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Research Article

A NOVEL AND HIGH-THROUGHPUT METHOD FOR THE SIMULTANEOUS DETERMINATION OF AMOXICILLIN AND CLAVULANIC ACID IN HUMAN PLASMA BY LIQUID CHROMATOGRAPHY COUPLED WITH TANDEM MASS SPECTROMETRY

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ABSTRACT

A simple, rapid, specific and sensitive liquid chromatography tandem mass spectrometric method has been developed and validated for the simultaneous determination of Amoxicillin and Clavulanic acid from 250µL of human plasma by Solid Phase Extraction. Hydrochlorothiazide was used as an internal standard. Quantified by the transition, 364.060→223.160, 198.061→136.000 for Amoxicillin and Clavulanic acid respectively (Fig 1 and 2) and detected by TSQ Quantum Discovery max triple quadrupole mass spectrometer. Detection was carried out by using ESI source in negative polarity. Chromatographic separation of analyte and internal standard were carried out by reverse phase C18 column at the flow rate of 0.5mL/min with mobile phase of Acetonitrile: 2 mM Ammonium Acetate (70:30) v/v. The assay of Amoxicillin and Clavulanic Acid were linear over the range of 0.103µg/mL to 6.822µg/mL and 0.046µg/mL to 3.026µg/mL respectively with a precision of ≤9.43 % and ≤11.75 % respectively, Mean extraction recovery obtained were 82.04% and 87.14% respectively. Samples were stable at room temperature for 6 hrs and also stable at three freeze-thaw cycle. The method has been used to perform pharmacokinetic and bioequivalence studies in human plasma.

Keywords: Amoxicillin, Clavulanic acid, LC-MS/MS, Solid Phase Extraction, Human Plasma.

INTRODUCTION

Amoxicillin ((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, $C_{16}H_{19}N_3O_5S.3H_2O$, MW 419.45 g/mol) 1 is an analog of ampicillin, derived from the basic penicillin nucleus, 6-aminopenicillanic acid. Amoxicillin trihydrate proved to be more stable at an acidic pH, while increasing the pH decreased its stability 2. For treatment of infection caused by β-lactamase-producing bacteria that are resistant to amoxicillin when administered alone, it is frequently combined with the β-lactamase inhibitor, clavulanic acid. Clavulanic acid administered as potassium salt (potassium (Z)-(2R, 5R)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]-heptane-2-carboxylate, $C_8H_8KNO_5$, MW 237.25 g/mol) 1 is originally produced by fermentation of Steptomyces clavuligerus. The pH of a 1% aqueous solution of Clavulanic acid potassium is 5.5 – 8.0 4 Maximum stability of potassium clavulanate sodium is experienced at neutral pH, while its decomposition rate was significantly higher at acidic and alkaline pH values 2.

Analytical methods for amoxicillin and clavulanic acid include microbiological assay 5 ultraviolet spectrometry 6 High performance liquid chromatographic (HPLC) methods were developed for more specific assay. Early HPLC methods involved pretreatment of amoxicillin and Clavulanic acid 6. Later HPLC methods using reversed-phase Chromatography with ultraviolet (UV) or amperometric detection 7 have been developed.

Recently, LCMS separation followed by selective mass spectrometric detection has become a method of choice8 used HPLC–tandem mass spectrometry in a pharmacokinetic study of an amoxicillin/clavulanate formulation; however, they did not describe the mass spectrometric conditions. LC–tandem mass spectrometry for determination of amoxicillin in plasma 8, amoxicillin and Clavulanic acid determination in animals [9].In this paper, authors present a simple, fast, and sensitive analytical method for simultaneous determination of amoxicillin and clavulanic acid in human plasma using HPLC with mass spectrometry.

MATERIALS AND METHDOS

Materials and Reagents

Amoxicillin and Clavulanic acid is provided by Maneesh exports at Mumbai. The Internal Standard Hydrochlorothiazide is provided by Maneesh Pharmaceuticals at Mumbai. Methanol (HPLC grade), Acetonitrile (Gradient grade), Orthophosphoric acid (GR grade), Ammonium Acetate (AR grade), Water (Ultra Pure Grade), Ammonia (GR Grade), Formic acid (ULC/MS), PLEXUS 30mg/1cc cartridges (Analchem) were used.

