# Determination of amoxicillin in human plasma by LC-MS/MS and its application to a bioequivalence study

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*Abstract:* - An analytical method based on solid phase extraction has been developed and validated for analysis of amoxicillin in human plasma using gemifloxacin as an internal standard. A COSMOSIL 5C18-PAQ column provided chromatographic separation of analytes followed by detection with mass spectrometry. The method involves simple isocratic chromatographic conditions and mass spectrometric detection in the positive ionization mode using an API-3000 system. The proposed method has been validated with linear range of 100–15000 ng/mL for amoxicillin. The intra-run and inter-run precision values are within 3.53% and 5.63% respectively for amoxicillin at the LOQ level. Total elution time was as low as 2 min. This validated method was used successfully for analysis of plasma samples from a bioequivalence study.

*Key-Words:* - Amoxicillin, Antibiotic, Automated solid phase extraction, Tandem mass spectrometry, Human plasma, Bioequivalence study.

## 1 Introduction

Amoxicillin is a semi synthetic antibiotic. It is an analog of ampicillin, with a broad spectrum of bactericidal activity against many gram-positive and gram-negative microorganisms. IUPAC name of the amoxicillin is (2S,5R,6R)-6-[(R)-(-)-2-amino-2-(p-hydroxyphenyl)acetamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid trihydrate. The presence of a benzyl ring in the side chain extends the antibacterial activity to gram-negative bacteria [1]. The action mechanism of these antibiotics has not been unequivocally established, but it is thought they may interfere with peptidoglycan bacterial cell wall synthesis in the effected organisms [2].

Amoxicillin shows high absorption after oral administration, and this is not altered by the concomitant ingestion with food. Amoxicillin reaches Cmax (8mg/mL) about 2 hours after administration, exhibits low binding with plasma proteins (17%), is quickly distributed through the body, and has an elimination half-life of 1 hour [3].

Amoxicillin is presently the most commonly used antibiotic. To understand the pharmacokinetic behavior of Amoxicillin in humans, a reliable quantitative method is needed. Several bioanalytical methods are reported to determine amoxicillin in body fluid by HPLC with UV [4-10] and fluorescence [11] detection.

Sensitive and selective methods based on LC-MS/MS methodologies have also been developed for determination of amoxicillin in bovine milk [12], pig tissues [13] and human plasma [14-15].

Different methods of sample preparation have been applied prior to the chromatographic analysis, mostly based on protein precipitation [4,16], liquid-liquid extraction [8,13,14], solid-

phase extraction [11,6,7,12,15] and other techniques like ultra filtration [5,9].

The method reported by Khuroo et al. describes solid phase extraction of amoxicillin in human plasma using LC-MS/MS. The lowest LOQ's reported by Khuroo et al., was 170 ng/mL and the linear dynamic range was 170-17000 ng/mL [15]. As per guidelines LOQ should be five-fold (32 times) below the $C_{max}$ value, but unfortunately the method reported by Khuroo et al. also failed to quantify LOQ of amoxicillin in human plasma which were observed in the present bioequivalence study.

The sensitive and selective LC–MS/MS method was reported by Wen et al. involved solvent precipitation procedure with methanol. Separation was achieved on a Lichrospher C18 column (150 mm x 4.6 mm ID, 5 µm) using methanol (containing 0.2% of formic acid) and water (containing 0.2% of formic acid) as a mobile phase by gradient elution at a flow rate of 1.0 mL/min. Linear dynamic range was 5-20000 ng/mL [16]. This method involved precipitation method with methanol which caused deposition of matrix on curtain plate of the mass spectrometer and needed cleaning of curtain plate frequently.

Hence, it is necessary to develop and validate a clean, rapid, selective and sensitive method which can be successfully applied to a bioequivalence study.

In the present paper we would like to present a simple solid phase extraction method for the quantification of amoxicillin using gemifloxacin as an internal standard using LC-MS/MS. The application of this validated method in analyzing samples from a bioequivalence study involving amoxicillin is also presented. The greatest advantage of this method over all those referenced is the simple and clean SPE procedure with short runtime. Linear dynamic range for amoxicillin was sufficient for application in bioavailability-bioequivalence studies.

# 2 Experimental

## 2.1 Chemicals and reagents

The reference standards of amoxicillin and gemifloxacin were obtained from Themis Laboratories (Mumbai, India). High purity water was prepared in-house using a Milli-Q A10 gradient water purification system (Millipore, Bangalore, India). LC grade methanol and methyl tertiary butyl ether were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). AR grade orthophosphoric acid and formic acid were procured from Merck (Mumbai, India). Drug free (blank) human plasma containing K3-EDTA was obtained in-house by enrolling healthy volunteers and taking their consent before bleeding. The plasma thus obtained was stored at –20 °C prior to use. Oasis HLB 30 mg/1 cc solid phase extraction (SPE) cartridges were procured from waters (Milford, Mass, USA).

## 2.2 Calibration curve and quality control samples

Two separate stock solutions of amoxicillin were prepared for bulk spiking of calibration curve and quality control samples for the method validation exercise as well as the subject sample analysis. The stock solutions of amoxicillin and gemifloxacin were prepared in methanol at free base concentration of 1000 µg/mL. Primary dilutions and working standard solutions were prepared from stock solutions by dilution with water:methanol (40:60, v/v). These working standard solutions were used to prepare the calibration curve and quality control samples. Blank human plasma was screened prior to spiking to ensure it was free of endogenous interference at retention times of amoxicillin and internal standard gemifloxacin. A nine point standard curve and four quality control samples were prepared by spiking the blank plasma with an appropriate amount of amoxicillin. Calibration samples were made at concentrations of 100, 200, 750, 1500, 3000, 6000, 9000, 12000 and 15000 ng/mL and quality control samples were made at concentrations of 300, 3800, 7600 and 12900 ng/mL for amoxicillin.

