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Highly selective, sensitive and precise Bioanalytical method for the estimation of amoxicillin and clavulanic acid in nano gram range in Albino rat's plasma

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ABSTRACT

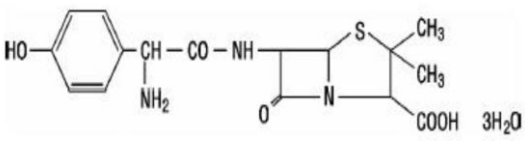
LC-MS/MS method for the determination of amoxicillin and clavulanic acid in Albino rat's plasma was developed and validated according to currently accepted FDA guidelines of Bioanalytical method validation. The retention times of amoxicillin, clavulanic acid and the internal standard were approximately 0.77, 0.86 and 1.17 minutes, respectively. The overall run time is 3 minutes. The correlation coefficient (r^2) is greater than or equal to 0.99. For the within-run accuracy and precision the coefficients of variation for lower limit of quantitation (LLOQ) low (LQC), medium (MQC) and high (HQC) quality control samples were 2.94, 1.31, 5.64 and 6.49 % for amoxicillin and 7.88, 6.79, 5.50, 7.33 for clavulanic acid respectively. The within-run percentages of nominal concentrations for LLOQ, LQC, MQC, and HQC were 94.77, 98.38, 103.18, 104.59 % for amoxicillin and 98.50, 99.83, 101.39 and 97.98 for clavulanic acid respectively.

1. INTRODUCTION

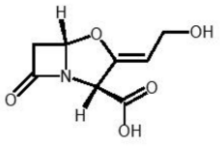
Pharmacokinetic and bio-equivalence studies require very precise and accurate assay methods that are well validated to quantify drugs in biological samples. The assay methods have to be sensitive enough to determine the biological sample concentration of the drug and/or its metabolite(s) for a period of about five elimination half-life after dosage of the drug. The assay methods also have to be very selective to ensure reliable data, free from interference of endogenous compounds and possible metabolites in the biological samples. In addition, methods have to be as robust and cost effective as possible, making of particular importance to bioequivalence studies. Above all, the assay methods must be able to withstand the scrutiny of national drug registration authorities who judge them on the basis of criteria established by international consensus.

Amoxicillin is a broad spectrum of semisynthetic antibiotic derived from the basic penicillin nucleus, 6-aminopenicillanic acid. It is a white, practically odourless, crystalline powder. Slightly soluble in water and in methanol; insoluble in carbon tetrachloride, and in chloroform. Clavulanic acid is produced by the fermentation of Streptomyces clavuligerus. Clavulanic acid is biosynthetically generated from the amino acid arginine and the sugar glyceraldehyde-3-phosphate.

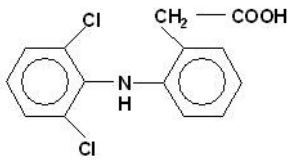
Diclofenac is a benzene-acetic acid derivative. Diclofenac, is a faintly yellowish white to light beige, virtually odourless, slightly hygroscopic crystalline powder. It is freely soluble in methanol, soluble in ethanol, and practically insoluble in chloroform and in dilute acid. Here diclofenac was used as internal standard.



Amoxicillin



Clavulanic acid



Diclofenac

2. MATERIALS AND METHODS

Acetonitrile, Methanol, Dichloromethane, Citric acid and Formic Acid were purchased form Merck, Mumbai. Water of HPLC grade from Milli-Q RO system was used. Working Standards of amoxicillin trihydrate, lithium clavulanate and diclofenac potassium were obtained from commercially. Blank plasma procured from blood bank.

MS/MS used was API 3200 with Analyst 1.4.1 software, Applied Biosystems, SCIEX, HPLC used in

this experiment was Prominence Degasser, DGU-20A3 Pump, LC- 20 AD Auto sampler, SIL HTC Column oven, CTO 20A, PDA detector, SPD M20A, Shimadzu make.

Optimization of the LC-MS/MS system: MS/MS is a much more specific and selective method of detection than UV. Interference by co-eluting components is not considered as significant a problem as with a UV detection system although the so-called "matrix effect" needs to be tested for. For this reason the whole development process was focused on mobile phase and extraction process optimization.

The LC- MS/MS instrument was calibrated with polypropylene glycol (PPG) standard in positive and negative ionisation mode. Infusion was done using 500 ng/ml of amoxicillin, clavulanic acid and IS separately in mobile phase. Using the spectra of the infused solutions, mass spectrometer parameters were optimized.

Preferred MS/MS detection conditions: LC-MS/MS detector at unit resolution in the multiple reaction monitoring (MRM) mode. The transition of the protonated molecular ions m/z 364, 198.2 and 293.78, product ion m/z 223.3, 136.2 and 250.1 for amoxicillin, clavulanic acid and IS respectively. Turbo Ion Spray ionisation (ESI) was used for ion production.

Compound parameters: The compound parameters namely Declustering potential (DP), Entrance potential (EP), Collision energy (CE), Collision cell entrance potential (CEP), Collision cell exit potential (CXP) were optimized separately for amoxicillin, clavulanic acid and Internal standard (IS).

