Original Article

An LC–MS–MS method for the simultaneous quantification of amoxicillin and clavulanic acid in human plasma and its pharmacokinetic application

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ABSTRACT

Objective: To develop a simple, sensitive and selective LC–MS–MS method for the simultaneous determination of amoxicillin and clavulanic acid in human plasma using amoxicillin D4 and ampicillin as internal standard (IS).

Method: Amoxicillin and clavulanic acid in plasma were concentrated by solid phase extraction and chromatographed on a C18 column using a mobile phase of acetonitrile: 2 mM ammonium acetate (80:20 v/v) under isocratic condition. Quantitation was performed on a triple quadrupole mass spectrometer by employing electrospray ionization technique and operating in multiple reaction monitoring (MRM) and negative ion mode with mass transitions 364.00/223.00, 198.00/136.00, 368.00/227.00 and 347.90/304.00 for Amoxicillin, Clavulanic acid, Amoxicillin D4 and Ampicillin respectively.

Results: The method was validated over a linear range of 50.43–31500.68 and 25.28–6185.18 ng/mL for amoxicillin and clavulanic acid respectively. The Lower Limit of Quantitation (LLOQ) were 50.43 and 25.28 ng/mL for amoxicillin and clavulanic acid respectively. Inter-batch and intra-batch coefficient of variation across three validation runs (LLOQ, LQC, MQC1, MQC and HQC) was less than 3.55% and 3.07% for amoxicillin and clavulanic acid respectively.

Conclusion: The method was validated and was suitable for the quantitation of amoxicillin and clavulanic acid from plasma samples in a pharmacokinetic study.

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1. Introduction

Co-amoxiclav (Fig. 1) is one of the potent broad spectrum antibiotics in the market today. It is made up of amoxicillin1,2 with beta-lactamase inhibitor clavulanic acid.3 It targets both Gram-positive and Gram-negative organisms especially those who have developed resistance to beta-lactam antibiotics.

Co-amoxiclav’s major component is amoxicillin, which is the
4-hydroxy analog of ampicillin. It acts on the bacterial cell walls by making them more porous. Despite its wide range of germicidal action, organisms produce the enzyme beta-lactamase. Beta-lactamase protects the bacteria from being attacked by amoxicillin. Clavulanic acid, a mild antibacterial agent, helps amoxicillin by competing and irreversibly binding to the bacterial cell wall. When this happens, the targeted bacteria cannot produce beta-lactamase and will become susceptible to amoxicillin.

Amoxicillin (AMX) and clavulanic acid (CLV) are fully dissociated in aqueous solution at physiological pH. Both components are rapidly and well absorbed by the oral route of administration. Absorption of amoxicillin/clavulanic acid is optimized when taken at the start of a meal. Following oral administration, amoxicillin and clavulanic acid are approximately 70% bioavailable.

To date several chromatographic methods, including LC–UV, LC–FL and LC–DAD, capillary electrophoresis and LC–MS–MS, have been developed for individual analysis of amoxicillin in biological fluids. LC–UV, FL, DAD and LC–MS–MS are not sufficiently sensitive (>500 ng/mL) and a large injection volume (>10 μL) and a large volume of plasma (>500 μL) are required for analysis. Among the other methods reported in the literature, reversed-phase liquid chromatography with UV detection involves protein precipitation method for simultaneous extraction of amoxicillin and clavulanic acid. An LC–MS–MS method for simultaneous analysis of amoxicillin and clavulanic acid in plasma has been reported; this method, however, requires three-step extraction and the LLOQ is too high for routine analysis.

We report, for the first time, a fully validated LC–MS/MS assay for the simultaneous quantification of amoxicillin and clavulanic acid in a small volume (200 μL) of human plasma with short run time. This assay was successfully applied to a pilot pharmacokinetic study in healthy volunteers after a single-oral administration of amoxicillin/clavulanic combination. The determined plasma concentrations of both amoxicillin and clavulanic acid were in the range of the expected values upon the literature data for HPLC–UV and LC–MS methods.