## 2.3 Sample preparation

A 0.5 mL aliquot of human plasma sample was mixed with 20 µL of internal standard working solution (35 µg/mL of gemifloxacin). To this 0.5 mL of 20% orthophosphoric acid was mixed. Sample mixture was loaded onto Oasis 30 mg/1 cc extraction cartridge pre-conditioned with 1 mL methanol followed by 1 mL water. The extraction cartridge was washed with 1 mL of methyl tertiary butyl ether. Amoxicillin and gemifloxacin were eluted with 1 mL of the mobile phase. 5 µL of the eluant was injected into the LC–MS/MS system through the autosampler.

## 2.4 Liquid chromatography and mass spectrometric conditions

### 2.4.1 Chromatography

Chromatographic separation was carried out on a Shimadzu HPLC system (Kyoto, Japan) using a COSMOSIL 5C18-PAQ $C_{18}$ column (50×4.6 mm i.d., 5 µm) purchased from Chromatopak (Thermo Scientific, India) employed under isocratic conditions. The mobile phase was 0.2% formic acid in water–0.2% formic acid in methanol (20:80, v/v) and set at a flow rate of 0.6 mL $min^{-1}$. The column was maintained at room temperature. The HPLC eluent was introduced directly into the positive electrospray ionization source and the total run time for each sample analysis was 2 min.

### 2.4.2 Mass spectrometry

The plasma concentration of amoxicillin was quantified using a Sciex API 3000 triple quadrupole mass spectrometer (Applied Biosystems, MDS Sciex, Concord, Canada) equipped with a Turbo Ion Spray interface to generate the positively charged ions. Ion spray voltage was 5500 V with a turbo gas temperature at 450°C. The operating conditions were optimized by flow injection of a mixture of all analytes and were as follows: nebulizing gas flow 13 L $min^{-1}$; auxiliary gas flow 8 L $min^{-1}$; collision activated dissociation (CAD) gas was set at 7 psi and curtain gas flow 15 L $min^{-1}$. Compound-dependent parameters for amoxicillin and gemifloxacin were: orifice voltage 50 eV, ring voltage 100 eV. Quantitation was performed in multiple reaction monitoring (MRM) mode employing collision energies of 16 eV for amoxicillin and 30 eV for gemifloxacin with dwell times of 250 ms for ion transitions *m/z* 366.2→ *m/z* 348.8 (amoxicillin) and *m/z* 390.0 → *m/z* 371.9 (gemifloxacin), respectively. Quadrupoles, Q1 and Q3 were set to unit resolution. Automated data acquisition and analysis were performed using the Analyst software (version 1.4.2).

For quantification peak area ratios of the target ions those of the internal standard were compared with weighted (1/x2) least squares calibration curves in which the peak area ratios of the calibration standards were plotted versus their concentrations.

## 2.5 Validation

A through and complete method validation of amoxicillin in human plasma was done following USFDA guidelines [17]. The method was validated for selectivity, sensitivity, linearity, precision and accuracy, recovery, dilution integrity, partial volume, matrix effect, reinjection reproducibility and stability. Selectivity was performed by analyzing the human blank plasma samples from six different sources (or donors) with two more lots, each of haemolyzed and lipemic to test for interference at the retention time of amoxicillin and gemifloxacin. The intrarun (within a day) and interrun (on three different days) accuracy was determined by replicate analysis of quality control samples ($n$ = 6) at the LOQ (limit of quantification), LQC (low quality control), MQC (medium quality control), M1QC (middle quality control), HQC (high quality control) and ULOQ (upper limit of quantification) levels. The %CV should be less than 15% and accuracy (%RE) should be within 15% except the LOQ where it should be within 20%.

Accuracy is defined as the percent relative error (%RE) and was calculated using the formula %RE = (E - T) (100/T) where E is the experimentally determined concentration and T is the theoretical concentration. Assay precision was calculated by using the formula %CV = (SD/M) (100) where M is the mean of the experimentally determined concentrations and SD is the standard deviation of M.

The extraction efficiencies of amoxicillin and gemifloxacin were determined by analysis of six replicates at low, medium, middle and high quality control concentrations for amoxicillin and at one concentration for the internal standard gemifloxacin. The percent recovery was evaluated by comparing the peak area of extracted analytes to the peak area of non extracted standards (sample spiked in mobile phase).

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations above upper limit of quantification (ULOQ), which may be encountered during real subject samples analysis. Dilution integrity experiment was carried out at 1.7 times the ULOQ concentration. Six replicates each of 1/2 and 1/4 concentrations were prepared and their concentrations were calculated by applying the dilution factor 2 and 4 against the freshly prepared calibration curve.

The partial volume experiment was performed to validate the method, for application in case of insufficient volume of plasma in real subject sample. Partial volume experiment was performed on middle quality control (M1QC) concentration level. Six replicates each of half and quarter volume of total volume of plasma required

for processing were prepared and their concentrations were calculated by applying the dilution factor 2 and 4 against the freshly prepared calibration curve.

To study the effect of matrix on analyte quantification with respect to consistency in signal suppression, matrix effect was checked with six different lots of plasma. Two replicates each of LQC and HQC were prepared from six different lots of plasma (total 24 QC samples) and checked for %CV which should be less than 10% at LQC and HQC level.

To check reinjection reproducibility, initially LQC and HQC samples were injected and after analysis of these samples system hardware was deactivated for two hours, after three hours again system hardware was activated and equilibrated. Then same samples were reinjected. Original values were compared with reinjected values with respect to %Change, which should be less than 10%.