DP (Declustering Potential): The DP parameter controls the potential difference between ground (usually the skimmer) and the orifice plate. It is used to minimize solvent cluster ions, which may attach to sample. The higher the voltage, the greater the amount of fragmentation. If the declustering potential is too high, the sample ion itself may fragment.

EP (Entrance Potential): The EP parameter controls the potential difference between the voltage on Q_0 and ground. The entrance potential guides and focuses the ions through the high-pressure and Q_0 region.

CE (Collision Energy): The CE parameter controls the potential difference between $Q_0$and Q_2 (collision cell). It is used only in MS/MS-type scan. This is the amount of energy that the precursor ions receive as they are accelerated into the collision cell, where they collide with gas molecules and fragment. In Q_1 and Q_3 scans the voltage applied to the collision cell is RO_2 (collision cell rod offset).

CEP (Collision Cell Entrance Potential): The CEP parameter controls the collision cell entrance potential, which is the potential difference between Q_0 and IQ_2. It focuses ions into Q_2 (collision cell). CEP is used in Q and MS/MS-type scans. Note that for Q3 scans, this voltage is called IQ_2 and by default is in fixed mode.

CXP (Collision Cell Exit Potential): The CXP parameter controls the potential difference between RO_2 and IQ_3. It is only used in Q_3 and MS/MS-type scans, where it transmits the ions into Q_3. In Q_1 scans, the voltage applied to IQ_3 is accessed through the IQ_3 parameter.

Table.1.Optimized parameters

Compound Name	DP	EP	CE	CEP	CXP
Amoxicillin	-21	-10	-20	-16	-13
Clavulanic acid	-11	-10	-11	-11	-5
Diclofenac (IS)	-25	-10	-16	-12	-6

Column Selection: Different reverse phase HPLC column tested for the analysis of amoxicillin and clavulanic acid were C-8 (100 x 4.6, 5µ), C-18 (100x 4.6, 3.7µ), (100 x 4.6, 5µ), (10x4.6, 3µ), (50x4.6, 2.5µ) of different makes and of different lots. The different makes are Hypersil column, Symmetry Column, Phenomenox column etc. Out of these columns discovery Waters - Symmetry C18 (50 x 4.6, 5u) was selected because of its better resolution and reproducibility.

Mobile phase optimization: For LC-MS/MS system, a volatile mobile phase is required, hence a buffer of 5mM ammonium acetate was prepared and acetic acid was added until the pH of the solution was adjusted to 5. Since electrospray ionisation is more efficient the higher the concentration of the organic modifier in the mobile phase, So methanol is used as organic modifier with ammonium acetate and found low sensitivity due to less ionization or suppression. To enhance the sensitivity and to get a symmetric peak, 0.1 % formic acid was used as mobile phase with Acetonitrile when using the above mobile phase sensitivity was good and the peak shape was satisfactory. Since the objective of this study was to develop a simple reliable method that would facilitate analysis of in rat plasma in a large number of samples over a relatively short period of time (Bioequivalence Studies), in a cost effective manner, the suitable mobile phase is 90 % Acetonitrile as organic modifier with 10% of 0.1% formic acid.

Effect of flow rate: To obtain some information about effect of flow rate the analysis was done at different flow rates with the optimized mobile phase. With an

isocratic run using 90% acetonitrile and 10% of 0.1% formic acid at a flow rate of 0.6ml per minute amoxicillin trihydrate eluted at 0.53 min with void volume and this retention time increased to 0.77 minutes when the flow rate was set at 0.5ml per minute and clavulanic acid eluted at 0.60 min with void volume and this retention time increased to 0.86 minutes when the flow rate was set at 0.5ml per minute. The flow rate was optimized as 0.5 ml per minute to avoid interference at low concentrations.

Effect of injection volume: The process of quantification at very low concentrations is a competition between signal and noise. In order to keep the instrument clean the amount of sample reaching into the mass spectrometer should be kept as low as possible. This means that, only very pure solvents should be used for the mobile phase and the amount of extract injected should be as small as possible, i.e. a small injection volume is preferable. A comparison was made between injecting 20 µL and 10 µL of a recons tituted extract of amoxicillin and clavulanic acid from a 500 ng/ml plasma sample. Since in both cases the amoxicillin and clavulanic acid peak had a similar signal to noise ratio (S/N = 15) it was decided that the injection volume of extracts should be fixed at 10 µL.

Selection of internal standard: Compounds such as cephalexin and dexibuprofen were tried for internal standard and there was no effective response. Finally diclofenac was tried as internal standard which showed effective response with good elution and recovery.

Extraction of analytes from plasma: Since the objective of this study was to develop a simple reliable method that would facilitate analysis of amoxicillin and clavulanic acid in rat plasma in a large number of samples over a relatively short period of time (bioequivalence studies), in a cost effective manner, it was decided to investigate a number of different extraction procedures. It was therefore decided to investigate this procedure as a first approximation. To do this, a pool of blank rat plasma was obtained and spiked with relevant concentrations of amoxicillin and clavulanic acid before extraction. The liquid liquid extraction procedure was attempted with some variations. Aliquots of 250 µL of spiked plasma in a glass tube was added with 50 µL of 300 ng/ml diclofenac potassium and tried with different extraction solvents. The ratio of the solvents and % recovery obtained is shown in table below.

