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Analysis of Amoxicillin and Clavulanic Acid by UPLC-MS/MS in Human Plasma for Pharmacokinetic Application

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ABSTRACT

A bioanalytical method for the simultaneous quantification of amoxicillin (AMX) and clavulanic acid (CLV) in human plasma using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) has been successfully developed and validated. The analytes and amoxicillin-d4 as internal standard were extracted from 100 µL plasma by solid phase extraction using Phenomenex Strata-X cartridges. Chromatographic separation was done on UPLC BEH C18 (50 mm × 2.1 mm, 1.7 µm) column using acetonitrile: 2.0 mM ammonium formate in water (85:15, v/v) as the mobile phase at a flow rate of 0.400 mL/min under isocratic condition. Mass spectrometric detection was by multiple reaction monitoring with an electrospray ionization source in the negative ionization mode. The response of the method was linear in the analytical range of 10.0-10000 ($r2 \geq 0.9997$) and 2.5-2500 ng/mL ($r2 \geq 0.9993$) for AMX and CLV respectively. Intra- and inter batch accuracy and precision (% CV) were in the range of 96.1-103.2 % and 1.48-5.88 respectively for both the analytes. The mean extraction recovery was 99.6 and 98.4 % for AMX and CLV respectively. Effect of matrix due to endogenous components on the quantitation and the stability of the analytes under different conditions were extensively studied. Dilution reliability and method ruggedness was also evaluated. The method as successfully applied to a bioequivalence study in 12 healthy subjects using 250 mg amoxicillin + 125 mg clavulanic acid fixed dose combination. The assay reproducibility was successfully demonstrated by reanalysis of 63 subject samples.

Keywords: Amoxicillin; clavulanic acid; UPLC-MS/MS; human plasma; solid phase extraction; bioequivalence

INTRODUCTION

Amoxicillin (AMX), a semi-synthetic, acid-stable and an orally absorbed drug. It belongs to a class of antibiotics called the aminopenicillin (β-lactam antibiotic). It is also known to be a 4-hydroxy analog of ampicillin. AMX is extremely effective against various infections caused by wide range of Gram-positive and Gram-negative microorganisms. AMX is prone to degradation by β-lactamase-producing bacteria, which are resistant to β-lactam antibiotics, such as penicillin [1, 2]. Because of this reason, it is often combined with β-lactamase inhibitor, clavulanic acid (CLV) to improve the antibacterial effect and to overcome bacterial resistance. It is a mild antibacterial agent. The name of this resulting drug combination is 'Co-amoxiclav' which reduces susceptibility to β-lactamase resistance, in which the major component is AMX [1]. This potent combination was introduced for the first time in Europe and United States in the year 1981 and 1984 respectively. It is generally used for the treatment of a wide range of bacterial infections, including upper and lower respiratory tract infections and infections of the skin and soft tissue structures [3].

When CLV is co-administered with AMX, there is no any substantial variation of the pharmacokinetics of either drug compared with their separate administration. AMX and CLV are well absorbed by the oral route of administration with peak serum levels appearing within 1-2 h. Protein binding of AMX is very less (17 %) and it is excreted primarily unchanged in the urine with a half-life of about 1 h [3]. Likewise, CLV is extensively metabolized in the liver with a similar half-life and is also slightly protein bound (20-30 %) [4].

Several chromatographic methods, including LC-UV, micellar LC and LC-MS/MS, have been developed for the analysis of AMX as a single analyte [5-7], in presence of its major active metabolites [8] and with other antibiotic drug in binary [9] and ternary combination [10] in different biological matrices. Similarly, number of HPLC-UV [11-14] and LC-ESI-MS/MS [15, 16] methods have been reported for the determination of CLV in various biological fluids. At the same time simultaneous analysis of AMX and CLV is also a subject of several reports [17-26]. Amongst these, three methods have employed HPLC-UV [17-19], while the rest are based on LC-MS/MS technique [20-26]. The simultaneous analysis of AMX and CLV has been done in various biological fluids including human plasma [17, 18, 20-25], human blood [19], and dog plasma [26]. However, several of these methods were either less sensitive [17, 18, 20, 21, 23], had long analysis time [17, 18] or involved large human plasma volume for processing [18, 25]. Additionally, few other methods based on UPLC-MS/MS are presented for the estimation of AMX and CLV along with several other antibiotics [27-29]. A comparative assessment of different methods developed for AMX and CLV in human plasma are presented in Table 1.

Based on the existing literature, the main objective of this work was to develop and validate a simple, reliable and rapid method with adequate sensitivity for the simultaneous estimation of AMX and CLV in human plasma by UPLC-MS/MS. The method presents an efficient extraction procedure based on solid phase extraction (SPE) with > 96 % extraction recovery for both the analytes. Systematic evaluation of matrix effect was performed by estimating the IS-normalized matrix factors, relative matrix effect in different plasma sources and through post-column analyte infusion. The proposed method was successfully applied to support a pharmacokinetic study of fixed dose combination of AMX and CLV (250 + 125 mg) tablet formulation in 12 healthy Indian subjects under fasting. The reproducibility in the measurement of study data has been demonstrated by reanalysis of selected incurred samples.

EXPERIMENTAL

Chemicals and materials

Reference standard of amoxicillin (AMX, 99.52 %), clavulanic acid (CLV, 99.67 %) and amoxicillin-d4 (AMX-d4, 99.36 %) were procured from Clearsynth Labs Pvt. Ltd. (Mumbai, India). HPLC grade methanol and acetonitrile were obtained from Mallinckrodt Baker, S.A.de C.V. (Estado de Mexico, Mexico). BioUltra grade ammonium formate and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phenomenex Strata-X (30 mg, 1 cc) solid phase extraction cartridges were obtained from Merck Specialties Pvt. Ltd. (Mumbai, India). Deionized water was obtained from Milli-Q water purification system from Millipore (Bangalore, India). Blank human plasma was procured from Supratech Micropath (Ahmedabad, India) and was stored at –70 °C until use.