As a part of the method validation, stability was evaluated in stock solutions and in plasma under different conditions, simulating the same conditions, which occurred during study sample handling and analysis. Stock solution stability was performed by comparing area response of stability sample of analyte and internal standard with the area response of sample prepared from fresh stock solutions. Stability studies in plasma were performed at LQC and HQC concentration level using six replicates at each level. Analyte was considered stable if the %Change is less than 15% as per USFDA guidelines. Analyte was tested using the quality control samples whenever appropriate. The stability of spiked human plasma stored at room temperature (bench top stability) was evaluated for 12 h. The stability of spiked human plasma stored at -70 °C in coolant (coolant stability) was evaluated for 24 h. The autosampler sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0), with the samples that were re-injected after keeping in the auto sampler at 10 °C for 24 h. The freeze–thaw stability was conducted by comparing the stability samples that had been frozen at –20 °C and thawed three times, with freshly spiked quality control samples. Six aliquots of each low and high concentration were used for the freeze–thaw stability evaluation. For long term stability evaluation the concentrations obtained after 15, 30, 45, 60 and 90 days intervals were compared with initial concentrations.

### 2.6 Application of method to a bioequivalence study

The validated method has been successfully applied to the analysis of amoxicillin concentrations in twenty four healthy, adult, human male volunteers which were enrolled in a bioequivalence study. One amoxicillin tablet (875 mg) was orally administered to volunteers rang ranging in age from 18 to 45 years as per the inclusion criteria for the study under fasting conditions. Volunteers who have used any prescribed medication during last 2 weeks or over the counter medicinal products during the last week preceding the first dose were excluded from the study. Seven days of wash-out period were kept between the two periods of the study.

The bioequivalence study was based on a balanced, open label, analyst blind, single centre, randomized, crossover, two treatment, two periods, two sequences and single dose design. A test formulation containing Amoxicillin 875 mg with the reference formulation Augmentin® was evaluated. The study was conducted according to current GCP guidelines after signed consent of the volunteers following approved by an authorized Ethics committee. In total, 20 time points after blood collection per period were evaluated post-dosing including the pre-dose sample. The blood samples were collected in separate vacutainers containing $K_3$-EDTA. The plasma from these samples was analyzed by LC-MS-MS.

Pharmacokinetic parameters were computed using the WinNonlin Software version 5.2 (Pharsight Corporation, California, USA) and 90% confidence interval was computed using SAS Software version 9.2 (SAS Institute, Cary, USA).

## 3 Results and discussion

### 3.1 Method development

During method development different options were evaluated to optimize detection parameters, chromatography and sample extraction.

#### 3.1.1 Mass spectra

The LC–MS/MS were tested to obtain sensitivity and signal stability during infusion of the analyte in the continuous flow of mobile phase to electrospray ion source operated both polarities at a flow rate of 10 µL/min. The amoxicillin gave better results in positive ion mode. The predominant peaks in the primary ESI spectra of amoxicillin and

gemifloxacin corresponds to the $MH^+$ ions at *m/z* 366.2 and 390.0 respectively. Product ions of amoxicillin and gemifloxacin scanned in