Instruments

The Liquid chromatography coupled with tandem Mass Spectrometer (LC-MS/MS) system consists of a Finnigan Surveyor Autosampler, Surveyor LC Pump Plus solvent delivery system and a column Oven (Thermo Electron Corporation) used for ion separation. The Mass spectrometer was Thermo Scientific TSQ Quantum discovery max Ultra triple stage quadrupole mass spectrometer used for ion detection. An Electron Spray Ionization (ESI) source was used. Data was collected and processed using LC Quan Version. 2.5.6 Data collection and integration software.

Chromatographic Condition

The Liquid Chromatographic separations were carried out by using Zorbax SB, C18, 50X4.6 mm, 5µ column (Agilent). Column temperature was held at 30°C. The auto sampler tray temperature was 10°C. The mobile phase is composed of Acetonitrile: 2 mM Ammonium Acetate (70:30) v/v with flow rate of 0.500 mL/min and the run time is 2.00 min. A typical injection volume was 10.0µL.

MS/MS Detection

Precursor ions for analyte and internal standard were determined from mass spectra obtained by the TSQ mass spectrometer. TSQ mass Spectrometer is includes an electronically-controlled, integrated syringe pump. The MS conditions for Amoxicillin, Clavulanic acid and the internal standard were optimized by separate infusion into the MS at a flow rate of 10µL/min constantly while adjusting MS parameters to achieve maximal intensity. Electro-spray ionization in negative ion mode (ESI-ve) was used for ionization and selective reaction monitoring (SRM) mode was chosen for detection. The optimized precursor ions pairs were m/z 364.060→223.160 for Amoxicillin, m/z 198.061→136.000 for Clavulanic acid and 295.960→268.920 for Hydrochlorothiazide. The optimized MS parameters were as follows: Ion Spray voltage: 5000volt, Sheath gas pressure: 30psi, Auxiliary gas pressure: 15psi, Capillary temperature: 350°C. Collision Pressure: 1.5psi. Peak areas were automatically integrated using LC Quan Version 2.5.6 (Thermo Corporation).

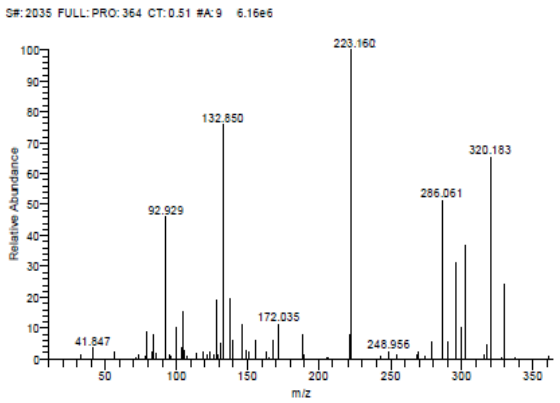

Fig 1: Product ion of Amoxicillin.