Table 1 Comparison of salient features of chromatographic methods developed for simultaneous determination of amoxicillin and clavulanic acid in human plasma

Sr. No.	Technique; linear range (ng/mL)	Extraction procedure; sample volume	Column; mobile phase; flow rate (mL/min); run time (min); retention time (AMX/CLV) in min	Application; matrix effect study; incurred sample reanalysis	Ref.
1.	HPLC-UV; AMX: 625-20000 CLV- 312.5-10000	PP; 100 µL	Lichrospher 100 RP8; acetonitrile-phosphate solution-tetramethyl ammonium chloride solution; 1.0; 10.0; 5.48/9.51	Pharmacokinetics study in 3 healthy volunteers with 500 mg AMX and 125 mg CLV; --; --	[17]
2.	HPLC-UV; AMX: 200-12000 CLV: 100-6000	LLE; 500 µL	Chromolith Performance (RP-18e); 0.02 M disodium hydrogen phosphate buffer-methanol (4:96, *v/v*); 1.3; 10.0; 3.8/5.2	Pharmacokinetics study in 12 healthy volunteers with 500 mg AMX and 125 mg CLV; --; --	[18]
3.	HPLC-ESI-MS; AMX: 125-8000 CLV: 62.5-4000	PP; 200 µL	Zorbax C8; formic acid-water-acetonitrile (2: 1000: 100, *v/v/v*); 0.4; 3.5; 2.80/2.05	Pharmacokinetic study in 30 healthy volunteers with 250 mg AMX and 125 mg CLV; --; --	[20]
4.	LC-MS/MS; AMX: 500-40000 CLV: 100-6000	LLE; 200 µL	Symmetry C18; acetonitrile -0.1% formic acid (90:10, *v/v*); 0.5; 3.0; 0.77/0.86	--; --; --	[21]
5	UPLC-MS; AMX: 10-40000 CLV: 10-10000	PP; 200 µL	Waters Acquity UPLC BEH C18; acetonitrile:water (95: 5, *v/v*); 0.5; 5.0; 0.92/2.83	Pharmacokinetic study in 24 healthy volunteers with 875 mg AMX and 125 mg CLV; --; --	[22]
6.	LC-MS/MS; AMX: 103-6822 CLV: 46-3026	SPE; 250 µL	Zorbax SB C18; acetonitrile: 2.0 mM ammonium acetate (70: 30, *v/v*); 0.5; 2.0; 0.86/0.76	--; --; --	[23]
7.	LC-MS/MS; AMX: 5-16000 CLV: 50-2000	LLE; 100 µL	Shim pack XR-ODS; water with 0.2 % formic acid and acetonitrile with 0.2 % formic acid; 0.40; 5.0; 1.22/3.25	Pharmacokinetic study in 12 healthy volunteers with 1000/500/250 mg AMX and 125/62.5/31.25 mg CLV; --; --	[24]
8.	LC-MS/MS; AMX: 50.43-31500.68	SPE; 950 µl	HyPURITY advanced C18; acetonitrile -2mM ammonium acetate (80: 20, *v/v*); 0.8; 1.5; 0.61/0.57	Bioequivalence study in 24 healthy volunteers with 875 mg AMX and 125 mg CLV;	[25]

	CLV: 25.28-6185.18			--; --	
9.	UPLC-MS/MS; AMO: 10-10000 CLV: 2.5-2500	SPE; 100 µl	Waters Acquity UPLC BEH C18; 2mM ammonium formate in water: acetonitrile (15:85, *v/v*); 0.350; 2.0; 1.57/1.13	Bioequivalence study in 12 healthy volunteers with 250 mg AMX and 125 mg CLV; Yes; Yes (% change within ± 12.0)	PS

AMX: amoxicillin, CLV: clavulanic acid; PP: protein precipitation; LLE: liquid-liquid extraction; SPE: solid phase extraction; PS: present study.

Liquid chromatographic and mass spectrometric conditions

Waters Acquity UPLC BEH C18 (50 mm × 2.1 mm, 1.7 mm) column maintained at 30 °C was used for chromatographic separation of the analytes. Elution of analytes and IS was carried out using a mobile phase consisting of acetonitrile: 2.0 mM ammonium formate in water (85:15, *v/v*), delivered at a flow rate of 0.400 mL/min. The pressure of the system was maintained at 6600 psi. Quantitative determination was performed on Waters Quattro Premier XE (USA) triple quadrupole mass spectrometer equipped with electro-spray ionization (ESI) in the negative ionization mode.

The source dependent parameters maintained for AMX, CLV and IS were, cone gas flow: 110 ± 10 L/h; desolvation gas flow: 800 L/h; capillary voltage: 3.0 kV, source temperature: 100 °C; desolvation temperature: 400 °C; extractor volts: 5.0 V. The pressure of argon used as collision activation dissociation gas was 0.124 Pa. The optimum values for compound dependent parameters like cone voltage and collision energy were set at -26 V and -15 eV for AMX, -32 and -10 eV for CLV, -28 V and -15eV for AMX-d4 respectively. Quadrupole 1 and 3 were maintained at unit mass resolution and the dwell time was set at 100 ms. MassLynx software version 4.1 was used to control all parameters of UPLC and MS.