Table.2.Ratio of solvents used and percentage recovery obtained

Solvents	Volume of the solvent	% recovery of analyte	
		Analyte	IS
TBME : Ethyl acetate (70:30)	3 ml	35%	15%
n Hexane : n Butanol (80:20)	3 ml	42%	28%
Ethyl acetate	3 ml	20%	45%
Dichloromethane	3 ml	55%	60%

The Solid phase extraction procedure was also carried using solid phase extraction cartridges of Strata-X 33µ m polymeric reversed phase (30mg/1ml) from phenomenex.

Solid phase extraction involves the following procedure: The cartridges were first conditioned using 1 mL of 100% Methanol. Then Equilibrated with 100% Water. Aliquot of 250 µl of spiked plasma with 5 0 µL of 200 ng/ml diclofenac potassium (Internal standard) was added. Then washed with 1 mL of 5% Methanol / 5%HCl. And finally eluted with 100% Methanol. Evaporate it in Low volume evaporator at 40ºC under nitrogen. Reconstitute the residue with 250 µL of Methanol: water (50:50). The Solid phase extraction method is costly and time consuming process, However since the selectivity of MS/MS detection is same as SPE when sample is extracted using LLE method since the objective of this study was to develop a simple reliable method that would facilitate analysis of amoxicillin and clavulanic acid in body fluids in a cost effective manner. So protein precipitation combined with LLE method using dichloromethane was selected because of its greater recovery.

The final optimized extraction procedure was as follows: Transfer 200 µL of plasma sample into a polypropylene vial. Add 50 µL of 300 ng/mL internal standard solution. Add 50 µ l of 0.1 M citric acid. Vortex for about 30 sec. Add 400 µ l of acetonitrile. Vortex for about 30 sec. Centrifuged for about 3 minutes at 4000 rpm and 10ºC. Collect the supernatant liquid in another glass tube. Add 200 µ l of Milli-Q. Vortexed for about 10 minutes. Add 1 ml of dichloromethane. Vortex for about 30 sec. Centrifuged for about 3 minutes at 4000 rpm and 10ºC. Transfer 200 µ l of the upper layer into a vial and used for analysis.

Table.3.Optimized chromatographic conditions

Mass	API 3200
Ion source	Turbo ion spray
Polarity	Negative ion mode
Detection ions	
Amoxicillin	364 amu (parent), 223.5 amu (product)
Clavulanic acid	198.2 amu (parent), 136.2 amu (product)
Diclofenac (IS)	296.1 amu (parent), 250.1 amu (product)
Column	Symmetry C18, 50x4.6, 5 µ
Column oven temperature	30±5ºC
Auto sampler temperature	10±2ºC
Mobile phase	0.5 mL/min
Volume of injection	10 µL
Retention time	
Amoxicillin trihydrate	0.77 minutes
Clavulanic acid	0.86 minutes
Diclofenac	1.17 minutes
Run time	3 minutes
MRM Conditions	
Curtain Gas (CUR)	15.0 psi
Collision Gas (CAD)	10 psi
Temperature (TEM)	4500 °C
Ion spray voltage (IS)	350 V
Ion Source Gas 1 (GS1)	55 psi
Ion Source Gas 2 (GS2)	10 psi
Interface heater (ihe)	ON
Resolution Q1	Unit
Q3	Unit

Mobile phase preparation:

Solvent A: Acetonitrile

Solvent B: To 100 mL of water add 0.100 mL of Formic acid, sonicate and filter the solution using 0.22µ membrane filter.

Premixing: The mobile phase was prepared by mixing Solvent A and Solvent B in the ratio of 90:10 v/v and ultrasonicated for 5 minutes.

Preparation of 50% Methanol in water solution (Diluent): To 50 mL of methanol, add 50 mL of water sonicate and filter the solution using 0.22µ membrane filter.

Needle wash: Methanol: water (30:70 v/v)

Seal wash: Water (MilliQ)

Preparation of Standards and Samples:

Amoxicillin trihydrate Standard Stock Solution: Weigh and transfer 200 mg of Amoxicillin trihydrate working standard in to a 100 ml standard flask and dissolved with 50 ml water and make the final volume with water (2 mg/mL).

Clavulanate Standard Stock Solution: Weigh and transfer 100 mg of Lithium clavulanate working standard in to a 100 ml standard flask and dissolved with 50ml water and make the final volume with water (1mg/mL).

Diclofenac Stock Solution (Internal Standard): Weigh and transfer 100mg of Diclofenac potassium working standard in to a 100 ml standard flask and dissolved with 50ml water and make the final volume with water (1mg/mL).

Spiking Solution for CC: Different concentrations of amoxicillin and clavulanic acid (10.0, 20.0, 80.0, 160.0, 240.0, 400.0, 600.0, 800.0 µg /mL for amoxicillin and 2.0, 4.0, 12.0, 24.0, 60.0, 80.0, 100.0, 120.00 µg/mL for clavulanic acid) were prepared from standard stock solution using mixture of Methanol and water (50:50) as diluent.