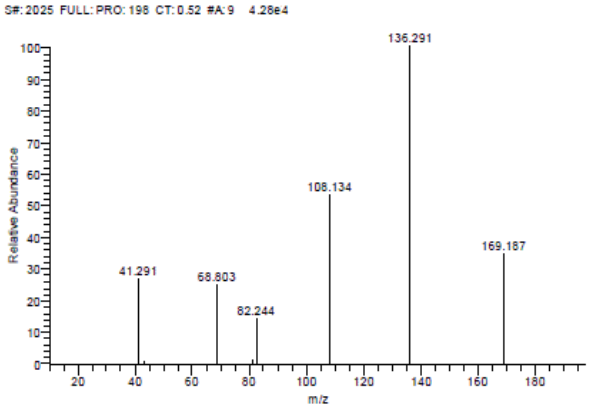

Fig 2: Product ion of Clavulanic acid

Preparation of Calibration standards and quality control samples

The calibration standards and the quality control (QC) samples were prepared from separate stock standard solutions. The concentration of Amoxicillin and Clavulanic acid used for preparing calibration standard was 1003.278 µg/mL and 1008.788 µg/mL, the concentration of Amoxicillin and Clavulanic acid used for preparing quality control was 1002.614 µg/mL and 1008.188 µg/mL were prepared in Ultra Pure Water. The Spiking solutions of calibration standards and quality control concentrations were prepared in Acetonitrile: water (1:1) %v/v. The calibration standard in human plasma samples were prepared from corresponding calibration standard's spiking solutions into blank human plasma to provide concentrations range between 0.103 µg/mL to 6.822 µg/mL for Amoxicillin and 0.046 µg/mL to 3.026 µg/mL for Clavulanic acid. The Quality control samples were prepared from corresponding Quality control spiking solutions in human blank plasma to attain the concentration of 0.105 µg/mL (LOQQC), 0.308 µg/mL (LQC), 3.082 µg/mL (MQC) and 5.815 µg/mL (HQC) for Amoxicillin and 0.047 µg/mL (LOQQC), 0.139 µg/mL (LQC), 1.389 µg/mL (MQC) and 2.621 µg/mL (HQC) for Clavulanic acid. For the spiking typically, the spiking solutions volume of 20µL were spiked into 980µL of human blank plasma. The Internal standard stock solution 999.039 µg/mL of Hydrochlorothiazide was prepared in methanol. Working internal standard solution 4.995 µg/mL was prepared in Acetonitrile: water (1:1) %v/v.

Sample Extraction

A 250.0µL aliquot of plasma samples was mixed with 25.0µL of internal standard working solution (4.995µg/mL) and pre-treatment is performed by adding 0.250mL of 2% Orthophosphoric acid and vortex-mix the samples for approximately 10 secs and apply the following SPE procedure. A commercially available cartridge (Analchem PLEXUS 30mg/1cc) was utilized for extraction. After conditioning and equilibrating the cartridge with 1 mL of methanol and water, the drugs were extracted into the cartridge by loading the pre-treated plasma samples. Then wash the cartridge using 2 x 1.0mL of 0.2% Formic acid in Ultrapure Water, in order to wash the unbound substance in the cartridge and reduce any interfering band in chromatograms. Finally the drug is eluted from the cartridge with 2 x 0.250mL of mobile phase. Then subject 10.0µL samples for chromatographic analysis.

Validation 10, 11

Selectivity and Specificity

Blank human plasma from eight different lots (including one Haemolysed and one Lipimic) were processed without Analyte and internal standard. And with the same eight lots LLOQ level is processed to evaluate the presence of any interference at the retention time of Analyte and Internal standard.

Matrix factor

Evaluate the matrix factor at low and high quality control concentrations, to ensure that the precision, selectivity and sensitivity are not compromised due to a change in matrix. Matrix factor can be termed as the quantitative measurement of the matrix effect. Prepare aqueous mixtures of internal standard and analyte at concentrations representing 100% extraction of internal standard and analyte at low and high QC concentrations. These shall serve as Reference Samples**.** Processed duplicate 8 different lots of blank matrices (from eight individuals, including, one Haemolysed and one Lipimic), without addition of IS. Eluted solution volumes were equally diluted with reference sample; it is compared with respective aqueous reference sample equally diluted with mobile phase.

Calibration Curve and Linearity

The eight-point calibration curve was constructed by plotting, peak area ratio of Amoxicillin, Clavulanic acid to their corresponding internal standard versus Amoxicillin, Clavulanic acid concentrations. A linear regression with weighing factor of linear $1/x^2$ was applied.

Intra and inter-day assay accuracy and precision

Intra-day precision and accuracy were determined by analysis of six replicates of each QC sample (n = 6) at LOQQC, LQC, MQC and HQC concentration levels extracted with a set of standards in one batch. The same procedure was repeated on different day with new samples to determine inter-day precision and accuracy.

Recovery

Recovery is carried out to evaluate the loss of drugs and/or internal standards during sample extraction. The drugs and internal standards area counts from extracted QC samples were compared with corresponding QC's reference sample to evaluate any loss of either drugs or internal standards. No acceptance criteria were applied to this parameter, but it is preferable to observe consistent recovery for all three QC levels except LOQQC.