2. Materials and methods

2.1. Chemicals and materials

The working standard of amoxicillin trihydrate, amoxicillin d-4, clavulanate potassium and ampicillin trihydrate were obtained from Vivan life science (Mumbai, India). Ammonium acetate (GR grade), ortho phosphoric acid (GR grade), methanol (HPLC grade), acetonitrile (HPLC grade) were purchased from Merck Pvt. Ltd. Mumbai, India. Water was deionized and purified by a Milli-Q System from Millipore (Bedford, MA, USA) Oasis HLB 1 c.c, 30 mg solid phase extraction cartridges were procured from Waters (India) Pvt. Ltd. The blank human plasma with sodium heparin anticoagulant was collected from Drs. Pathology Labs, Mumbai, India.

2.2. Instrumentation

A Shimadzu HPLC system with a 5 μm HyPURITY advance C18 column (50 × 4.6 mm) was used for separation. The mobile phase was prepared by the addition of 2 mM ammonium acetate to acetonitrile (20:80 v/v). The flow rate was 0.8 mL/min. An API 4000 triple Quadrupole Mass Spectrometer (Applied Biosystem-SCIEX, Canada), equipped with a Turbo Ion spray source, was used for the LC–MS–MS analyses. The data processing was carried out using Analyst 1.5 software. The MS was operated in negative ion detection mode. Nitrogen was used as nebulizing turbo spray. The temperature of the vaporizer was set at 400 °C and the ESI needle voltage was 4500 V. The declustering potential was set at 50 for AMX, AMX–D4, AMP and 30 for CLV. Collision energy for AMX, CLV, AMX–D4 and AMP was –25, –10, –15 and –15 V respectively. The mass spectrometer was operated at unit mass resolution with a dwell time of 200 ms per transition. Quantification was performed using multiple reactions monitoring (MRM) of the...
transition ions \( m/z \ 364.00 \rightarrow m/z \ 223.00, m/z \ 198.00 \rightarrow m/z \ 136.00, m/z \ 368.00 \rightarrow 227.00 \) and \( m/z \ 347.90 \rightarrow m/z \ 304.00 \) for AMX, CLV, AMX-D4 and AMP respectively.

2.3. Preparation of stock and working solutions

The stock solutions of AMX, CLV (1 mg/mL) and IS (1 mg/mL) were prepared, for calibration standards and quality control (QC) samples, by dissolving appropriate amounts of the compounds in methanol. Stock solutions of AMX and CLV were subsequently serially diluted with mobile phase to obtain working solutions which were then added to plasma to yield concentrations in the range 50.43–31500.68 and 25.28–6185.18 ng/mL. A working solution for each IS (60.0 \( \mu \) g/mL) was prepared in water. All solutions were stored at 2–8 °C.

2.4. Preparation of calibration standard, quality control samples

To 950 \( \mu \) L of drug free plasma, 50 \( \mu \) L of working solutions of AMX, CLV were added to yield final concentrations of 50.43, 100.81, 1047.84, 2526.68, 5250.16, 10500.47, 20600.49 and 31500.68 ng/mL for AMX and 25.28, 50.61, 201.75, 510.52, 1078.54, 2118.32, 4215.49 and 6185.18 ng/mL for CLV in plasma. QC samples of 50.55, 150.30, 9411.75 and 18823.24 ng/mL of AMX, 26.99, 76.98, 2368.62 and 4737.23 ng/mL of CLV were also prepared in a similar manner using the same stock solutions.

2.5. Sample preparation

To all the calibration standards (0.2 mL) or QC samples (0.2 mL) taken in polypropylene tubes, 50 \( \mu \) L of internal standard was added and vortexed for 30 s. 0.25 mL of 2.00% ortho phosphoric acid in water was added to the plasma samples, vortexed for 30 s. The samples were transferred to a 1 cc/30 mg Oasis HLB SPE column, which had been conditioned with 1.0 mL methanol, followed by 1.0 mL water. After application of the samples, the SPE column was dried for 1.0 min by applying positive pressure at maximum flow rate. The column was eluted with 1.0 mL mobile phase. The SPE eluates were transferred into 1 mL LC vials for injection of 10 \( \mu \) L into the LC system.

