceutical dosage forms. Fluorescence detection requires complicated sample pretreatment involving pre-column derivatization of the analyte. Assay procedures making use of electrochemical detection is often very time consuming, both in the sample preparation steps and the chromatography. The United States Pharmacopoeaia (USP) method [18] describes a high pH mobile phase (pH = 11) as well as a specific column (Gamma alumina) which is quite expensive and difficult to obtain commercially as many of the column manufacturers do not supply this column. Also, the USP method employs amperometric electrochemical detection, which is not available in many laboratories. Therefore, there is a need for a convenient and effective method for determination of AZI in pharmaceutical dosage forms. Liquid chromatography with UV detection has been already employed for the analysis of AZI either in tablets [19] or in raw material [20] or in both [21]. Mass spectrometry methods may have the highest sensitivity, but the determination process is complex [22]. Various extraction methods have been reported for extraction of Azithromycin form plasma like, protein precipitation [23,24], pressurized liquid extraction (PLE) [25], liquid-liquid extraction [26,27] and solid phase extraction [28,29].

The objective of the study was to develop simple, fast, sensitive, selective, accurate and economic method for quantification of Azithromycin in human plasma following liquid-liquid extraction by LC-MS/MS. Unlike other LC-MS methods, the presented method has the advantages of short run time (about 0.5 min) which made it possible to analyze more than 400 plasma samples per day and a wide range of linearity (5-1500 ng/mL). Sildenafil citrate (Figure 2), designated chemically as 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1*H*pyrazolo[4,3-*d*]pyrimidin-5-yl)-4-ethoxyphenyl] sulfonyl]-4-methylpiperazine citrate was used as internal standard (IS).

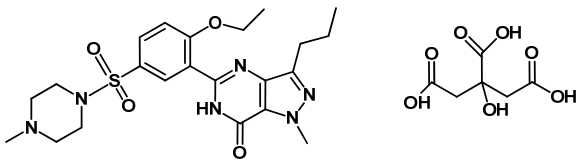

Figure 2. Chemical structure of sildenafil citrate (IS).

2. Experimental

2.1. Chemicals and reagents

Azithromycin reference standard (B.N: 1182090426; purity: 97.80%) was obtained from CSPC, OUYI Pharmaceutical Co. Ltd (Shijiazhuang, China). Sildenafil citrate (B.N. 586032009SC; purity: 99.20%) was employed as an internal standard (IS) obtained from BDR Pharmaceuticals International PVT. ITD (Mumbai, India). Water for HPLC was purchased from Merck, Germany. Formic acid and acetonitrile were of HPLC grade and purchased from Sigma-Aldrich Chemie Gmbh (Munich, Germany). Sodium carbonate and *tert*-butyl methyl ether were supplied by (Romil Ltd., London, UK). The control human plasma sample was procured from VACSERA (Cairo, Egypt).

2.2. Equipment

Acquity Ultra-Performance Liquid Chromatographic-MS/MS (UPLC-MS/MS) system equipped with UPLC C18 (50 × 2.1, 5 µm) column, tandem mass triple-quadruple detector and electro spray ionization (ESI) probe was used for the assay (Waters, USA). The system included a vacuum degasser, a quaternary pump, a thermostatted auto-sampler and a column oven compartment. Data acquisition and data integration were done using Empower™ Ver.2 Chromatography Data Software (CDS) solutions.

2.3. Chromatographic conditions

Separations were performed on UPLC C18 (50 × 2.1, 5 µm) column. A mobile phase consisting of a mixture of acetonitrile-0.1 %formic acid (80:20, *v*:*v*) was delivered at a flow rate of 0.5 mL/min into the mass spectrometer. Aliquot of 7.5 µL of the processed samples were injected into the column, which was kept at 30 °C. Quantification was achieved with MS-MS detection in positive ion mode for both the internal standard and azithromycin using Waters ACQUITY TQD (triple quad detector) MS/MS (Foster City, CA, USA). The compound parameters viz. the collision energy (CE) and Cone voltage (CV) were 38 and 40 volt for azithromycin and 30 and 50 volt for sildenafil citrate. Detection of the ions was carried out in the multiple-reaction monitoring mode (MRM), by monitoring *m/z* 749.62/82.87 for azithromycin ion and *m/z* 475.34/99.75 for sildenafil citrate ion (IS). Data acquisition and data integration were done using Empower™ Ver. 2 CDS software solutions.