Spiking Solution for QC: Different concentrations of amoxicillin and clavulanic acid (10.0, 30.0, 400.0, 600.0 µg /mL for amoxicillin and 2.0, 4.0, 60.0 and 90.0 for clavulanic acid) were prepared from standard stock solution.

Preparation of Calibration Curve Standards (CC): The calibration standards of amoxicillin and clavulanic acid (0.50, 1.0, 4.0, 8.0, 12.0, 20.0, 30.0, 40.0 µg/mL for amoxicillin and 0.100, 0.200, 0.600, 1.20, 3. 0, 4.0, 5.0, and 6.0 µg/mL for clavulanic acid) were prepared

from spiking solution using blank plasma as diluent. The aliquots of the prepared solutions were transferred to different vials and stored at –70 ± 5ºC until processing.

Preparation of Quality Control (QC) Samples: The quality control samples of amoxicillin and clavulanate {0.500 µg/mL Lower limi t of quality control (LLOQ), 1.5 µg/mL low quality control (LQC), 20.0 µg/mL mid quality control and 30.0 µg/mL High quality control (HQC) samples for amoxicillin and 0.100 µg/mL Lower limit of quality control (LLOQ), 0.200 µg/mL low quality control (LQC), 3.0 µg/mL mid quality control and 4.5 µg/mL High qua lity control (HQC) samples for clavulanic acid were prepared from spiking solution using blank plasma as diluent. The aliquots of the prepared solutions were transferred to different vials and stored at –70 ± 5 º C until processing.

Preparation of Plasma Samples: At the time of analysis, the samples were removed from the deep freezer and kept in the room temperature and allowed to thaw. The resulting solution was processed by employing the procedure given below.

Transfer 200 µL of plasma sample into a polypropylene ria vial. Add 50 µL of 300 ng/mL internal standard solution. Add 50 µ l of 0.1 M citric acid. Vortex for about 30 sec. Add 400 µ l of acetonitrile. Vortex for about 30 sec. Centrifuged for about 3 minutes at 4000 rpm and 10ºC. Collect the supernatant liquid in another glass tube. Add 200 µ l of Milli-Q. Vortexed for about 10 minutes. Add 1 ml of dichloromethane. Vortex for about 30 sec. Centrifuged for about 3 minutes at 4000 rpm and 10ºC. Transfer 200 µ l of the upper layer into a vial and used for analysis.

System Suitability Sample: Perform the system suitability test using the chromatographic device during the following cases. At the start of each batch of method validation and subject sample analysis. After completion of repairs of major malfunctions (any malfunction which may affect the output of the chromatography) of chromatographic systems in the middle of the method validation or project. After change of column in the middle of a project. If, any components of the chromatographic device is replaced in the middle of a project. Prepare un-extracted standard equivalent to middle level of calibration curve concentration and internal standard. Inject six times on the chromatographic device as per Bioanalytical method (BM). If the system suitability does not pass with six injections perform another six more injections until the system suitability test passes. Record the retention times and responses of the analyte and internal standard present. Evaluate the system suitability by inbuilt system suitability software or manually calculating the mean, standard deviation and coefficient of variation for the retention time and area. The % CV of area ratio of drug and internal standard is ≤3% for single analyte and for ≤5% for multiple analyte. The % CV of retention time of drug and internal standard is 2%. If results do not comply with acceptance criteria, check for system malfunctions and undertake suitable remedial actions and perform the system suitability again.

Method of analysis: The standard solutions, CC standard, QC samples are injected with the optimized chromatographic conditions and the chromatograms were recorded. The quantification of the chromatogram is performed using peak area ratios (response factor) of the drug to internal standard. The calibration curves are constructed for spiked plasma containing amoxicillin and clavulanate and internal standard during validation.

Analytical batch organization: Validation batches contain extracts of calibration standards to obtain a calibration line. Extracts of quality controls to assess the accuracy and precision of the assay method. Prepare the quality control samples 1.8 times of ULOQ with blank plasma to assess the validity of diluting samples with concentrations higher than the highest calibration standard if such samples were to occur during the processing of study samples. Extracts of blank plasma samples to which no internal standard has been added to monitor possible carryover effects from previous injections and for the possible appearance of other interfering peaks. Extracts of zero samples; these are extracts of blank plasma samples spiked with the internal standard. Extracts of on-instrument stability samples to assess the stability of the analytes in the reconstituted extracts while the samples are being assayed. Freeze and thaw stability samples to assess the stability of the analyte in samples that have undergone more than one freeze and thaw cycle. Bench-top stability samples to assess the stability of the analytes in the thawed plasma samples while they are standing at room temperature on the bench-top awaiting extraction.

Parameters assessed during validation were System suitability, Selectivity, Sensitivity, Ruggedness, Recovery, Linearity, Precision, Accuracy, Stability and Matrix effect.

System suitability: System suitability was performed by injecting 6 sets of known concentrations of aqueous mixture for analyte and IS, CV% for retention time (RT) and area ratio (Analyte area/IS area) were calculated.

Selectivity: Selectivity was assessed by analysing blank plasma samples obtained from six different sources with six samples at LLOQ concentrations spiked using the biological matrix of any one source.