Calibration standards and quality control samples

The standard stock solutions of AMX (1000 µg/mL) and CLV (500 µg/mL) were prepared by dissolving their requisite amounts in methanol. Further, working solutions were prepared using intermediate solutions of 250.0 µg/mL & 10.0 µg/mL for AMX and 100.0 & 20 µg/mL for CLV in methanol: water (50:50, *v/v*) respectively. Calibration curve standards (CSs) were made at the following concentrations 10.0, 20.0, 50.0, 100.0, 200.0, 500.0, 1000, 2000, 5000, 10000 ng/mL and 2.50, 5.00, 10.0, 25.0, 50.0, 100.0, 250.0, 500.0, 1250, 2500 ng/mL for AMX and CLV respectively. The quality control (QC) samples were prepared at five levels, viz. 8500/2000 ng/mL (HQC, high quality control), 4500/750.0 and 750.0/200.0 ng/mL (MQC-1/2, medium quality control), 30.0/7.50 ng/mL (LQC, low quality control) and 10.0/2.50 ng/mL (LLOQ QC, lower limit of quantification quality control) for AMX/CLV respectively.

Separate stock solutions of the internal standard (100.0 µg/mL for AMX-d4) was prepared by dissolving accurately known amounts of IS in methanol. The working solution was prepared from its stock solutions in methanol: water (50:50, *v/v*) at 5000 ng/mL concentration. Standard stock and working solutions used for spiking were stored at 5 °C, while CSs and QC samples in plasma were kept at -70 °C until use.

Sample extraction protocol

To an aliquot of 100 µL plasma sample, 25 µL of internal standard was added and vortexed for 10s. Further, 100 µL of 1 % (*v/v*) formic acid in water was added and vortex mixed for another 30s. The samples were centrifuged at 14000 × g for 5 min at 10 °C and loaded on Phenomenex Strata-X (30 mg, 1 cc) cartridge, which was pre-conditioned with 1.0 mL methanol followed by 1.0 mL 1 % (*v/v*) formic acid in water. The samples were washed with 1.0 mL 1 % (*v/v*) formic acid in water followed by 1.0 mL of water. Thereafter, the cartridges were dried for 1 min under nitrogen (1.72 x 10^5 Pa) at 2.4 L/min flow rate. Both the analytes and IS were eluted using 1.0 mL of mobile phase into pre-labeled vials, followed by evaporation to dryness. The dried residue was reconstituted with 250 µL of mobile phase, briefly vortexed for 15 s and 5 µL was used for injection in the chromatographic system using an autosampler.

Validation procedures

The method was validated as per the USFDA guidelines to establish the accuracy and precision of the method [30]. The details of the parameters studied were similar to our previous report [31].

System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture of analytes and IS at the start of each batch during method validation. System performance was studied by injecting one extracted blank (without analytes and IS) and one extracted LLOQ sample with IS at the beginning of each analytical batch and before re-injecting any sample during method validation. The carryover effect of the auto-sampler was evaluated by sequentially injecting extracted blank plasma → ULOQ sample → extracted blank plasma → LLOQ sample→ extracted blank plasma at the start and end of each batch.

Selectivity of the method was assessed for potential matrix interferences in eight batches of blank human plasma by extraction and inspection of the resulting chromatograms for interfering peaks. The batches comprised of 5 normal lots of K_3EDTA, 1 heparinized, 1 haemolysed and 1 lipemic plasma. Interference of commonly used medications by human volunteers was also checked. This included paracetamol, chlorpheniramine maleate, diclofenac, caffeine, acetylsalicylic acid and ibuprofen. Their stock solutions (100 µg/mL) were prepared by dissolving requisite amount in methanol:water (50:50, *v/v*). Further, working solutions were prepared in the mobile phase and 5 µL was injected to check for any possible interference at the retention time of analytes and IS.

For linearity of the method, five calibration curves were plotted covering the range of 10.0-10000 ng/mL for AMX and 2.5-2500 ng/mL for CLV using least square regression and $1/x^2$ as a weighting factor. The area response ratio for analyte/IS obtained from multiple reaction monitoring was used for regression analysis. The acceptance criterion for a calibration curve was a correlation coefficient (r^2) ≥ 0.99 and the lowest standard on the calibration curve was accepted as the assay sensitivity expressed as LLOQ.

Intra-batch accuracy and precision was determined by analyzing six replicates of QC samples along with calibration

curve standards on the same day, while the inter-batch accuracy and precision were assessed by analyzing five precision and accuracy batches on three consecutive days. The precision (% CV) at each concentration level from the nominal concentration was expected to be not greater than 15 % and the accuracy to be within ±15 % as per USFDA guidelines [30], except for the LLOQ where it can be 80-120 % of the nominal concentration. Reinjection reproducibility was also checked by re-injecting one entire validation batch.

Ion suppression/enhancement effect was studied through post column analyte infusion experiment. Briefly, standard solutions containing the analytes (at ULOQ level) and IS were infused post column via a 'T' connector into the mobile phase. Aliquots of 10 µL of extracted control (blank) plasma were then injected into the column and MRM chromatograms were acquired for analytes and IS to check any possible interference due to endogenous and exogenous plasma components.

The extraction recovery for the analytes and IS was calculated by comparing the mean area response of samples (n=6) spiked before extraction to that of extracts with post-spiked samples (spiked after extraction) at four QC levels. Matrix effect, expressed as matrix factors (MFs) was assessed by comparing the mean area response of post-spiked samples with samples prepared in mobile phase. IS-normalized MFs (analyte/IS) were calculated to access the variability of the assay due to matrix effects. Relative matrix effect was assessed from the precision (% CV) values of the slopes of the calibration curves prepared from eight plasma lots, which included haemolysed and lipemic plasma. To prove the absence of matrix interference, % CV should not be greater than 3-4% [32].