2.4. Preparation of stock solutions, secondary and working solutions

2.4.1. Stock solution of azithromycin for calibration and quality control

A stock standard solution of azithromycin (100 µg/mL) was prepared by accurate weighing and transferring 10 mg of azithromycin into a 100 mL volumetric flask and diluting to volume with the mobile Phase.

2.4.2. Working solutions of azithromycin

Different working standard solutions of azithromycin (B, C, D, E and F) were prepared by transferring (10 mL, 1 mL, 100 µL, 10 µL and 1 µL, respectively) of stock solution each into a 100 mL volumetric flask and diluting to volume with mobile phase to give a final concentrations of (10 µg/mL, 1 µg/mL, 100 ng/mL, 10 ng/mL and 1 ng/mL, respectively).

2.4.3. Stock solution of internal standard for calibration and quality control

A stock standard solution of sildenafil citrate (100 µg/mL) was prepared by accurately weighing and transferring 10 mg of sildenafil citrate into a 100 mL volumetric flask and diluting to volume with mobile phase.

2.4.4. Working solution of internal standard

A working standard solution of sildenafil citrate (0.5 µg/mL) was prepared by transferring 0.5 mL of stock solution into a 100 ml volumetric flask and diluting to volume with mobile phase. Human plasma was chromatographed prior to use to determine possible interference with azithromycin or internal standard.

Table 1. The optimum Multiple Reaction Mode parameters for both drug and IS.

No	Drug / IS	Precursor (Da)	Product (Da)	Dwell (sec)	Cone voltage (V)	Collision energy (V)
1	Sildenafil (IS)	475.34	99.75	0.100	50	30
2	Azithromycin	749.62	82.87	0.100	40	38

No significant interferences were observed in the lots of human plasma used for the preparation of calibration standards and quality control samples.

2.5. Preparation of calibration curve and quality control solutions

Standard curve was produced by preparing six plasma standards over the range of 5.0-1500.0 ng/mL for azithromycin. Standards were prepared in triplicates. Similarly, quality control (QC) samples were also prepared as a bulk based on an independent weighing of standard drug, at concentrations of 5.0 (Lower Limit of Quantitation, LLOQ), 10.0 (low; Low Quality Control, QCL), 400.0 (middle; Medium Quality Control, QCM) and 1200.0 ng/mL (high; High Quality Control, QCH) as a single batch at each concentration. Each calibrations or QC solution was prepared by spiking 50 µL of the corresponding stock solution into 450 µL of human plasma, then 100 µL of Na_2CO_3 (30%, *w*:*v*) was spiked. Then extraction with 6 mL *tert*-butyl methyl ether was done. The mixture was vortexed for thirty seconds and centrifuged at 4000 rpm for five minutes. The organic layer was then separated and evaporated in Epindorff evaporator then the residue was reconstituted with 250 µL of IS dissolved in mobile phase and injected.

2.6. Bio-analytical method validation

The method was validated in accordance with international regulations [30]. The parameters determined were selectivity, specificity, matrix effect, linearity, precision, accuracy, recovery, stability and dilution integrity.

3. Results and discussion

3.1. Method development

3.1.1. MS-MS tuning

Mass parameters were tuned in both positive and negative ionization modes for the drug. Good response was found in positive ionization mode for both the drug and IS. The MRM state file parameters were optimized to maximize the response for both the drug and IS as shown in Table 1. The product ion mass spectrum of azithromycin and IS are presented in Figure 3.