Sensitivity: Sensitivity is determined by limit of quantitation by analyzing six replicates of LLOQ that can be measured with acceptable accuracy and precision.

Ruggedness: Ruggedness of the method was evaluated by changing Column with same make and configuration using one accuracy precision batch (6 replicates of LLOQ, LQC, MQC & HQC).

Recovery: Recovery of the developed method was evaluated by analysing six replicates for analyte along with internal standard by comparing the analytical results for extracted samples at three concentrations (equivalent to LQC, MQC and HQC) with unextracted samples that represent 100% recovery. The % recovery of analyte and IS were calculated using appropriate chromatographic conditions.

Linearity: To determine the linearity of the selected range a calibration curve consists of a blank sample (matrix sample processed without internal standard), blank with IS and 8 non-zero standards covering the expected range (coded as CC1 through to CC7/CC8) were analysed. A linear equation will be determined to produce the best fit for the concentration/response relationship. Selection of weighting and use of regression equation were justified.

Accuracy: Accuracy of the method is determined by analyzing six replicates of LLOQ, LQC, MQC, and HQC in different occasions.

Precision: Precision of the method is determined by analyzing six replicates of LLOQ, LQC, MQC, and HQC samples. Inter batch precision and intra batch precision also evaluated.

Stability: Freeze and thaw stability

Six replicates of each LQC, MQC, and HQC stored at -70oC were thawed completely unassisted at room temperature and refrozen immediately to -70oC. This cycle was repeated three times with 12 hour intervals and the samples were extracted and analysed with freshly prepared calibration curve and quality control samples.

Bench top stability: The stability of samples on the bench i.e., when kept outside the freezer were studied to know the stability of samples at room temperature. Six replicate of LQC & HQC were kept at room temperature for 6 hrs these samples were processed and analyse with a freshly processed calibration curve.

Stock solution stability (Short term stability): The stability of stock solutions of analyte and IS at room temperature for 6 hours was evaluated by comparing with fresh solutions (zero time solutions) response.

Stock solution stability (Long term stability): The stock solutions are refrigerated for the relevant period, and the stability of the solution were compared with the instrument response with that of freshly prepared solutions.

Long term stability: Long term stability of plasma sample at -70ºC were estimated by analysing six replicates of stored LQC and HQC samples with a freshly prepared calibration curve standards.

Dilution integrity: Dilution integrity test was done by diluting, 1.8 times the ULOQ concentration in the ratio of 50:50 and 25:75 with matrix blank. This test was performed using 6 replicates. Concentration obtained was multiplied with dilution factor 2 (or) 4 to get the actual concentration.

Matrix effect: It has been noted that coeluting, undetected endogenous matrix components may reduce the ion intensity of the analyte and adversely affect the reproducibility and accuracy of the LCMS/MS assay.

In order to determine whether this effect (called the Matrix Effect) is present or not, 6 different plasma pools were extracted and then spiked with standard solution concentration equal to LQC (post extracted spiked sample). Prepared standard solution concentration equal to LQC. These samples were injected and response of samples and standard were compared.

3. RESULTS AND DISCUSSION

An LC-MS/MS method for the determination of amoxicillin and clavulanic acid in Albino rat's plasma was developed and validated according to currently accepted FDA guidelines of Bioanalytical method validation. The following parameters were tested;

Chromatography: The mass spectrum of amoxicillin, clavulanic acid and diclofenac parent ion and product ions are given in figure 1, 2, 3, 4, 5 and 6. A typical chromatogram obtained from a processed blank Albino rat's plasma sample is presented in Figure 7 and Representative chromatograms of the lower limit of quantitation and upper limit of quantitation samples are given in Figures 8 and 9 respectively.

The retention times of amoxicillin, clavulanic acid and the internal standard were approximately 0.77, 0.86 and 1.17 minutes, respectively. The overall run time is 3 minutes.

Calibration curves: Calibration curves are found to be consistently accurate and precise over the 0.500 µg/ml to 40 µg/mL for amoxicillin and 0.100 µg/ml to 6.0 µg/mL for clavulanic acid respectively. The correlation coefficient (r2) is greater than or equal to 0.99, back-calculations were made from the calibration curves to determine amoxicillin and clavulanic acid concentrations of each calibration standard. Data's are presented in Tables 1 and a typical calibration curve is presented in Figure 10.

Weighing factor of regression method: To determine whether to fit the data for the calibration curves by weighed or unweighed linear regression, the functional dependence of the natural logarithm of standard deviation of the analyte/internal standard area ratio on

natural logarithm of sample concentration was evaluated.

Within-run accuracy and precision: Within-run accuracy and precision evaluations were performed by analyzing replicate concentrations of amoxicillin and clavulanic acid in Albino rat's plasma. The run consisted of a calibration curve plus 6 replicates of each of lower limit of quantitation (LLOQ) low (LQC), medium (MQC) and high (HQC) quality control samples.