Stability tests were conducted for stock solutions of analytes and IS for short term and long term stability at 25 °C and 5 °C respectively. All stability results for spiked plasma samples were evaluated by measuring the area response ratio (analyte/IS) of stability samples against freshly prepared comparison standards. QC samples at HQC and LQC levels were prepared to check for bench top, wet extract (autosampler), processed sample, freeze-thaw (-20 °C and -70 °C) and long term (-20 °C and -70 °C) stabilities of anaytes. The acceptance criterion was ±10.0 % deviation (from the nominal value) for stock solutions and ±15.0 % deviation for all other storage conditions.

To verify the ruggedness of the proposed method, it was done on two precision and accuracy batches. The first batch was analyzed on two different columns (same make but different batch no.), while the second batch was analyzed by two different analyst who were not part of method validation. Dilution integrity experiment was conducted by diluting the stock solution prepared as spiked standard at 2 times ULOQ concentration (20000 ng/mL) for AMX and (5000 ng/mL) for CLV with monitored blank human plasma. The precision and accuracy for dilution integrity standards at 1/5th and 1/10th dilutions for AMX and CLV were determined by analyzing the samples against freshly prepared calibration curve standards.

Application of the method

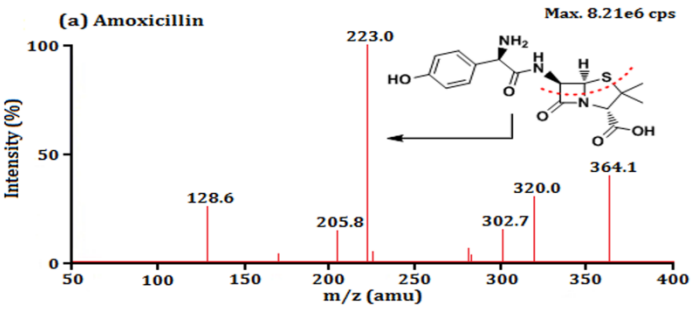
The bioequivalence study was conducted with a single fixed dose of a test (250 mg amoxicillin + 125 mg clavulanic acid tablets from a Generic Company) and a reference (AUGMENTIN®, 250 mg amoxicillin + 125 mg clavulanic acid as potassium salt tablets from GlaxoSmithKline Research Triangle Park, NC 27709, USA) formulation to 12 healthy Indian subjects under fasting conditions. The study was conducted as per International Conference on Harmonization and USFDA guidelines [33]. The subjects were informed about the objectives and possible risks involved in the study and a written consent was obtained. The subjects were orally administered a single dose of test and reference formulations with 240 mL of water. Blood samples were collected at 0.00 (pre-dose), 0.16, 0.33, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 2.75, 3.00, 3.25, 3.50, 4.00, 5.00, 6.00, 8.00, 10.0, 12.0, 14.0, 16.0, 20.0 and 24.0 h after oral administration of the dose for test and reference formulation in labeled K_3EDTA-vacuettes. For the purpose of bioequivalence analysis, Cmax, $AUC_{0\text{-}24h}$ and $AUC_{0\text{-}inf}$ were considered as primary variables. The pharmacokinetic parameters of AMX and CLV were estimated by non-compartmental analysis using WinNonlin® software version 5.3 (Pharsight Corporation, Sunnyvale, CA, USA).

An incurred sample re-analysis (ISR, assay reproducibility test) was also conducted by random selection of subject samples. The selection criteria included samples which were near the C_{max} and the elimination phase in the pharmacokinetic profile of the drug. The results obtained were compared with the data obtained earlier for the same sample using the same procedure. The percent change in the values should not be more than ±20 % [34].

RESULTS AND DISCUSSION

Method development

Reported methods for the analysis of AMX and CLV have optimized mass parameters in the positive [20] as well as negative ionization [24] modes as AMX possesses a carboxylic acid and a primary amino group, while CLV has carboxylic acid functionality. Thus, in the present work mass parameters were tuned using ESI in positive as well as negative ionization modes for optimum and consistent response for both the analytes. It was found that AMX gave comparable response in both the ionization modes, while CLV showed much higher response in the negative mode. Thus, to avoid polarity switch both the analytes were analyzed in the negative ionization mode. The Q1 MS full scan mass spectra contained deprotonated precursor $[M\text{-}H]^-$ ions at *m/z* 364.1, 197.5 and 368.0 for AMX, CLV and IS respectively. The most abundant and consistent product ions in Q3 MS spectra were observed at *m/z* 223.0, 135.9 and 226.9 for the analytes and IS respectively as shown in Figure 1a-c. The product ion fragment at *m/z* 223.0 and 226.9 for AMX and AMX-d4 corresponded to the possible removal of formic acid and iso-butyl formamide, while the fragment at *m/z* 135.9 for CLV was formed due to elimination of water and carboxylic acid group from the precursor ion. The source dependent and compound dependent parameters were suitably optimized to obtain a consistent and sufficient response for both the analytes. A dwell time of 100 ms gave was adequate date points for the quantitation of the analytes and IS.