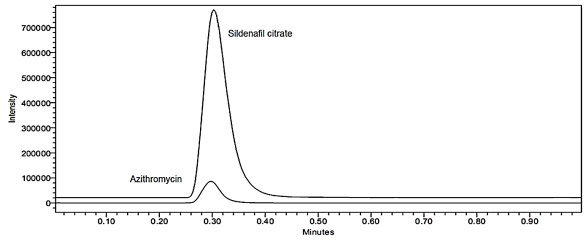

Figure 3. Mass spectrum of pure azithromycin and sildenafil citrate (IS).

3.1.2. Chromatographic separation

Separation was attempted using various combinations of acetonitrile and buffers with varying contents of each component on different columns like C8 and C18 of different types like Chromolith, Hypersil, Zorbax, Kromasil and Intertsil *etc*. Use of 0.1 % formic acid helped in achieving good response for MS detection by facilitating ionization of the ions. A mobile phase consisting of acetonitrile and 0.1% formic acid (80:20, *v*:*v*) was found suitable for both the drug and IS. The chromatographic separation was performed at room temperature on Waters C18 column (50 mm x 2.1 mm, 5 µm) which gave a good peak shape and response even at lower limit of quantitation level. A flow-rate of 0.5 mL/min produced a good peak shape and permitted a runtime to 0.5 min.

3.1.3. Plasma sample extraction

Different methods of sample extraction including protein precipitation with methanol or acetonitrile and liquid-liquid extraction (LLE) with different solvents commonly used were attempted. Better recoveries were obtained with LLE using *tert*-butyl methyl ether. Besides, LLE can be helpful in producing a spectroscopically clean sample and avoiding the introduction of nonvolatile materials onto the column and MS system and also minimized the experimental cost. Clean samples are essential for minimizing ion suppression and matrix effect in LC-MS/MS analyses. *Tert*-butyl methyl ether was found to be optimal, which can produce a clean chromatogram for a blank plasma sample and yield the highest recovery for the drug from the plasma.

3.2. Method validation

3.2.1. Linearity

Six calibration curves each consisting of a zero, non-zero and calibration standards prepared in human plasma were chromatographed. The concentrations of calibration standards cover the range (5 -1500 ng/mL), the linearity was evaluated by calculating the linear regression (correlation coefficient, r^2), and by evaluating the back calculated concentrations of the calibration standards. The lower limit of quantification (LLOQ) of azithromycin is (5 ng/mL).

Calibration curves are found to be consistently accurate and precise over the calibration range of 5-1500 ng/mL. The mean correlation coefficient (r^2) is equal to 0.999. Back calculations were made from the calibration curves to determine drug concentrations of each calibration standard. Data are presented in Table 2. A typical calibration curve is presented in Figure 4.

3.2.2. Selectivity

The selectivity of the method was examined by analyzing a blank human plasma extract and a blank plasma sample spiked with IS only (Figure 5). As shown in this figure, no significant interferences were observed in the lots of human plasma used for the preparation of calibration standards and quality control samples.

3.2.3. LLOQ

The lowest limit of reliable quantification for the drug was set at the concentration of the LLOQ which is the lowest quality control level with a coefficient of variation less than 20 %. LLOQ is 5 ng/mL with a signal to noise ratio of 6.1.

Table 2. Back-calculated standards from each calibration curve.

Calibration no	Nominal concentrations (ng/mL)							
	5	25	50	100	250	500	1000	1500
1	5.414	24.873	49.112	111.359	256.809	513.472	1004.263	1558.137
2	5.165	25.455	45.562	111.180	253.650	524.688	979.228	1513.784
3	5.240	24.936	42.811	110.972	246.615	515.740	1004.699	1406.883
4	6.054	22.758	42.811	104.843	240.341	507.540	981.047	1513.023
5	5.868	24.936	42.811	110.999	246.615	515.740	1004.699	1554.451
6	5.266	22.000	43.350	134.416	248.320	496.705	1004.699	1538.047
N	6	6	6	6	6	6	6	6
Mean	5.501	24.160	44.410	113.961	248.725	512.314	996.439	1514.054
SD (±)	0.370	1.416	2.539	10.332	5.813	9.427	12.642	55.92411
CV (%)	6.723	5.860	5.717	9.066	2.337	1.840	1.269	3.693667
% Nominal	110.022	96.640	88.819	113.961	99.490	102.463	99.644	100.9369

Table 3. Intra-day accuracy and precision.