For the within-run accuracy and precision the coefficients of variation for LLOQ, LQC, MQC, & HQC samples were 2.94, 1.31, 5.64 and 6.49 % for amoxicillin and 7.88, 6.79, 5.50, 7.33 for clavulanic acid respectively. The within-run percentages of nominal concentrations for LLOQ, LQC, MQC, and HQC were 94.77, 98.38, 103.18, 104.59 % for amoxicillin and 98.50, 99.83, 101.39 and 97.98 for clavulanic acid respectively. Results are presented in Table 2 and 3.

Between-run accuracy and precision: The between-run accuracy and precision evaluation were assessed by the repeated analysis of Albino rat's plasma samples containing different concentrations of amoxicillin and clavulanic acid on separate occasions. A single run consisted of a calibration curve plus 6 replicates of each of lower limit of quantitation (LLOQ) low (LQC), medium (MQC) and high (HQC) quality control samples. For between-run accuracy and precision the coefficients of variation for LLOQ, LQC, MQC, & HQC samples were 6.41, 2.32, 5.42 and 3.03 % for amoxicillin and 6.26, 2.98, 5.74, 6.43 for clavulanic acid respectively. The within-run percentages of nominal concentrations for LLOQ, LQC, MQC, and HQC were 95.33, 100.89, 98.92, 101.36 % for amoxicillin and 102.67, 102.00, 101.89 and 101.19 % for clavulanic acid respectively.

Recovery: Recovery of amoxicillin and clavulanic acid was evaluated by comparing mean analyte responses of six processed samples of low (LQC), medium (MQC) and high (HQC) quality control samples to mean analyte responses of six appropriately diluted pure standard solutions. Mean recovery values are 82.57, 84.35 and 81.21 % for amoxicillin low, medium and high quality control levels, 89.78, 91.88 and 92.07 for clavulanic acid low, medium and high quality control levels respectively. The global mean recovery for amoxicillin and clavulanic acid were 82.71 % and 91.25 %, respectively. For the internal standard, mean internal standard responses of eighteen processed samples was compared to the mean internal standard responses of eighteen appropriately diluted pure internal standard solutions. Mean recovery value for the internal standard is 80.96%.

Matrix selectivity: Randomly selected blank Albino rat's plasma sources were carried through the sample processing procedure and chromatographed to determine the extent to which endogenous Albino rat's plasma components may contribute to chromatographic interference with the analyte or the internal standard. No significant interference was observed in 6 different sources of Albino rat's plasma samples.

Lower limit of quantitation (lloq) / sensitivity: The lower limit of quantitation, i.e. the lowest standard level with a coefficient of variation less than 20 %, is 0.500 µg/mL with a coefficient of variation of 8.20 % and a percentage of nominal concentration of 97.20 % for amoxicillin and 0.100 µg/mL with a coefficient of variation of 10.15 % and a percentage of nominal concentration of 97.45 % for clavulanic acid.

Dilution integrity: Six replicates of the dilution quality control samples (DQC) were respectively diluted two and four times in Albino rat's plasma prior to sample processing and analysis. The calculated concentrations for amoxicillin 50:50, 25:75 ratio, including the dilution factor, yielded coefficients of variation of 5.87 and 7.82 % respectively, Percentages of nominal concentrations are 98.17 and 101.52 %, respectively. The calculated concentrations for clavulanic acid 50:50, 25:75 ratio, including the dilution factor, yielded coefficients of variation of 9.67 and 9.14 % respectively, percentages of nominal concentrations are 97.55 and 98.18 %, respectively.

Matrix effect: Six different plasma samples were extracted and then spiked with standard solution concentration equal to LQC (post extracted spiked sample). Prepared standard solution concentration equal to LQC. These samples were injected and response of samples and standard were compared. No matrix effect was found in 6 different sources of Albino rat's plasma tested.

Stability:

Freeze - thaw stability at -70 °C: Samples were prepared at low (LQC) and high (HQC) quality control levels, aliquoted and frozen at -70 °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 6 replicates of stability samples and analyzed in a single run. Amoxicillin and clavulanic acid was found to be stable in Albino rat's plasma after three thaw cycles at -70 °C with % stability of 102.384 % and 103.05 % for amoxicillin LQC and HQC samples respectively. 101.583 % and 94.42 % for clavulanic acid LQC and HQC samples respectively.

Bench top stability: Samples were prepared at low (LQC) and high (HQC) quality control levels. Six replicates of low and high quality control samples were left at room temperature (bench top) for approximately 6 hours (stability samples). A calibration curve and 6

replicates of low and high quality control samples (comparison samples) were freshly processed with all stability samples and analyzed in a single run. Concentrations were calculated to determine % stability over time. Amoxicillin and clavulanic acid was found to be stable in Albino rat's plasma for 6 hours at room temperature with % stability 100.88 % and 103.58 % for amoxicillin LQC and HQC samples and 102.62 % and 103.05 % for clavulanic acid LQC and HQC samples respectively.

Short-term stability of analyte in solution at room:

Temperature: Solutions of amoxicillin and clavulanic acid were prepared and aliquoted. Some of the aliquots were immediately stored at (2-10 °C) refrigerator (comparison samples) and the others were kept at room temperature for 6 hours and then stored at (2-10 °C) (stability samples). Replicates of stability samples and comparison samples were diluted at approximately the same analyte concentration and analyzed in a single run, analyte responses were used to determine % stability over time. Amoxicillin and clavulanic acid was found to be stable for 6 hours at room temperature with a % stability 98.82 % and 99.03 % respectively.