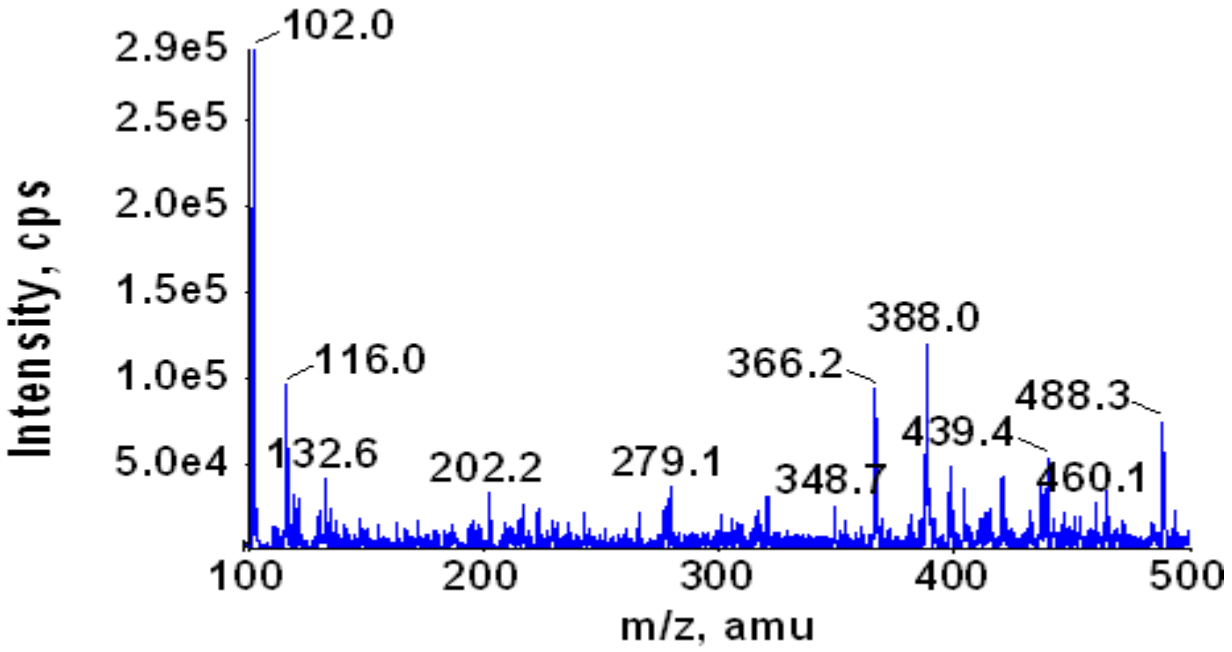


**Fig.1 Parent ion mass spectrum of amoxicillin**

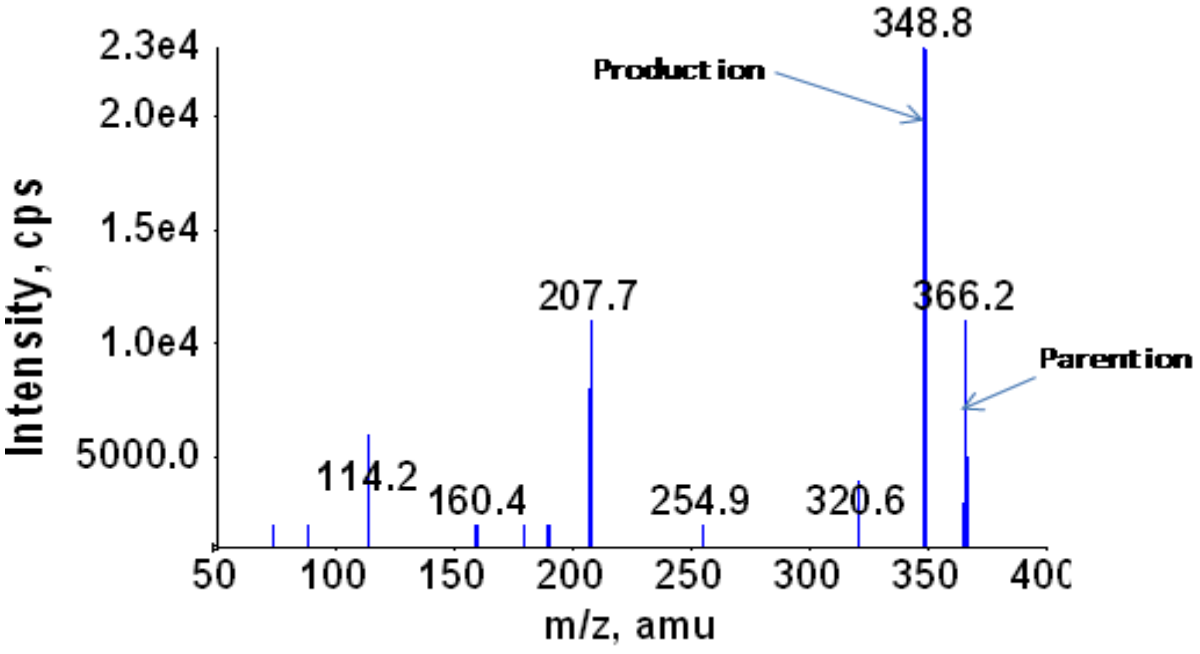


**Fig.2 Product ion mass spectrum of amoxicillin.**

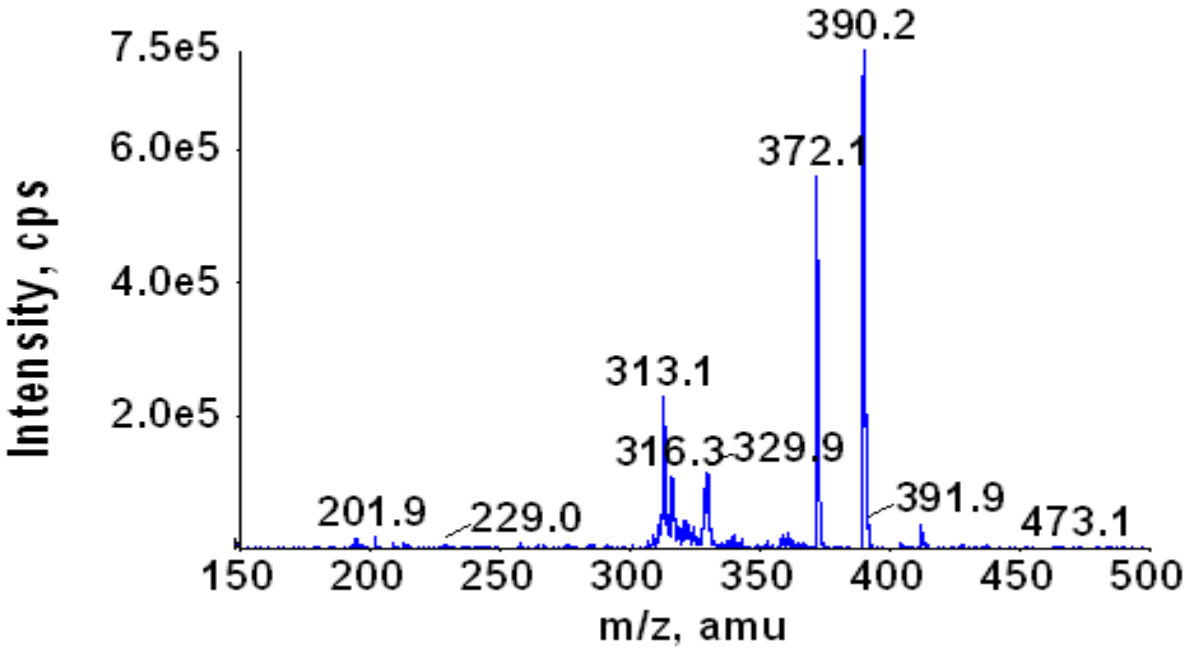


**Fig.3 Parent ion mass spectrum of gemifloxacin**

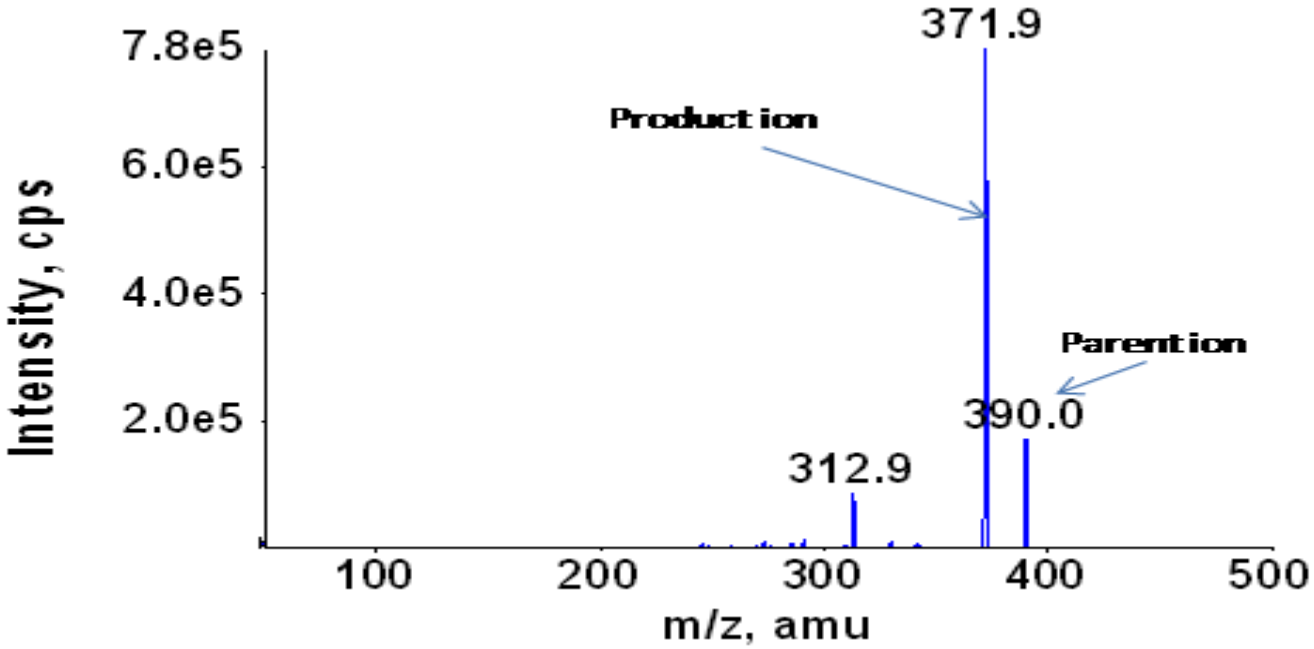


**Fig.4 Product ion mass spectrum of gemifloxacin**

quadrupole 3 after a collision with nitrogen in quadrupole 2 had an *m/z* of 348.8 and 371.9 respectively.

### 3.1.2 Chromatography

Initially, a mobile phase containing acetic acid solution and acetonitrile in varying combinations was tried in which poor peak shape was observed. The mobile phase containing 2mM ammonium acetate:acetonitrile (20:80v/v) and 2mM ammonium acetate:methanol (20:80v/v) exhibited better separation, but response was very low, which was insufficient to quantify LOQ. We had tried mobile phase 0.2% formic acid in water solution in combination with 0.2% formic acid in methanol and 0.2% formic acid in acetonitrile in varying combinations. Use of 2mM ammonium acetate

with 0.2% formic acid in water in combination with methanol improves peak shape but response was low. Finally we received the best signal for amoxicillin and gemifloxacin using a mobile phase containing 0.2% formic acid in water solution in combination with 0.2% formic acid in methanol (20:80 v/v) as there was almost a two fold increase in its area count as compared to the mobile phase containing 0.2% formic acid in water solution in combination with acetonitrile. Moreover, a marked improvement in the peak shape of amoxicillin and gemifloxacin was also observed using this mobile phase combination.

Short length columns such as, COSMOSIL 5C18-PAQ (50 mm x 4.6 mm, 5 µm), Symmetry Shield RP18 (50 mm x 2.1 mm, 3.5 µm), Inertsil C18 (50 mm x 4.6 mm, 5 µm), HyPURITY C18 (50 mm x 4.6 mm, 5 µm) and HyPURITY Advance (50 mm x 4.0 mm, 5 µm) were tried during the method development. In Symmetry Shield RP18 columns poor chromatography was observed. Inertsil C18, HyPURITY Advance and HyPURITY C18 columns gave a relatively good peak shape but the response was low. The best signal was obtained using the COSMOSIL 5C18-PAQ (50 mm x 4.6 mm, 5 µm) column. It gave satisfactory peak shapes for all the analytes and a flow rate of 0.6 mL/min reduced the run time to 2.0 min. Introducing such a high flow directly in to the ionization source affects evaporation of solvents which further causes improper ionization and reduces response, hence splitter was utilized to control direct flow in the ionization source.