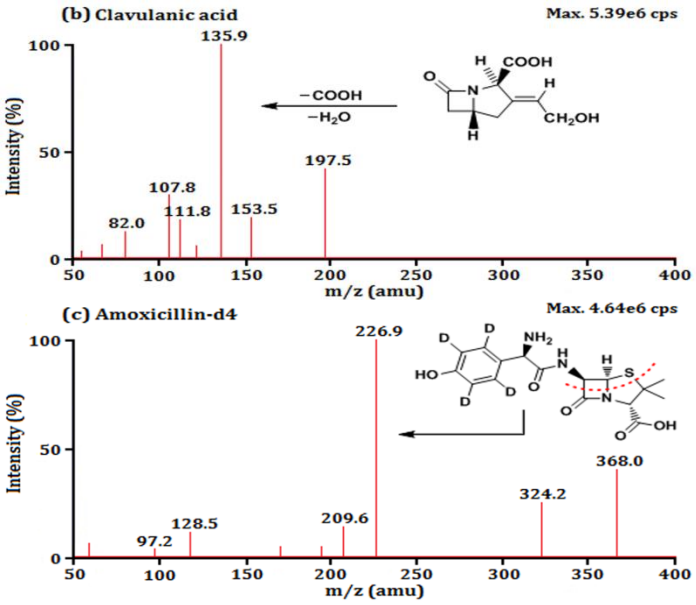


Figure 1 Product ion mass spectra of (a) amoxicillin (*m/z* 364.1 → 223.0) (b) clavulanic acid (*m/z* 197.5 → 135.9) (c) amoxicillin-d4, IS (*m/z* 368.0 → 226.9) in the negative ionization mode, scan range 50-400 amu.

The analytes were chromatographically separated on Waters Acquity UPLC BEH C18 (50 mm × 2.1 mm, 1.7 µm) analytical column under isocratic conditions to obtain adequate response and acceptable peak shape. Several existing methods have used acetonitrile together with either formic acid or ammonium acetate as an additive for mobile phase selection. Acetonitrile has been found to be more compatible than methanol with ESI and gives better ionization efficiencies [20]. Thus different combinations of acetonitrile and acidic buffers (ammonium formate/formic acid, ammonium acetate/acetic acid) having different ionic strengths (1-10 mM) in the pH range of 3.0-6.5 were tested. Although acceptable chromatography in terms of baseline separation was observed in all the systems, the peaks shapes and response was much superior in acetonitrile and ammonium formate solvent system. Further optimization was carried out to finalize their composition by changing the ratio (organic: aqueous) from 50:50 to 90:10 (*v/v*). After several trials, acetonitrile-2.0 mM ammonium formate in water (85: 15, *v/v*), delivered at a flow rate of 0.400 mL/min was optimized to give sharp peak shapes, adequate retention and response. AMX-d4 used as an internal standard worked well in maintaining the ionization efficiency of the analytes and for overall performance of the method. AMX and CLV were baseline resolved within 2.0 min with a retention time was 1.57 and 1.13 min respectively and a resolution factor of 2.09. The reinjection reproducibility (% CV) of retention times for the analytes was ≤ 0.97 % for 60 injections on the same column. The capacity factors, which describe the rate at which the analyte migrates through the column, were 2.83 and 1.41 for AMX and CLV respectively. Representative MRM ion chromatograms in Figure 2 of (a) blank plasma spiked with IS, (b) AMX and CLV at LLOQ and (c) a real subject sample at C_{max} demonstrates the selectivity of the method to differentiate and quantify the analytes from endogenous components in the plasma matrix or other components in the sample. Further, there was no interference at the retention times of analytes and IS.

All three conventional extraction techniques, protein precipitation (PP) [17, 20, 22], liquid-liquid extraction (LLE) [18, 21, 24] and SPE [23, 25] have been employed for the simultaneous analysis of AMX and CLV from human plasma. Initial trials conducted using different protein precipitants like acetonitrile, methanol and trichloroacetic acid resulted in poor and inconsistent recoveries, especially for CLV (30 to 40 %). Further LLE with common organic diluents, namely dichloromethane, methyl *tert*-butyl ether and *n*-hexane provided somewhat improved recovery compared to PP but matrix interference was also observed. Thus SPE trials were carried out on Phenomenex Strata-X (30 mg, 1 cc) cartridge, which resulted in highly consistent recovery for both the analytes. However, it was found that addition of 100 µL, 1 % (*v/v*) formic acid prior to sample loading on SPE markedly improved the recovery at LLOQ and LQC levels for both the analytes. To establish a method with very low quantitation levels (10.0 ng/mL for AMX and 2.50 ng/mL for CLV), a concentration step was required after elution of samples with 1.0 mL of the mobile phase solution. The extracts obtained were clear with no matrix interference and the recovery was consistent at all QC levels.

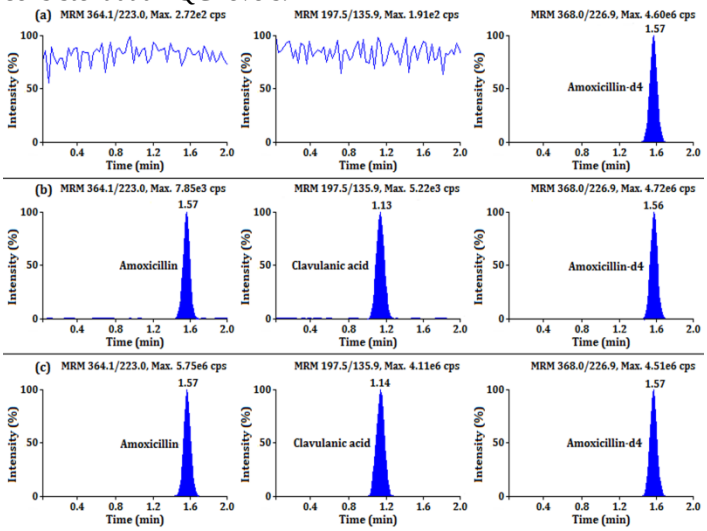


Figure 2 MRM ion-chromatograms of (a) blank plasma with amoxicillin-d4, IS (b) amoxicillin (*m/z* 364.1 → 223.0) and clavulanic acid (*m/z* 197.5 → 135.9) at LLOQ and IS (*m/z* 368.0 → 226.9) (c) amoxicillin and clavulanic acid in subject sample at C_{max} after oral administration of 250 mg amoxicillin + 125 mg clavulanic acid fixed dose formulation.