Stability

Stability of both drugs in different matrices and under different conditions was evaluated. The detailed tests are described below. Stability was assessed by comparing the mean concentration of the stored QC samples with the mean concentration of freshly prepared QC samples. Drug stability in pooled human blank plasma is a function of the storage conditions, the chemical properties of the drug and the matrix. The following tests were performed to evaluate the stability, Short-term and Long-term Stock solution stability, Bench top stability, Freeze and thaw stability, Autosampler stability, Wet Extract Stability, Long Term stability In Matrix.

RESULT AND DISCUSSION

Method development

The main objective was to develop and to validate a novel, rapid, selective and high-throughput LCMS/MS method for the simultaneous determination of Amoxicillin and Clavulanic acid. During MS tuning and compound optimization, it was found that

Amoxicillin is better detected in the positive ion mode and negative ion mode. But Clavulanic acid was better detected in the negative ion mode. For simultaneous detection, both the molecules were tuned in the negative ion mode.

In the optimization of chromatographic condition, more critical and practical problems were resolved during the stage of method development. Clavulanic acid had sensitivity and repeatability problem in most of the mobile phases which was reported as earlier. A mobile phase composition of Acetonitrile: 2mM Ammonium Acetate (70:30) v/v was found to be more optimistic. With the same phase conditions very low quantification levels were achieved (0.103 µg/mL for Amoxicillin and 0.046 µg/mL for Clavulanic acid), which was lower than that of the published LCMS method. More over a mass spectrometer with Triple quadrupole was used for achieving the more selective detection, whereas the published LCMS method was based on single quadrupole detection. Triple quadrupole systems have more advantages compare to single quadrupole like higher selectivity, better signal to noise ratio, wider linear range of quantification, better accuracy and reproducibility especially at low concentrations and more reliable identification of detected analytes using Multiple Reaction Monitoring (MRM) in comparison to Selected Ion Monitoring (SIM). Hydrochlorothiazide was chosen as an internal standard and found to show good repeatability and consistency with the optimized chromatographic conditions. Column Dimensions are function of the shorter analysis time. Different chemistries of columns with different dimensions have been tried, but most of the columns exhibit matrix effect as well as low recovery for Amoxicillin and improper peak shape for Clavulanic acid. Finally, Zorbax SB, C18, 50X4.6 mm, 5µ column, was used where in reproducibility and matrix effect problems were eliminated. Both the analytes were eluted faster and the run times were as low as 2.00 min, which is very lower than the reported methods.

Reported Solid Phase extraction procedure for the simultaneous determination of Amoxicillin and Clavulanic acid were not available. Authors have proposed a new extraction process with advantages of less matrix effect, repeatability and good peak shape. By eliminating the individual methods for extraction, analyst was able to extract the drug simultaneously in one proposed Solid Phase extraction Protocol; where in the final eluent was directly injected into column. The method developed was novel, rapid, selective and high-throughput for the simultaneous determination of Amoxicillin and Clavulanic acid.

Validation

Selectivity and Specificity

No interference from the blank plasma at the retention time of the Analyte and Internal standard.

Matrix Factor

Observed % CV for matrix factor is 5.63 %, 6.66 % and 3.33 % for LQC, 11.81 %, 13.13 % and 13.97 % for HQC for Amoxicillin, Clavulanic acid and Internal standard respectively. All eight matrix lots showed very similar matrix effect for both analyte and their corresponding internal standard.

Calibration Standard and Linearity

For three consecutive batches, the calibration curves showed an overall accuracy of 94.09 % - 109.15 % with % CV of 0.42 % - 7.65 % for Amoxicillin and 94.54 % - 108.12 % with % CV of 0.00 % - 13.38 % for Clavulanic acid. The calibration standard linearity has a regression Coefficient of 0.9957 and 0.9961 for Amoxicillin and Clavulanic acid. The detailed results are shown in Tables 1 and 2. The Calibration Standards met the acceptance criteria.