2.6. Method validation

Validation was carried out according to the US Food and Drug Administration (FDA) Bioanalytical Method Validation Guidance. Accuracy, precision and linearity of the calibration curve were determined. Intra- and inter-day precision were carried out on three different days. Each validation run consisted of a minimum of one set of calibration standards and six sets of QC samples at four concentrations. Recoveries of AMX, CLV, AMX-D4 and AMP in aqueous solutions were determined at lower limit of quantification (LLOQ QC), low QC (LQC), medium QC (MQC) and high QC (HQC) levels. The stabilities of the stock solution, bench top, autosampler solutions, long term and freeze–thaw stability were carried out.

2.7. Selectivity, specificity and matrix effect

For specificity, six different lots of blank plasma were evaluated for any interference at the retention times of AMX, CLV, AMX-D4 (IS) and AMP (IS). Selectivity was carried out by analyzing the six blank plasma samples spiked with AMX and CLV (LLOQ level) and IS. Matrix effect was assessed by comparing the mean area responses of samples spiked after extraction with those of standard solutions in mobile phase at low and high QC levels.

2.8. Calibration curve

The linearity of the method was evaluated using bulk spiked plasma samples in the concentration range as mentioned above using the method of least squares. Five such linearity curves were analyzed. Each calibration curve consisted of a blank sample, a zero sample (blank + IS) and eight concentrations. Samples were quantified using the ratio of peak area of analyte to that of IS. A weighted linear regression (1/concentration) was performed with the nominal concentrations of calibration levels. Peak area ratios were plotted against plasma concentrations.

2.9. Recovery

The extraction efficiency of AMX and CLV was evaluated by comparing the mean peak responses of three QC samples 150.30, 9411.75 and 18823.24 ng/mL of AMX and 76.98, 2368.62 and 4737.23 ng/mL of CLV concentrations to the mean peak responses of three standards of equivalent concentration. Similarly, the recovery of IS was evaluated by comparing the mean peak responses in the three quality control samples to mean peak responses of three standards at a concentration of 9411.62 ng/mL of AMX-D4 and 2368.62 ng/mL of AMP.

2.10. Precision and accuracy

Intra-day accuracy and precision were evaluated from replicate analyses \( n = 6 \) of quality control samples containing AMX and CLV at different concentrations (LLOQ QC, LQC, MQC and HQC) on the same day. Inter-day accuracy and precision were also assessed from the analysis of the same QC samples on three separate occasions in replicate \( n = 6 \). QC samples were analyzed against calibration standards.

2.11. Effect of dilution on sample integrity

During the course of study the probability of encountering samples with concentrations above the upper limit of quantitation (ULOQ) could not be ruled out and therefore dilution with drug free plasma is necessary to bring them within the calibration range. To establish the effect of dilution on the integrity of samples, six aliquots of 63001.36 ng/mL and 12370.35 ng/mL of AMX and CLV respectively were prepared. The samples were subjected to twofold dilution \( n = 6 \) and fivefold dilution \( n = 6 \) with drug free human plasma to bring them within the calibration range. The samples were processed, analyzed and the concentrations obtained were compared with theoretical values.
Fig. 2 – Typical chromatograms of (a) Blank plasma of amoxicillin (b) Blank plasma of clavulanic acid (c) LLOQ of amoxicillin (d) LLOQ of clavulanic acid (e) Amoxicillin D4 and (f) Ampicillin.
2.12. Stability

Evaluation of the stability of samples was based on the comparison of various samples against freshly prepared samples of the same concentration. Percentage difference between the back calculated concentrations obtained for the sample under investigation and freshly prepared sample was evaluated. Six aliquots, each of LQC and HQC concentrations were used for stability study.