Assay results

System suitability, system performance and auto-sampler carryover

The precision (% CV) for system suitability test was observed in the range of 0.37 to 0.84 % for the retention time and 0.65 to 1.45 % for the area response of the analytes and IS. The signal to noise ratio for system performance was ≥ 15 for both the analytes. Carry-over evaluation was performed in each analytical run so as to ensure that it does not affect the accuracy and the precision of the proposed method. There was practically negligible carry-over (≤ 0.59 %) in extracted double blank plasma (without analyte and IS) after subsequent injection of highest calibration standard (aqueous and extracted) at the retention time of analytes and IS.

Linearity, lower limit of quantification and accuracy & precision

The five calibration curves were linear over the concentration range of 10.0-10000 and 2.5-2500 ng/mL for AMX and CLV respectively, with a correlation coefficient (r^2) ≥ 0.9993 for both the analytes. The mean linear equations obtained were *y* =

(0.000218 ± 0.000009) x – (0.000064 ± 0.000012) and y = (0.001289 ± 0.000027) x + (0.000048 ± 0.000011) for AMX and CLV respectively. The accuracy and precision (% CV) observed for the calibration curve standards ranged from 98.2 to 101.8 % and 1.18 to 2.98 % for AMX and 97.1 to 103.0 % and 1.12 to 2.95 % for CLV respectively. The lowest concentration (LLOQ) in the standard curve that can be measured with acceptable accuracy and precision was 10.0 and 2.5 ng/mL for AMX and CLV respectively in plasma at a signal-to-noise ratio (S/N) of ≥ 15. The LOD values were 2.56 and 0.85 ng/mL for AMX and CLV respectively at S/N ≥ 5. The intra-batch and inter-batch precision and accuracy were established from validation runs performed at five QC levels and the results are presented in Table 2.

Table 2 Intra-batch and inter-batch precision and accuracy for amoxicillin and clavulanic acid

Nominal conc. (ng/mL)	Intra-batch (n = 6; single batch)			Inter-batch (n = 30; 6 from each batch)		
	Mean conc. found (ng/mL)	% CV	Accuracy %	Mean conc. found (ng/mL)	% CV	Accuracy %
Amoxicillin						
8500	8312	2.26	97.8	8670	2.66	102.0
4500	4644	3.05	103.2	4322	3.52	96.1
750.0	767.3	3.97	102.3	739.4	2.79	98.6
30.0	29.65	4.29	98.8	30.33	5.26	101.9
10.0	10.11	5.10	101.1	9.941	4.39	99.4
Clavulanic acid						
2000	2045	1.48	102.3	1966	2.26	98.3
750.0	729.8	3.03	97.3	725.4	2.87	96.7
200.0	202.1	2.94	101.0	198.9	2.80	99.4
7.50	7.37	4.59	98.3	7.718	3.30	102.9
2.50	2.49	5.15	99.6	2.546	5.88	101.8

CV: Coefficient of variation; n: Number of replicates

Table 3 Extraction recovery and matrix effect for amoxicillin and clavulanic acid

QC level	Area response (replicate, n = 6)			Extraction recovery, % (B/A)		Matrix factor		
	A (post-extraction spiking)	B (pre-extraction spiking)	C (neat samples in mobile phase)	Analyte	IS	Analyte (A/C)	IS	IS-normalized
Amoxicillin								
LQC	2846	2759	2934	96.2	97.6	0.97	1.01	0.96
MQC-2	70069	70895	70482	101.2	102.4	0.99	0.97	1.02
MQC-1	420064	429814	427951	102.3	100.8	0.98	0.95	1.03
HQC	800594	790365	790169	98.7	99.8	1.01	1.02	0.99
Clavulanic acid								
LQC	1954	1905	1911	97.1	97.6	1.02	0.98	1.04
MQC-2	51315	52056	51833	101.4	102.4	0.99	1.01	0.98
MQC-1	194632	192369	198251	98.8	100.8	0.98	0.97	1.01
HQC	529654	510312	523964	96.3	95.1	1.01	0.98	1.03

CV: coefficient of variation; n: Number of replicates; LQC: low quality control; MQC: medium quality control; HQC: high quality control

Extraction recovery and matrix effect

The extraction recovery of analytes from SPE ranged from 96.2-102.3 for AMX and 96.3-101.4 % for CLV. The mean recovery of AMX-d4 was 100.2 %. The presence of endogenous or exogenous components in biological fluids can lead to ion suppression or enhancement in the measurement of analyte signal, giving rise to matrix effect. The chromatogram in **Figure 3a-c** show negligible ion suppression or enhancement at the retention time of analytes and IS. The absolute matrix effect, expressed as matrix factor (MF) was evaluated at four QC levels. The MFs were calculated from the peak area response for the analytes and their IS separately and their ratios were then used to find the IS-normalized MF, which ranged from 0.96-1.04 across four QC levels for both the analytes (Table 3). Further, relative matrix effect was assessed in eight different plasma sources. The precision (% CV) in the measurement of slope of calibration lines was 3.92 and 3.13 for AMX and CLV respectively as shown in Table 4.