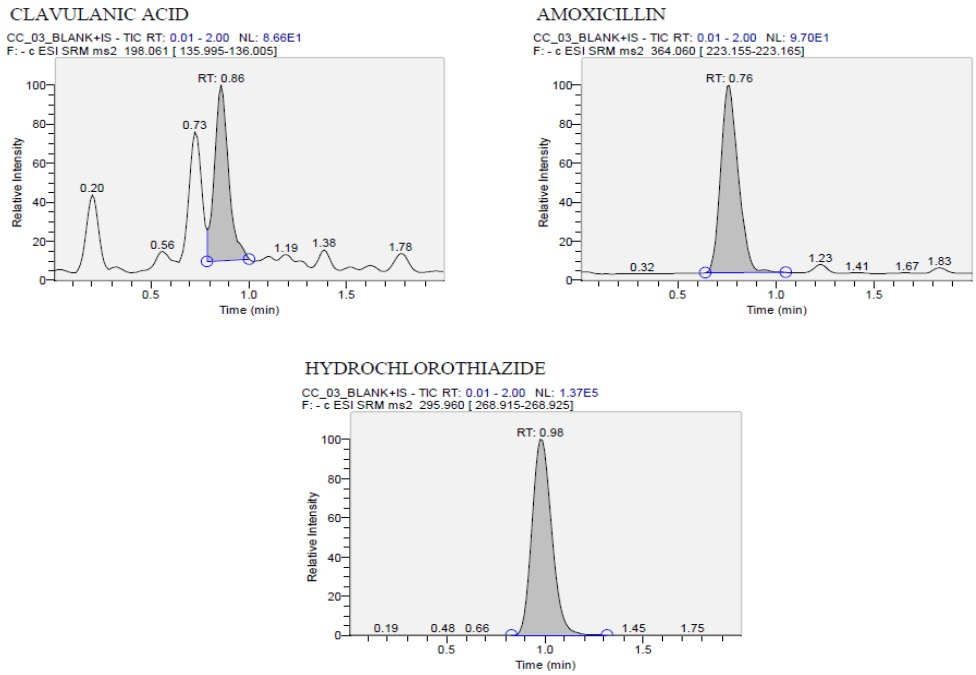

Fig 3: Blank+ IS

Table 1: Accuracy and precision of calibration standards for Amoxicillin

Theoretical concentration (µg/mL)								
Nominal Conc.	**0.103**	**0.206**	**0.344**	**0.688**	**2.292**	**3.82**	**5.458**	**6.822**
Batch 01	0.109	0.187	0.328	0.683	2.341	3.745	6.100	6.593
Batch 02	0.102	0.218	0.329	0.663	2.264	3.767	5.764	6.848
Batch 03	0.107	0.203	0.314	0.651	2.352	3.776	6.008	6.839
Mean	0.106	0.202	0.323	0.665	2.319	3.762	5.957	6.760
Precision	3.40	7.65	2.59	2.43	2.07	0.42	2.91	2.14
Accuracy	102.91	98.38	94.09	96.75	101.18	98.50	109.15	99.09

Table 2: Accuracy and precision of calibration standards for Clavulanic acid

Theoretical concentration (µg/mL)								
Nominal Conc.	**0.046**	**0.092**	**0.153**	**0.305**	**1.017**	**1.695**	**2.421**	**3.026**
Batch 01	0.047	0.087	0.155	0.308	1.034	1.694	2.597	2.802
Batch 02	0.047	0.096	0.152	0.267	1.013	1.741	2.642	3.025
Batch 03	0.047	0.113	0.141	0.29	1.007	1.734	2.614	3.045
Mean	0.047	0.098	0.149	0.288	1.018	1.723	2.617	2.957
Precision	0.00	13.38	4.94	7.13	1.39	1.47	0.87	4.56
Accuracy	102.17	107.25	97.6	94.54	100.1	101.65	108.12	97.73