Bench top stability was studied on samples kept at ambient temperature (20–30 °C) for 6 h 26 min. The processed samples were kept in the autosampler at 5 °C for 59 h 33 min and then injected to determine the stability in the autosampler. The freeze–thaw stability of samples stored at −80 °C was studied after subjecting the samples to five freeze–thaw cycles. The long term stability of the samples were determined after storing the samples at −80 °C for 28 days.

In order to determine the stability of AMX and CLV in solution, the working solution was kept at 2–8 °C for 9 days 22 h. Thereafter, the mean areas of AMX and CLV from six replicate chromatographic runs were compared to that of the mean area of a freshly prepared solution of the same concentration.

2.13. Pharmacokinetic studies

For the pharmacokinetic studies co-amoxiclav a single dose of 875/125 mg tablets was administered orally. 24 healthy adult male volunteers who gave written informed consent took part in this study. The study was approved by Ethics Committee of Institutional Review Board. The volunteers were selected on predetermined inclusion/exclusion criteria. Males had a mean age of 27.19 ± 6.32 years, mean weight of 60.87 ± 7.07 kg, mean height of 167.87 ± 5.53 cm and a body mass index mean of 21.57 ± 1.93 kg/cm². The volunteers who were included in the study have not taken any other medication for at least two weeks beforehand. Blood samples were taken by using vacutainers, precoated with sodium heparin, at 0.00, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 2.75, 3.00, 3.25, 3.50, 3.75, 4.00, 5.00, 6.00, 7.00, 8.00, 10.00, and 12.00 h after

<table>
<thead>
<tr>
<th>Nominal conc. (ng/mL)</th>
<th>Mean conc. a (ng/mL)</th>
<th>% CV</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC1</td>
<td>50.43</td>
<td>50.423 ± 1.29</td>
<td>1.27</td>
</tr>
<tr>
<td>CC2</td>
<td>100.81</td>
<td>107.654 ± 2.84</td>
<td>1.32</td>
</tr>
<tr>
<td>CC3</td>
<td>1047.84</td>
<td>1061.002 ± 12.87</td>
<td>1.21</td>
</tr>
<tr>
<td>CC4</td>
<td>2526.68</td>
<td>2721.006 ± 14.76</td>
<td>0.54</td>
</tr>
<tr>
<td>CC5</td>
<td>5250.16</td>
<td>5380.875 ± 31.31</td>
<td>0.58</td>
</tr>
<tr>
<td>CC6</td>
<td>10500.47</td>
<td>10310.734 ± 8.88</td>
<td>0.09</td>
</tr>
<tr>
<td>CC7</td>
<td>20600.49</td>
<td>20712.001 ± 207.96</td>
<td>1.00</td>
</tr>
<tr>
<td>CC8</td>
<td>31500.68</td>
<td>30939.627 ± 133.93</td>
<td>0.43</td>
</tr>
</tbody>
</table>

a Data represent the mean ± S.D of five observations.

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![Image](image-url)
Intra- and inter-day precision and accuracy data for amoxicillin and clavulanic acid in human plasma.

Analysis of plasma calibration standards of maximum concentration (CMAX), tMAX was obtained directly/C0

3.1. Method development

Liquid–liquid extraction with dichloromethane, diethyl ether, n-hexane, terbutyl methyl ether and mixtures of these solvents was evaluated. The extraction efficiency of the drug was found to be poor and also interference at the retention time of drug was observed. Poor extraction efficiency was also observed using precipitation. Hence solid phase extraction (SPE) technique was used with Oasis HLB extraction cartridges.

Samples were retrieved from the deep freezer then thawed and vortexed. Each 0.2 mL of sample was transferred to pre-labeled tubes which contained extraction buffer. The tubes were vortexed for about 10 s and centrifuged at 4000 rpm and 10 °C for 2 min. HLB extraction cartridges (1 cc, 30 mg) were arranged in solid phase extraction manifolds to condition the cartridge with 1.0 mL of methanol followed by 1.0 mL of water. The conditioned cartridges were loaded with prepared samples and the cartridges were then subjected to positive pressure. The contents were eluted from the cartridge by the addition of 0.5 mL of mobile phase into pre-labeled tubes then vortexed for 10 s and transferred to the HPLC vials to inject 10 µL of the sample.