Short-term stability of internal standard in solution at room:

Temperature: Solutions of internal standard were prepared and aliquoted. Some of the aliquots were immediately stored at 2-10 °C (comparison samples) and the others were kept at room temperature for 6 hours. Replicates of stability samples and comparison samples were diluted at approximately the same internal standard concentration and analyzed in a single run. Internal standard responses were used to determine % stability over time. Internal standard is found to be stable in methanol for 6 hours at room temperature with% stability 96.84 %.

Long-term stability of analyte in solution at (2-10 °C): Solutions of amoxicillin and clavulanic acid were prepared, aliquoted and stored at -refrigerator (stability samples). Replicates of stability samples and comparison samples were diluted at approximately the same analyte concentration and analyzed in a single run. Analyte/internal standard response ratios were used to determine % stability over time, amoxicillin and clavulanic acid is found to be stable in methanol for 30 days at refrigerated condition with % stability 99.02 and 97.58 % respectively.

Long-term stability of analyte in matrix at -70°C: Samples were prepared in Albino rat's plasma at low (LQC) and high (HQC) quality control levels, aliquoted and then stored at -70°C (stability samples). A calibration curve and 6 replicates of low and high quality control samples (comparison samples) were freshly processed with 6 replicates of stability samples and analyzed in a single run. Mean concentrations of the stability samples were compared to the mean concentrations of comparison samples, amoxicillin and clavulanic acid is found to be stable in Albino rat's plasma for 90 days at -70 °C with % stability of 102.18 % and 99.32 % for amoxicillin LQC and HQC samples and 99.38 % and 107.68 % clavulanic acid LQC and HQC samples respectively.

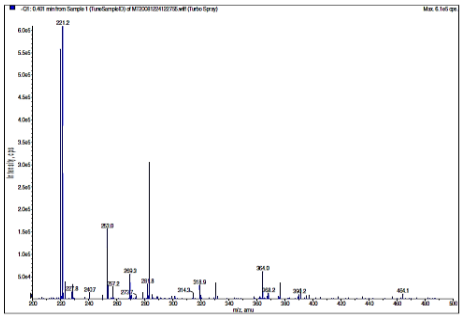


Figure 1: Mass spectrum of amoxicillin (parent ion scan)

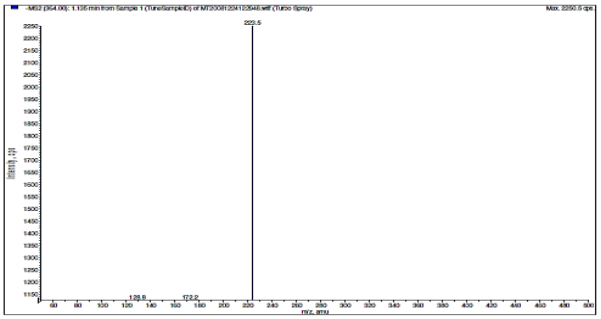


Figure 2: Mass spectrum of amoxicillin (product ion scan)

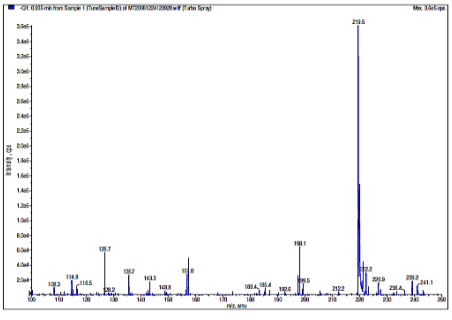


Figure.3.Mass spectrum of clavulanic acid (parent ion scan)

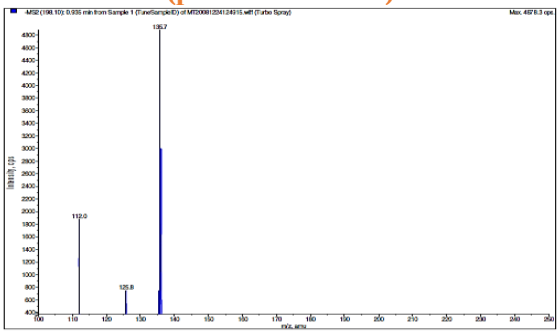


Figure.4.Mass spectrum of clavulanic acid (Product ion Scan)

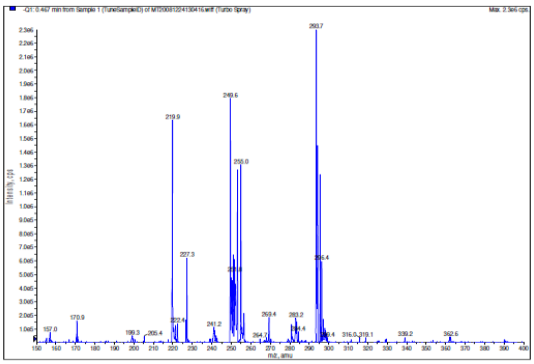


Figure.5.Mass spectrum of diclofenac (parent ion scan)