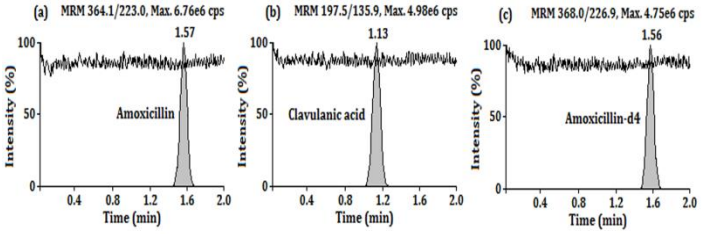


Figure 3 Post column analyte infusion MRM LC-MS/MS chromatograms for (a) amoxicillin, (b) clavulanic acid and (c) amoxicillin-d4.

Table 4 Relative matrix effect in eight different lots of human plasma for amoxicillin and clavulanic acid

Plasma lot	Slope of calibration curve	
	Amoxicillin	Clavulanic acid
Lot-1	0.000220	0.001250
Lot-2	0.000213	0.001308
Lot-3	0.000221	0.001255
Lot-4	0.000218	0.001311
Lot-5	0.000211	0.001320
Lot-6 (heparinized)	0.000221	0.001241
Lot-7 (haemolysed)	0.000222	0.001273
Lot-8 (lipemic)	0.000197	0.001355
Mean	0.000215	0.001289
±SD	0.000008	0.000040
%CV	3.92	3.13

SD: Standard deviation; CV: coefficient of variation

Stability results, method ruggedness and dilution reliability

Stock solutions kept for short-term and long-term stability as well as spiked plasma solutions showed no evidence of degradation under all studied conditions. No significant degradation of analytes was observed during sample storage and sample processing. The detailed results for stability studies are presented in Table 5.

The precision (% CV) and accuracy values for two different columns for method ruggedness ranged from 2.4 to 3.9 % and 95.2 to 104.4 % respectively across five QC levels. For the experiment with different analysts, the results for precision and accuracy were within 1.6 to 2.9 % and 97.3 to 102.4 % respectively at these levels. For dilution reliability experiment the precision and accuracy values for 1/5th and 1/10th dilution ranged from 1.2-2.5 % and 95.8-103.1 % for both the analytes respectively.

Table 5 Stability of amoxicillin and clavulanic acid in plasma under different conditions (n = 6)

Storage conditions	Amoxicillin			Clavulanic acid		
	Nominal conc. (ng/mL)	Mean stability sample (ng/mL)± SD	% Change	Nominal conc. (ng/mL)	Mean stability sample (ng/mL) ± SD	% Change
Bench top stability at 25 °C, 12 h	8500	8325 ± 104.9	-2.06	2000	1959 ± 50.19	-2.05
	30.00	31.30 ± 0.51	4.34	7.500	7.625 ± 0.195	1.67
Freeze & thaw stability at -20 °C	8500	8655 ± 60.7	1.82	2000	1925 ± 46.40	-3.75
	30.00	29.17 ± 0.41	-2.78	7.500	7.322 ± 0.235	-2.38
Freeze & thaw stability at -70 °C	8500	8879 ± 40.2	4.46	2000	2071 ± 49.98	3.55
	30.00	29.36 ± 0.28	-2.13	7.500	7.184 ± 0.158	-4.21
Processed sample stability at 25°C , 22 h	8500	8990 ± 126.3	5.76	2000	2030 ± 56.16	1.50
	30.00	30.59 ± 0.86	1.97	7.500	7.921 ± 0.261	5.61
Autosampler stability at 4°C, 72 h	8500	8123 ± 104.4	-4.44	2000	2042 ± 70.11	2.09
	30.00	30.98 ± 0.99	3.28	7.500	7.696 ± 0.192	2.61
Wet extract stability at 2-8°C, 48 h	8500	8088 ± 102.2	-4.85	2000	1908 ± 36.47	-4.60
	30.00	29.75 ± 0.783	-0.82	7.500	7.315 ± 0.125	-2.46
Long term stability at -20 °C, 165 days	8500	8251 ± 82.50	-2.93	2000	2039 ± 64.42	1.95
	30.00	28.59 ± 0.62	-4.70	7.500	7.750 ± 0.120	3.33
Long term stability at -70 °C, 165 days	8500	8931 ± 151.5	5.07	2000	2105 ± 57.97	5.26
	30.00	29.70 ± 0.78	-1.00	7.500	7.225 ± 0.145	-3.67

SD: Standard deviation, n: Number of replicates

$$\%Change = \frac{Mean\ stability\ samples - Mean\ comparison\ samples}{Mean\ comparison\ samples} \times 100$$

Application of the method in healthy subjects and incurred sample reanalysis results

The plasma concentration *vs.* time profile for AMX and CLV under fasting is shown in Figure 4. The results show that the newly developed method has the required sensitivity to measure plasma concentration of the analytes after oral administration of test and reference formulations. The mean pharmacokinetic parameters evaluated after oral administration of combination tablet are summarized in Table 6. The T_{max} and C_{max} values obtained for both the analytes in the present work were comparable with previous report with identical dose strength in 30 healthy subjects [20]. Further, no statistically significant differences were found between the two formulations in any parameter. The ratios of mean log-transformed parameters (C_{max}, $AUC_{0\text{-}24h}$ and $AUC_{0\text{-}inf}$) and their 90 % CIs were all within the defined bioequivalence range of 80-125 % (Table 7). These observations confirm the bioequivalence of the test sample with the reference product in terms of rate and extent of absorption.