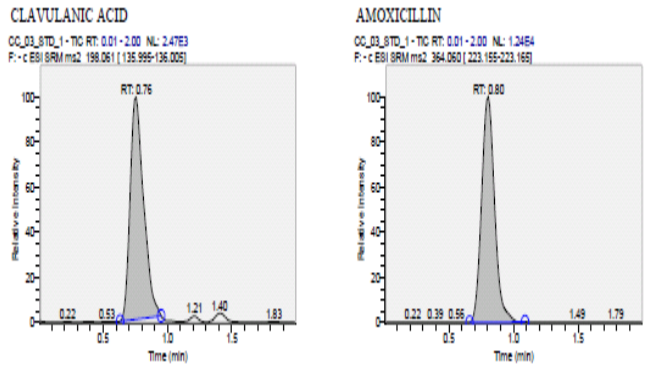

Fig 4: Standard 1

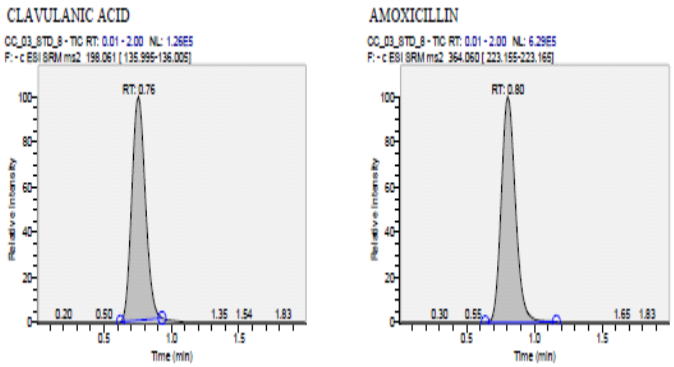

Fig 5: Standard 8

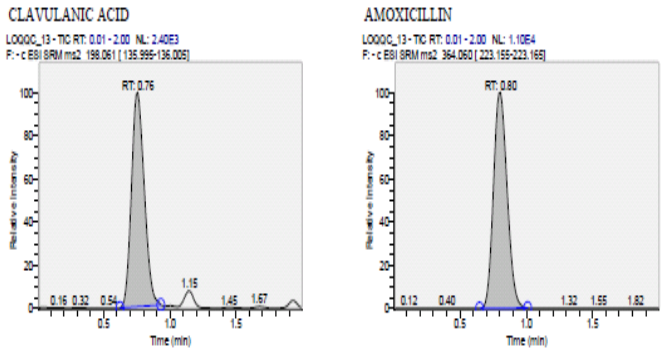

Fig 6: LLOQ QC

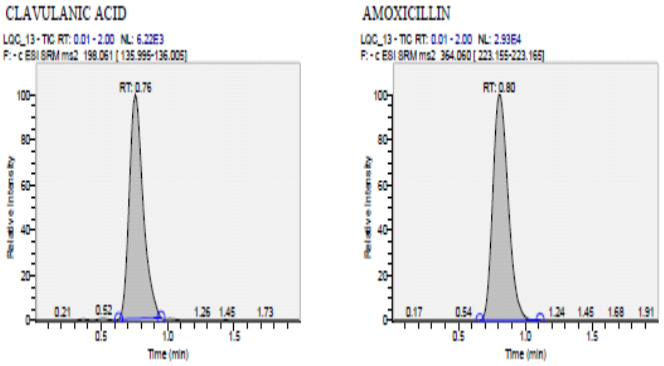

Fig 7: LQC

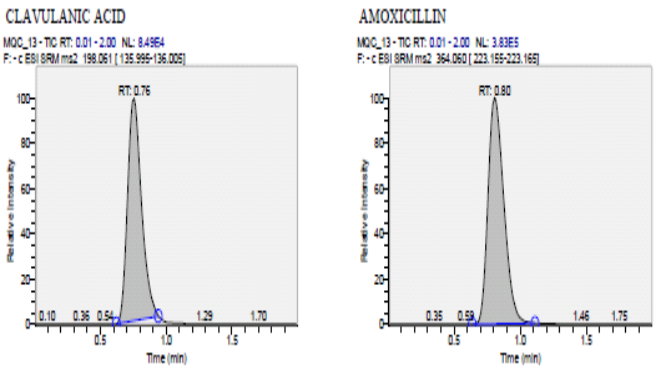

Fig 8: MQC

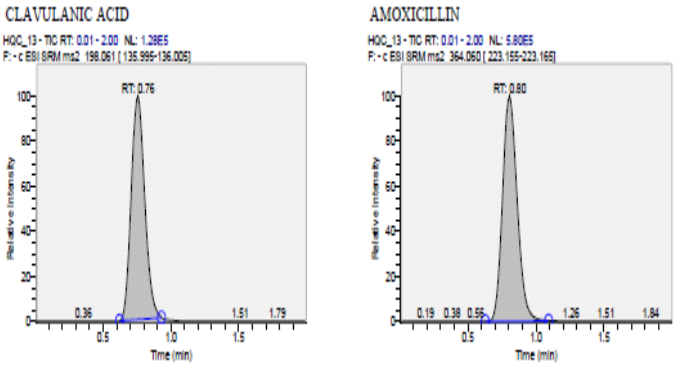

Fig 9: HQC

Accuracy and Precision

Table 3 show inter-day and intra-day assay precision and accuracy for the amoxicillin and clavulanic acid. The method was found to be highly accurate and precise for Amoxicillin and Clavulanic acid. RSD for intra-day and inter-day assay were obtained for all QC levels including LOQQC were met the acceptance criteria.

Recovery

Table 4 show the overall recovery of 82.04 % for Amoxicillin, 87.14 % for Clavulanic acid, and 79.41 % for Hydrochlorothiazide were obtained. Amoxicillin, Clavulanic acid and Hydrochlorothiazide shows consistent recovery results for all three QC levels.

Table 3: Intra and Inter-Day Accuracy and Precision for Amoxicillin and Clavulanic

Analytes Parameter	Amoxicillin	Clavulanic acid
	Intra-Batch Inter-Batch	**Intra-Batch Inter-Batch**
Accuracy (%)	91.02 - 99.41 95.33 – 96.76	93.97 – 117.73 97.55 - 112.29
Precision (%)	2.36 - 11.70 5.34 – 9.43	2.99 – 18.59 5.82 - 11.69

Table 4: Recovery of Analyte and IS

Recovery (%)			
QC Level	**Amoxicillin**	**Clavulanic acid**	**Hydrochlorothiazide**
LQC	80.78	89.24	80.81
MQC	84.81	89.03	77.00
HQC	80.52	83.16	80.42
% Recovery	82.04	87.14	79.41
%CV	2.93	3.96	2.64

Stability

Stability of Amoxicillin, Clavulanic acid in human plasma under different conditions was evaluated. The detailed results are shown in Table 5 and 6 as seen from the table, three freeze/thaw cycles, 6hrs room temperature storage and 41hrs autosampler stability has been established. In addition, 8 days stability for standard stock solutions and wet extract stability shown for 41 hrs were established. All of these demonstrate the ruggedness of the method.