Run number	QCL 10 ng/mL		QCM 400 ng/mL		QCH 1200 ng/mL	
	Conc. found (ng/mL)	% Nominal conc.	Conc. found (ng/mL)	% Nominal conc.	Conc. found (ng/mL)	% Nominal conc.
1	11.23	112.3	384	96.00	1202	100.17
2	10.24	102.36	399	99.75	1125	93.75
3	9.63	96.32	374	93.50	1136	94.67
4	9.32	93.21	386	96.50	1189	99.08
5	9.75	97.45	401	100.25	1187	98.92
6	8.52	85.21	422	105.50	1120	93.33
N	6	6	6	6	6	6
Mean	9.78	97.81	394.33	98.58	1159.83	96.65
SD (±)	0.91		16.86		36.70	
CV (%)	9.29		4.28		3.16	

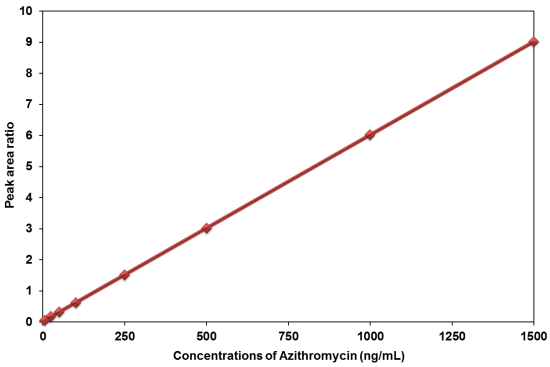

Figure 4. Standard Calibration curve of azithromycin in human plasma.

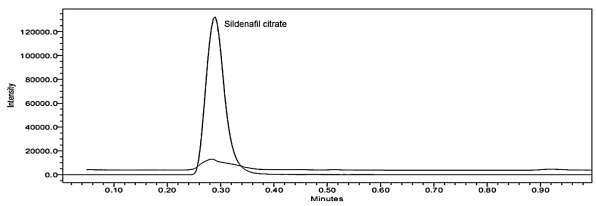

Figure 5. Mass spectrum of blank plasma compared to that containing only IS.

3.2.4. Accuracy and precision

3.2.4.1. Intra-day accuracy and precision

The intra-day accuracy and precision evaluations were assessed by repeated analysis of human plasma samples containing different concentrations of Azithromycin on separate occasions, same day. A single run consisted of a calibration curve plus 6 runs of low (QCL), medium (QCM) and high (QCH) quality control samples, the intra-day coefficients of variation ranged between 9.29, 4.28 and 3.16%. The intra-day percentages of nominal concentration ranged between 97.81, 98.58 and 96.65%. Results are presented in Table 3.

3.2.4.2. Inter-day accuracy and precision

Inter-day accuracy and precision evaluations were performed by analyzing three sets of QCL, QCM and QCH quality control samples of Azithromycin in human plasma. One set was extracted and analyzed on one day (Day I). The other two sets were kept frozen, thawed, extracted and analysed on a next day (Day II) for the second set, and third day (Day III) for the last set.

The inter-day coefficients of variation ranged between 14.50, 4.70 and 3.40%.

The inter-day percentages of nominal concentration ranged between 90.90, 97.30 and 98.90%. Results are presented in Table 4.