### 3.1.3 Extraction

Prior to LC injection, the co-extracted proteins should be removed from the prepared solution. Several organic solvents were employed to extract analytes from the plasma sample. In the tested solvents (ethyl acetate, chloroform, hexane, dichloromethane, methyl tertiary butyl ether and acetonitrile) samples were not clean and poor chromatography observed.

In the state of nonionic forms, the strong binding of analytes to the copolymer of the SPE cartridge enabled sufficient clean-up and suitable eluting solution helped to elute analytes with more efficiency. So the method was optimized to achieve maximum extraction efficiency. The amoxicillin is approximately 17% protein-bound, so prior to extraction it is necessary to release the amoxicillin from protein binding. So orthophosphoric acid was added in the sample before extraction. for breaking protein binding. Different types of cartridges such as Phenomenex (Strata-X, Strata-X-C, Strata-XCW, Strata-XAW) and Oasis HLB were tried. Finally we choose an Oasis HLB 30 mg/1 cc cartridge. It was difficult to find a compound which could ideally mirror the analytes to serve as a good IS. Several compounds were investigated to find a suitable IS, and finally gemifloxacin of the same class of compound was found most appropriate for the present purpose. There was no significant effect of IS on analyte recovery, sensitivity or ion suppression. The results of method validation using gemifloxacin as the IS were acceptable in this study based on FDA guidelines. The high recovery and selectivity was observed in the solid phase extraction method which was used in the present work.

These optimized detection parameters, chromatographic conditions and extraction procedure resulted in reduced analysis time with accurate and precise detection of amoxicillin in matrix.

## 3.2 Method validation

### 3.2.1 Selectivity and sensitivity

Representative chromatograms obtained from blank plasma, plasma spiked with internal standard and plasma spiked with LOQ standard for amoxicillin and gemifloxacin is presented in Fig. 5.