Table 5: Stability of Amoxicillin

Parameter	Bench Top Stability	Auto Sampler Stability	Wet extract Thaw Stability	Freeze and Stability
QC Levels	LQC HQC	LQC HQC	LQC HQC	LQC HQC
Precision	9.44 3.25	5.84 1.77	5.41 3.65	6.2 1.2
% Stability	103.38 103.62	94.02 96.36	96.13 99.98	96.73 100.19

Table 6: Stability of Clavulanic Acid

Parameter	Bench Top Stability	Auto Sampler Stability	Wet extract Stability	Freeze and Thaw Stability
QC Levels	LQC HQC	LQC HQC	LQC HQC	LQC HQC
Precision	11.16 2.75	9.53 1.92	6.42 3.43	6.14 1.28
% Stability	104.42 104.96	98.72 98.00	100.64 100.50	97.98 98.78

CONCLUSION

A rapid and simple LC-MS/MS method has been described for simultaneous determination of amoxicillin and clavulanic acid in human plasma. This method is linear over the range of 0.103µg/mL to 6.822µg/mL for amoxicillin and 0.046µg/mL to 3.026µg/mL for Clavulanic acid. Using Zorbax SB, C18 (50x4.6mm), 5µ column (Agilent), the chromatographic elution step is undertaken in a short time with high resolution. The total run time is 2.00 min. Hence, this method is useful for the high-throughput analysis of subject samples. Expected recoveries were observed in the present processing technique for all three QC levels. With the usage of solid phase extraction procedure, the matrix effect was reduced and also there is no relative matrix effect in quantitative analysis. There is no stability problem in storage condition at -20° C for long term analysis of subject samples. The values obtained from system suitability demonstrated the suitability of the system for the analysis of the amoxicillin and clavulanic acid. In addition, the use of a simple sample preparation instead of more complex extraction procedures makes this method suitable for pharmacokinetic and bioequivalence studies of amoxicillin and clavulanic acid simultaneously in human plasma.

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