3.2.5. Extraction efficiency

Recovery of Azithromycin was evaluated by comparing mean analyte responses of two processed samples of low (QCL), medium (QCM) and high (QCH) quality control samples to mean analyte responses of the same concentrations with spiked samples in previously extracted blank plasma. Mean recovery values are 89.08, 97.02 and 95.03% at low, medium and high quality control levels, respectively. Results are presented in Table 5.

3.2.6. Dilution integrity

Three replicates of the high quality control (QCH) were diluted five times in human plasma prior to sample processing and analysis. The calculated concentrations, including the dilution factor, yielded coefficients of variation of 4.48% for QCH. Percentages of nominal concentration are 100.67% Results are presented in Table 6.

3.2.7. Stability studies

3.2.7.1 Post-preparative stability at 10 °C

Samples prepared at low (QCL), medium (QCM) and high (QCH) quality control levels were aliquoted and submitted to the sample processing procedure and kept at 10 °C (stability samples).

Table 4. Inter-day accuracy and precision.

Day	Run number	QCL 10 ng/mL		QCM 400 ng/mL		QCH 1200 ng/mL	
		Conc. found (ng/mL)	% Nominal conc.	Conc. found (ng/mL)	% Nominal conc.	Conc. found (ng/mL)	% Nominal conc.
I	1	8.3	83.2	385.0	96.3	1203.0	100.3
	2	8.4	84.0	386.0	96.5	1205.0	100.4
	3	9.0	90.0	397.0	99.3	1220.0	101.7
	4	10.0	100.0	375.0	93.8	1159.0	96.6
	5	10.8	108.0	402.0	100.5	1156.0	96.3
	6	11.3	113.0	406.0	101.5	1148.0	95.7
II	1	10.4	103.6	410.0	102.5	1149.0	95.8
	2	10.0	100.0	412.0	103.0	1157.0	96.4
	3	11.2	112.0	416.0	104.0	1169.0	97.4
	4	9.0	90.0	384.0	96.0	1158.0	96.5
	5	7.0	70.0	365.0	91.3	1126.0	93.8
	6	8.0	80.0	372.0	93.0	1136.0	94.7
III	1	9.9	98.5	379.0	94.8	1187.0	98.9
	2	8.5	85.0	377.0	94.3	1214.0	101.2
	3	7.6	76.0	388.0	97.0	1256.0	104.7
	4	7.4	74.0	397.0	99.3	1236.0	103.0
	5	7.9	79.0	346.0	86.5	1245.0	103.8
	6	8.9	89.0	406.0	101.5	1233.0	102.8
N		6.0	6.0	6.0	6.0	6.0	6.0
Mean		9.1	90.9	389.1	97.3	1186.5	98.9
SD (±)		1.3		18.4		40.7	
CV (%)		14.5		4.7		3.4	

Table 5. Recovery of azithromycin.

Analyte samples	Theoretical true spiked in matrix analyte response			Extracted sample analyte response		
	Analyte 1	Analyte 2	Analyte 3	Analyte 1	Analyte 2	Analyte 3
Sample 1	18562	987541	2854126	16598	945876	2854712
Sample 2	17451	965147	2963514	14856	952147	2685471
Sample 3	21365	996321	2745214	19657	963214	2596874
N	3	3	3	3	3	3
Mean	19126.00	983003	2854284.667	17037.00	953745.667	2712352.33
SD (±)	2017.03	16074.81421	109150.0865	2430.42	8778.85883	131004.058
CV (%)	10.55	1.64	3.82	14.27	0.92	4.83
Concentration (ng/mL)	10	400	1200	10	400	1200
Mean recovery (%)				89.08	97.02	95.03

Table 6. Results of dilution integrity.

Analyte samples	Conc. vound (ng/mL)	% Nominal concentration
Sample 1	1156.00	96.33
Sample 2	1204.00	100.33
Sample 3	1264.00	105.33
N	3	3
Mean	1208.00	100.67
SD (±)	54.11	
CV (%)	4.48	

Three replicates of those quality control samples were freshly processed with a calibration curve and analyzed in a single run to serve as time zero (comparison samples). After 100 hours, a calibration curve was freshly processed and analyzed with all stability samples in a single run. Concentrations were calculated to determine % change after 100 hours when compared to time zero. Azithromycin is found to be stable for 100 hours at 10 °C following sample processing with % changes of -9.71, -7.40 and -2.68%. Results are presented in Table 7.