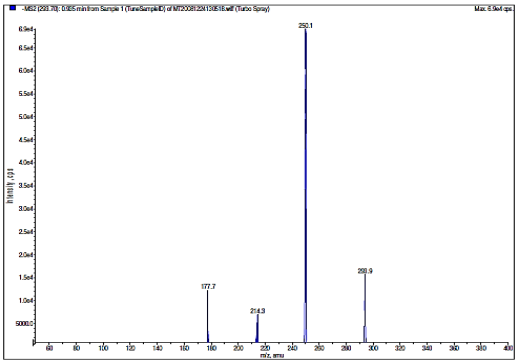


Figure.6.Mass spectrum of diclofenac (product ion scan)

Table.4.Within - run accuracy and precision for amoxicillin

S.No	LLOQ (0.5µg)		LQC (1.5 µg)		MQC (20 µg)		HQC (30 µg)	
	Concentration found	Accuracy	Concentration found	Accuracy	Concentration found	Accuracy	Concentration found	Accuracy
1	0.478	95.60	1.451	96.73	20.04	100.20	32.54	108.47
2	0.485	97.00	1.455	97.00	22.44	112.20	28.25	94.17
3	0.457	91.40	1.478	98.53	19.56	97.80	32.56	108.53
4	0.456	91.20	1.501	100.07	19.55	97.75	30.14	100.47
5	0.488	97.60	1.481	98.73	21.54	107.70	30.87	102.90
6	0.479	95.80	1.488	99.20	20.69	103.45	33.91	113.03
Mean	**0.47**		**1.48**		**20.64**		**31.38**	
%CV	**2.94**		**1.31**		**5.64**		**6.49**	
Nominal	**94.77**		**98.38**		**103.18**		**104.59**	

Table.5.Within - run accuracy and precision for clavulanic acid

S.No	LLOQ (0.1µg)		LQC (0.2 µg)		MQC (3.0 µg)		HQC (4.5 µg)	
	Concentration found	Accuracy	Concentration found	Accuracy	Concentration found	Accuracy	Concentration found	Accuracy
1	0.098	98.00	0.213	106.50	3.145	104.83	4.125	91.67
2	0.089	89.00	0.211	105.50	3.110	103.67	4.015	89.22
3	0.092	92.00	0.198	99.00	3.240	108.00	4.587	101.93
4	0.102	102.00	0.189	94.50	2.987	99.57	4.286	95.24
5	0.099	99.00	0.179	89.50	2.756	91.87	4.871	108.24
6	0.111	111.00	0.208	104.00	3.012	100.40	4.571	101.58
Mean	**0.100**		**0.20**		**3.04**		**4.41**	
%CV	**7.88**		**6.79**		**5.50**		**7.33**	
Nominal	**98.50**		**99.83**		**101.39**		**97.98**	

Table.6.Between - run accuracy and precision for amoxicillin

S.No	LLOQ (0.5µg)	LQC (1.5 µg)	MQC (20 µg)	HQC (30 µg)
	Concentration found	Concentration found	Concentration found	Concentration found
Accuracy & Precision Day 1 Analyst 1	0.47	1.48	20.64	31.38
Accuracy & Precision Day 1 Analyst 2	0.51	1.51	20.13	30.29
Accuracy & Precision Day 2 Analyst 1	0.45	1.55	18.58	29.55
Mean	0.48	1.51	19.78	30.41
% CV	6.41	2.32	5.42	3.03
% Nominal	95.33	100.89	98.92	101.36

Table.7.Between - run accuracy and precision for clavulanic acid

S.No	LLOQ (0.1µg)	LQC (0.2 µg)	MQC (3.0 µg)	HQC (4.5 µg)
	Concentration found	Concentration found	Concentration found	Concentration found
Accuracy & Precision Day 1 Analyst 1	0.100	0.2	3.04	4.41
Accuracy & Precision Day 1 Analyst 2	0.098	0.211	3.24	4.36
Accuracy & Precision Day 2 Analyst 1	0.110	0.201	2.89	4.89
Mean	0.10	0.20	3.06	4.55
% CV	6.26	2.98	5.74	6.43
% Nominal	102.67	102.00	101.89	101.19

4. CONCLUSION

Based on the data presented in this report, it can be concluded that the present method is validated for the estimation of amoxicillin and clavulanic acid in Albino rat's plasma over concentration range of 0.500 µg to 40 µg/mL and 0.100 µg to 6.0 µg/mL respectively. The precision and accuracy are very much within the prescribed limits in this concentration range. Expected recoveries were observed in the present processing technique for LQC, MQC, and HQC. The drug is found to be very stable to the effect of three freeze-thaw cycles and up to 6 hours on the bench-top. The values obtained from system suitability studies demonstrated the suitability of the system for the analysis of the amoxicillin and clavulanic acid in plasma. Limit of quantitation for amoxicillin and clavulanic acid is 0.500 µg/mL and 0.100 µg/mL respectively, which shows that the developed method has adequate sensitivity and also more than 50 samples, can be processed at a time without affecting the assay values. The long-term stability is established for these molecules for the required period of subject samples analysis. The method can be applied for bioavailability studies and for analyzing samples in animal study.

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