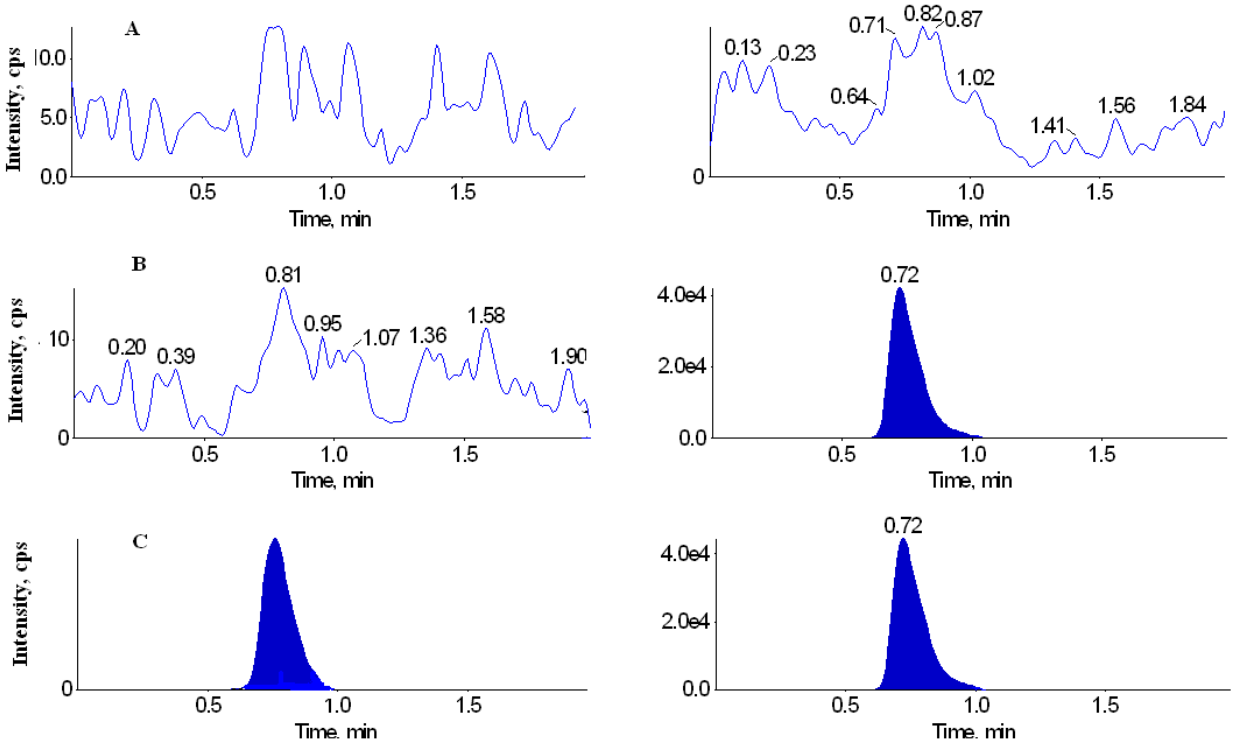


**Fig.5 Representative chromatograms of amoxicillin (left) and gemifloxacin (right) in human plasma. (A) Blank plasma (B) Plasma spiked with internal standard and (C) LLOQ**

The mean %interference observed at the retention time of analytes between eight different lots of human plasma including haemolyzed and lipemic plasma containing K3-EDTA as the anti-coagulant calculated was 0.00% for amoxicillin and gemifloxacin, which was within acceptance criteria. Six replicates of extracted samples at the LOQ level in one of the plasma samples having least interference at the retention time of amoxicillin was prepared and analyzed. The %CV of the area ratios of these six replicates of samples was 6.68% for amoxicillin confirming that interference does not affect the quantification at LOQ level. Utilization of selected product ions for each compound enhanced mass spectrometric selectivity. The product ions of m/z 366.2 and 390.0 were hence concluded to be specific for amoxicillin and gemifloxacin.

The LOQ for amoxicillin was 100 ng/mL. The intra-run precision and intra-run accuracy (%RE) of the LOQ plasma samples containing amoxicillin was 3.53 and -1.81, respectively. We obtained the mean $C_{max}$ of amoxicillin, for test and reference formulations were 8521.98 and 8696.75 ng/mL, respectively. As per guidelines LOQ should be below 32 times (five-fold) of $C_{max}$. So for amoxicillin, the LOQ was easily, quantified using the present method. All the values obtained below 100 ng/mL for amoxicillin were excluded from statistical analysis as they were below the LOQ values validated for amoxicillin.

### 3.2.2 Linearity, precision and accuracy, recovery

The peak area ratios of calibration standards were proportional to the concentration of amoxicillin in each assay over the nominal concentration range of 100–15000 ng/mL. The calibration curves appeared linear and were well described by least-squares linear regression lines. As compared to the 1/x weighing factor, a weighing factor of $1/x^2$ properly achieved the homogeneity of variance and was chosen to achieve homogeneity of variance. The correlation coefficient was ≥0.9900 for amoxicillin. The observed mean back-calculated concentration with accuracy (%RE) and precision (%CV) of four linearities analyzed during method validation are given in table 1. The deviation of the back calculated values from the nominal standard concentrations were less than 15% except LOQ where it should be less than 20%. This validated linearity range justify the concentration observed during real sample analysis.

**Table 1 Summary of calibration curve standards**

| Nominal concentration (ng/mL) | Mean back calculated concentration (ng/mL) | %CV | %RE |
|---|---|---|---|
| 101.46 | 101.08 | 1.28 | -0.38 |
| 202.92 | 205.78 | 2.66 | 1.41 |
| 751.57 | 721.55 | 0.90 | -3.99 |
| 1503.14 | 1545.28 | 1.65 | 2.80 |
| 3006.28 | 3014.00 | 1.75 | 0.26 |
| 6012.55 | 6101.60 | 2.04 | 1.48 |
| 9018.83 | 9096.99 | 0.98 | 0.87 |
| 12025.10 | 11834.46 | 1.42 | -1.59 |
| 15031.38 | 14902.28 | 1.61 | -0.86 |

CV: coefficient of variation; RE: relative error.