3.2.7.2. Short-term stability of azithromycin in matrix at room temperature

Samples were prepared at low (QCL), medium (QCM) and high (QCH) quality control levels. Three replicates of low, medium and high quality control samples were left at room (stability samples). Three replicates of those quality controls samples were freshly processed with a calibration curve and, analyzed in a single run to serve as time zero (comparison samples). After 24 hours, a calibration curve was freshly processed and analyzed with all stability samples in a single run. Concentrations were calculated to determine % change over the time when compared to time zero. Azithromycin is found to be stable in human plasma for 24 hours at room temperature with % changes of -9.34, -9.03 and -1.77%. Results are presented in Table 7.

3.2.7.3. Freeze and thaw stability at -20 °C

Samples were prepared at low (QCL), medium (QCM) and high (QCH) quality control levels, aliquoted and frozen at -20 °C. Some of the aliquots of quality control samples were subjected to three freeze-thaw cycles (stability samples). A calibration curve and quality control samples were freshly prepared and processed with 3 replicates of stability samples and analysed in a single run. Azithromycin is found to be stable in human plasma after three freeze-thaw cycles at, -20 °C with coefficients of variation of 7.78, 1.71 and 1.48% and nominal concentrations of 103.66, 95.75 and 95.61% for QCL, QCM and QCH, respectively. Results are presented in Table 7.

3.2.7.4. Long-term stability of azithromycin in matrix at -20 °C

Stability samples were prepared in human plasma at low (QCL), medium (QCM) and high (QCH) quality control levels and stored at -20 °C (stability samples).

Table 7. Summary of validation results.

Parameters	Value
Linearity	$r \geq 0.999$
Calibration curve range	5-1500 ng/mL
Inter-day accuracy	QC % nominal conc., 90.90, 97.30 and 98.90%
Inter-day precision	QC coefficients of variation, 14.50, 4.70 and 3.40%
Intra-day accuracy	QC % nominal conc., 97.81, 98.58 and 96.65%
Intra-day precision	QC coefficients of variation, 9.29, 4.28 and 3.16%
Recovery of analyte	QC means, 89.08, 97.02 and 95.03%
Lower limit of quantification	5 ng/mL
Dilution integrity accuracy	QC % nominal. cone., 100.67%
Dilution integrity precision	QC coefficient of variation, 4.48%
Post-preparative stability at 10 °C	Mean % change, -9.71, -7.40 and -2.68%
Short-term stability of analyte in matrix at -20 °C	Mean % change after 24 hours, -9.34, -9.03 and -1.77%
Long-term stability of analyte in matrix at -20 °C	Mean % change after 52 days, -11.12, -7.09 and -3.05%
Freeze and thaw stability of analyte in matrix at -20 °C	QC % nominal conc., 103.66, 95.75 and 95.61%

A calibration curve and 3 replicates of low and high quality control samples (comparison samples) were freshly processed with three replicates of stability samples and analysed in a single run. Concentrations were calculated to determine % change over time. Azithromycin is found to be stable in human plasma for 52 days at -20 °C with % changes of -11.12, -7.09 and -3.05%. Results are presented in Table 7.

4. Conclusion

The developed LC-MS/MS assay for azithromycin is rapid, selective, and suitable for routine measurement of subject samples. This study reports a high throughput liquid-liquid extraction method for extraction of azithromycin in human plasma using LC-MS/MS. The developed method provided a short run time of about 0.5 minutes that can afford the analysis of more than 400 plasma samples per day. The present method also provided excellent specificity and linearity with an LLOQ of 5 ng/mL for azithromycin.

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