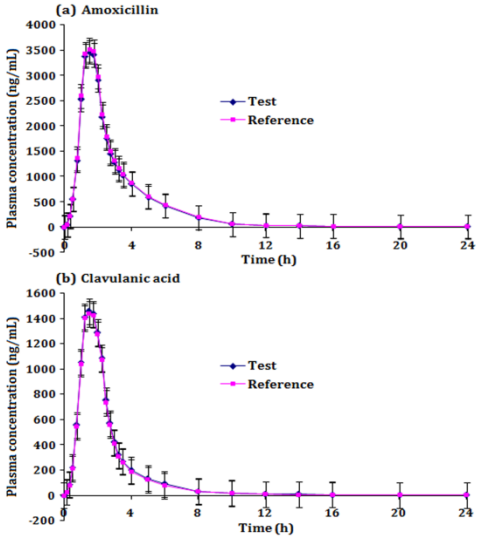


Figure 4 Mean plasma concentration-time profile of amoxicillin and clavulanic acid after oral administration of test (250 mg sumatriptan + 125 mg naproxen sodium tablets from a Generic Company) and a reference (AUGMENTIN®, 250 mg amoxicillin + 125 mg clavulanic acid as a potassium salt from GlaxoSmithKline, USA) formulation to 12 healthy volunteers.

Table 6 Mean pharmacokinetic parameters following oral administration of 250 mg amoxicillin + 125 mg clavulanic acid combination formulation in 12 healthy Indian subjects under fasting.

Parameter	Amoxicillin(Mean ±SD)		Clavulanic acid (Mean ±SD)	
	Test	Reference	Test	Reference
C_{max} (ng/mL)	3512.2 ± 360.1	3466.1 ± 329.3	1433.7 ± 154.4	1455.6 ± 165.8
T_{max} (h)	1.57± 0.31	1.59 ± 0.23	1.47± 0.38	1.52 ± 0.22
$t_{1/2}$ (h)	2.57 ± 0.27	2.50 ± 0.20	2.33± 0.17	2.40± 0.23
AUC $_{0\text{-}24h}$(h.ng/mL)	9619.3 ± 888.6	9377.8 ± 769.9	3244.3 ± 258.1	3350.6 ± 289.7
AUC $_{0\text{-}inf}$ (h.ng/mL)	10133.3 ± 951.3	9846.7 ± 868.2	3406.8 ± 333.8	3535.1 ± 361.2
Kel (1/h)	0.270 ± 0.033	0.277 ± 0.051	0.297 ± 0.026	0.279 ± 0.031

C_{max}: Maximum plasma concentration; T_{max}: Time point of maximum plasma concentration; $t_{1/2}$: Half life of drug elimination during the terminal phase; $AUC_{0\text{-}t}$: Area under the plasma concentration-time curve from zero hour to 24h; $AUC_{0\text{-}inf}$: Area under the plasma concentration-time curve from zero hour to infinity; K_{el}: Elimination rate constant; SD: Standard deviation

Table 7 Comparison of treatment ratios and 90% CIs of natural log (Ln)-transformed parameters for test and reference formulations in 12 healthy subjects under fasting.

Parameter	Ratio (test/reference),%		90% CI (Lower – Upper)		Power		Intra subject variation, % CV	
	AMX	CLV	AMX	CLV	AMX	CLV	AMX	CLV
C_{max} (ng/mL)	101.3	98.5	97.1-103.8	95.8-102.1	0.9997	0.9998	5.84	4.34
$AUC_{0\text{-}24h}$ (h.ng/mL)	102.6	96.8	99.5-104.1	93.6-98.2	0.9993	0.9994	6.24	5.03
$AUC_{0\text{-}inf}$ (h.ng/mL)	102.9	96.3	99.9-105.2	93.1-98.6	0.9998	0.9996	7.02	6.32

AMX: Amoxicillin; CLV: Clavulanic acid; CI: confidence interval; CV: coefficient of variation

In order to prove the method reproducibility, 63 study samples were selected which were near the C_{max} and the elimination phase in the pharmacokinetic profile of the drugs. These samples were reanalyzed and the results were compared with the initial study results. The % change in the results was within ±12 % for both the analytes, which is within the acceptance criterion. The graphical representation of the results is shown in Figure 5.

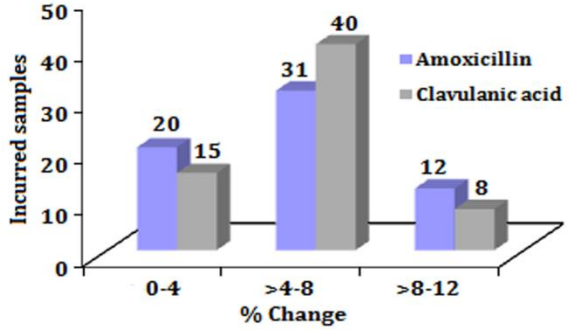


Figure 5 Graphical representation of results for 63 reanalyzed samples of amoxicillin and clavulanic acid.

CONCLUSIONS

The proposed validated method for the estimation of AMX and CLV in human plasma is highly selective, accurate and precise. The method offers significant advantages over those previously reported, in terms of lower sample requirements, sensitivity and analysis time. The efficiency of solid phase extraction and a chromatographic run time of 2.0 min per sample make it an attractive procedure in high-throughput bioanalysis of this antibiotic combination. The linear dynamic range established was adequate to measure the plasma concentration of AMX and CLV in a clinical study involving healthy subjects. In addition, matrix effect and stability of analytes in plasma was extensively studied. Further, incurred sample reanalysis results proved the reproducibility of the proposed method.

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