The inter-run precision and accuracy were determined by pooling all individual assay results of replicate ($n$ = 6) quality control over three separate batch runs analyzed on three different days. The inter-run precision and inter-run accuracy (%RE) was ≤5.63% and ≤-8.43% respectively for amoxicillin. The intra-run precision and accuracy were determined by pooling all individual assay results of replicate ($n$ = 6) quality control of two separate batch runs analyzed on the same day. The intra-run precision (%CV) and intra-run accuracy (%RE) was ≤3.53% and ≤-3.99% respectively for amoxicillin. All these data presented in Table 2 indicate that the method is precise and accurate.

**Table 2 Intra-run and inter-run precision and accuracy ($n$ = 6) of amoxicillin in human plasma**

| Run | Concentration added (ng/mL) | Mean concentration found (ng/mL) | %CV | %RE |
|---|---|---|---|---|
| Intra | 102.12 | 100.27 | 3.53 | -1.81 |
| | 300.35 | 300.10 | 3.24 | -0.08 |
| | 3754.37 | 3643.88 | 1.72 | -2.94 |
| | 7508.75 | 7209.04 | 1.53 | -3.99 |
| | 12764.87 | 12475.94 | 1.63 | -2.26 |
| Inter | 102.12 | 96.63 | 5.63 | -5.38 |
| | 300.35 | 289.65 | 3.62 | -3.56 |
| | 3754.37 | 3527.34 | 2.61 | -6.05 |
| | 7508.75 | 6875.84 | 3.82 | -8.43 |
| | 12764.87 | 11847.038 | 4.40 | -7.19 |

CV: coefficient of variation; RE: relative error.

**Table 3 Absolute recovery for amoxicillin and gemifloxacin**

| Analytes | Level | A | B | %Recovery | %CV |
|---|---|---|---|---|---|
| LQC | 22129 | 8322 | 37.77 | 8.44 | |
| Amoxicillin | M1QC | 271084 | 103411 | 38.15 | 2.32 |
| | MQC | 543420 | 194786 | 35.85 | 1.66 |
| | HQC | 895241 | 333162 | 37.21 | 0.75 |
| | LQC | 731810 | 373229 | 51.08 | 4.16 |
| Gemifloxacin | M1QC | 749219 | 375201 | 50.10 | 2.00 |
| | MQC | 750613 | 356526 | 47.51 | 1.63 |
| | HQC | 755997 | 352230 | 46.65 | 3.96 |

A: Area of unextracted sample; B: Area of extracted sample; Mean recovery was found to be 37.25% for amoxicillin and 48.83 for gemifloxacin; CV: coefficient of variation.

Six aqueous replicates (samples spiked in mobile phase) at low, medium, middle and high quality control concentration levels for amoxicillin were prepared for recovery determination and the areas obtained were compared versus the areas obtained for extracted samples ( shown in Table 3) of the same concentration levels from a precision and accuracy batch run on the same day. The mean recovery for amoxicillin was 37.25% with a precision of 4.85%. The mean recovery for gemifloxacin was 48.83% with a precision of 4.81%. This indicates that the extraction efficiency for the amoxicillin as well as gemifloxacin was consistence and reproducible.

### 3.2.3 Dilution integrity and partial volume

The mean back calculated concentrations for 1/2 and 1/4 dilution samples were within 85-115% of their nominal. The %CV for 1/2 and 1/4 dilution samples were 1.52% and 1.53% respectively. The mean back calculated concentrations for half and quarter partial volume samples were within 85-115% of their nominal. The %CV for half and quarter partial volume samples were 2.16% and 1.31% respectively.

### 3.2.4 Matrix effect, reinjection reproducibility and stabilities.

The assessment of matrix effect constitutes an important and integral part of validation for quantitative LC–MS/MS for supporting pharmacokinetics studies. The results found were well within the acceptable limits as the %CV of the area ratios of post spiked recovery samples at LQC and HQC were 4.45% and 2.47% respectively for amoxicillin which were within 10% for amoxicillin. Hence minor suppression of analyte signal due to endogenous matrix interferences did not affect the quantification of amoxicillin.

Reinjection reproducibility exercise was performed to check weather the instrument performance remains unchanged after hardware deactivation due to any instrument failure during real subject sample analysis. %Change is less than 4.13% for LQC and HQC level concentration; hence batch can be reinjected in case of instrument failure during real subject sample analysis.

Stock solution stability was performed to check stability of amoxicillin and gemifloxacin in stock solutions prepared in water : methanol (80:20) and stored at 2-8 °C in refrigerator. The freshly prepared stock solutions were compared with stock solutions prepared before 30 days. The %Change for amoxicillin and gemifloxacin were 2.22% and 1.99% respectively indicates that stock solutions were stable at least for 30 days.

Bench top, coolant and autosampler stability for amoxicillin was investigated at LQC and HQC levels. The results revealed that amoxicillin was stable in plasma for at least 12 h at room temperature, 24 h in coolant at -70 °C and 24 h in an autosampler at 10 °C. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with amoxicillin at LQC and HQC levels did not affect their stability. The long term stability results also indicated that amoxicillin was stable in matrix for up to 90 days at a storage temperature of –20 °C. The results obtained from all these stability studies are tabulated in Tables 4.

**Table 4 Stability results for montelukast**

| Stability | level | A | %CV | B | %CV | %Change |
|---|---|---|---|---|---|---|
| Autosampler | LQC | 294.70 | 4.86 | 274.82 | 3.32 | -6.74 |
| (24 h, 10 °C) | HQC | 12084.80 | 1.95 | 11791.98 | 1.72 | -2.42 |
| Bench top | LQC | 294.70 | 4.86 | 286.95 | 6.02 | -2.63 |
| (12 h at Room temp.) | HQC | 12084.80 | 1.95 | 11781.571 | 1.99 | -2.51 |
| Coolant | LQC | 294.70 | 4.86 | 284.47 | 4.70 | -3.47 |
| (24 h, -70 °C) | HQC | 12084.80 | 1.95 | 648.66 | 1.57 | -4.60 |
| Reinjection | LQC | 313.07 | 4.85 | 321.67 | 4.85 | 2.75 |
| (2 h, ambient temp.) | HQC | 12526.17 | 0.92 | 13042.88 | 1.79 | 4.13 |
| 3rd freeze-thaw cycle | LQC | 306.96 | 4.54 | 302.62 | 6.46 | -1.41 |
| | HQC | 12043.18 | 2.92 | 12009.97 | 2.85 | -0.28 |
| Long term | LQC | 297.07 | 3.50 | 341.46 | 0.64 | 14.94 |
| (90 days, -20 °C) | HQC | 12390.67 | 1.32 | 12702.09 | 4.27 | 2.51 |

A: comparison sample concentration (ng/mL); B: stability sample concentration (ng/mL); CV: coefficient of variation; h: hours, temp.: temperature.

### 3.2.5 Application of the Analytical Method to Pharmacokinetic Studies

Statistical data analysis included determination of Cmax (maximum observed drug concentration during the study), AUC0–t (area under the plasma concentration- time curve measured to the last quantifiable concentration, using the trapezoidal rule), AUC0–inf (AUC0–t plus additional area extrapolated to infinity, calculated using the formula AUC0-t + Ct/Kel, where Ct was the last measurable drug concentration), Tmax (time to observe maximum drug concentration), Kel (apparent first-order terminal rate constant calculated from a semi-log plot of the plasma concentration versus time curve, using the method of least square regression) and T1/2 (terminal half-life as determined by quotient 0.693/Kel).

**Table 5 Pharmacokinetic parameters of amoxicillin using non-compartmental analyses**

| Parameters | Amoxicillin Mean ± SD | |
|---|---|---|
| | Test | Reference |
| $C_{max}$ (ng/mL) | 8521.98 ± 3371.87 | 8696.75 ± 3053.90 |
| $AUC_{0-t}$ (ng x h/mL) | 37330.73 ± 12634.43 | 37915.47 ± 12659.06 |
| $AUC_{0-inf}$ (ng x h/mL) | 37926.25 ± 12619.85 | 38390.10 ± 12654.11 |
| $T_{max}$ (h) | 2.65 ± 0.99 | 2.59 ± 0.90 |
| $K_{el}$ ($h^{-1}$) | 0.41 ± 0.10 | 0.45 ± 0.15 |
| $T_{1/2}$ (h) | 1.81 ± 0.46 | 1.68 ± 0.47 |

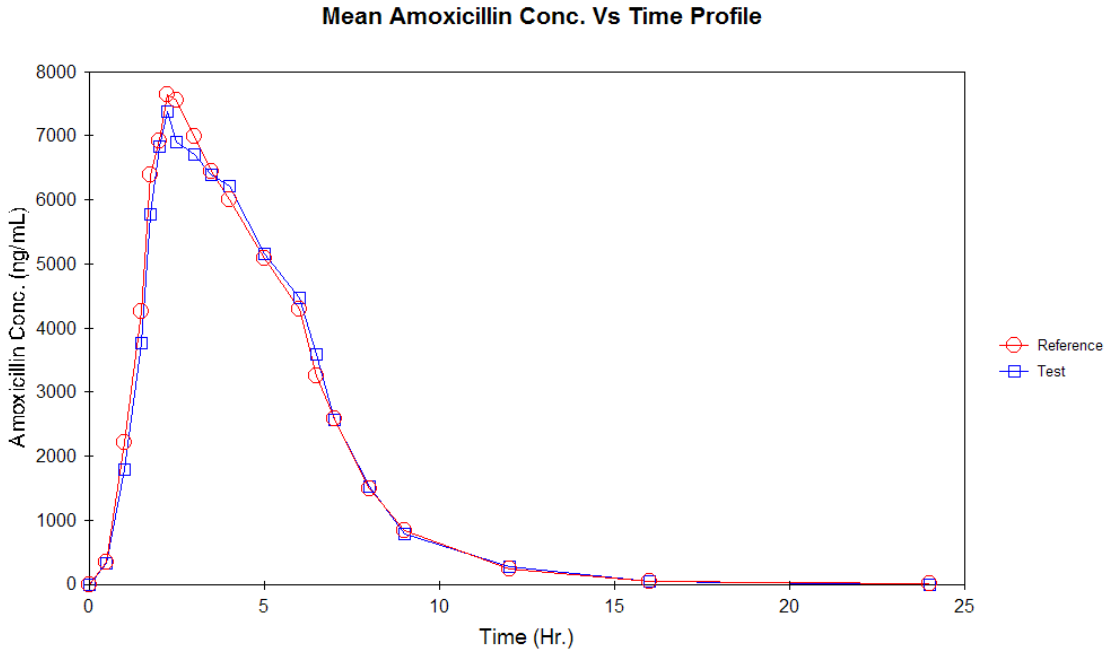


**Fig.6 Mean concentration versus time profile of amoxicillin in human plasma from 24 subjects receiving a single oral dose of 875 mg amoxicillin tablet as test and reference**

These variables calculated post-study are tabulated in Table 5. The mean Cmax data obtained justified the selected LOQ levels as they were at least less than five half-lives of the obtained Cmax values.

The mean concentration versus time profile of amoxicillin in human plasma is shown in Fig.6.

The 90% confidence intervals based on the ratios of means Cmax, AUC0–t and AUC0–inf fell within the acceptance range of 0.8–1.25, demonstrating the bioequivalence of the two formulations of amoxicillin.

## 4 Conclusions

The developed LC-MS/MS assay for amoxicillin is rapid, selective and suitable for routine measurement of subject samples. In the present study solid phase extraction method was reported for extraction of amoxicillin in human plasma using LC-MS/MS. The solid phase extraction method provided excellent sample clean up and high extraction efficiency has been successfully applied to the bioequivalence study of a tablet containing 875 mg amoxicillin as an oral dose in 24 healthy human volunteers under fasting conditions.

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