3.2. Method validation

No significant interfering peaks were observed at the retention time of either analyte or internal standard in six different lots of drug free human plasma samples. Chromatograms of extracted blank, LLOQ sample and internal standard are shown in Fig. 2. The matrix effect for both amoxicillin and clavulanic acid was calculated as a percentage of the comparison of area response obtained with the post extracted and the aqueous samples and was found to be more than 98.00% at LQC and HQC levels which implies that there is no matrix effect in the extracted samples on comparison with aqueous samples. All calibration curves were found to be linear over the range of 50.43–31500.68 and 25.28–6185.18 ng/mL. The mean correlation coefficient was 0.9998 for AMX and 0.9997 for CLV. The back calculated concentrations of calibration standards for AMX and CLV are presented in Tables 1 and 2 respectively.

The inter-batch assay accuracy for amoxicillin and clavulanic acid ranged between 97.29–103.56 and 97.28–101.22% respectively, whereas intra-batch accuracy ranged between 100.38–103.99 and 95.48–102.17%. The inter-batch precision for amoxicillin and clavulanic acid ranged between 2.97–3.55 and 1.73–2.03% and intra-batch precision ranged between 1.06–3.07 and 1.88–2.89%. The results are presented in Table 3.

The extraction efficiency of AMX from human plasma at the concentrations of LQC, MQC and HQC was found to be 54.06, 55.33 and 54.65%. The extraction efficiency of CLV from human plasma at the concentrations of LQC, MQC and HQC was found to be 47.18, 50.23 and 47.23%. The results are presented in Table 4. The mean recovery for AMX-D4 (IS) was

### Table 2 – Analysis of plasma calibration standards of clavulanic acid.

<table>
<thead>
<tr>
<th>Nominal conc. (ng/mL)</th>
<th>Mean conc.* (ng/mL)</th>
<th>% CV</th>
<th>Accuracy %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC1</td>
<td>25.28</td>
<td>0.76</td>
<td>100.19</td>
</tr>
<tr>
<td>CC2</td>
<td>50.61</td>
<td>1.68</td>
<td>99.87</td>
</tr>
<tr>
<td>CC3</td>
<td>201.75</td>
<td>1.50</td>
<td>99.89</td>
</tr>
<tr>
<td>CC4</td>
<td>510.52</td>
<td>1.69</td>
<td>102.24</td>
</tr>
<tr>
<td>CC5</td>
<td>1078.54</td>
<td>1.49</td>
<td>100.82</td>
</tr>
<tr>
<td>CC6</td>
<td>2118.32</td>
<td>3.07</td>
<td>99.92</td>
</tr>
<tr>
<td>CC7</td>
<td>4215.49</td>
<td>3.81</td>
<td>99.35</td>
</tr>
<tr>
<td>CC8</td>
<td>6185.18</td>
<td>1.76</td>
<td>98.22</td>
</tr>
</tbody>
</table>

*Data represent the mean ± S.D. of five observations.

### Table 3 – Intra- and inter-day precision and accuracy data for amoxicillin and clavulanic acid in human plasma.

<table>
<thead>
<tr>
<th></th>
<th>Mean conc.* (ng/mL)</th>
<th>% CV</th>
<th>Accuracy %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amoxicillin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LLOQ QC (50.55 ng/mL)</td>
<td>49.178 ± 2.31</td>
<td>2.97</td>
<td>97.29</td>
</tr>
<tr>
<td>LQC (150.30 ng/mL)</td>
<td>150.348 ± 4.56</td>
<td>2.57</td>
<td>100.03</td>
</tr>
<tr>
<td>MQC (9411.75 ng/mL)</td>
<td>9746.479 ± 127.16</td>
<td>3.55</td>
<td>103.56</td>
</tr>
<tr>
<td>HQC (18823.24 ng/mL)</td>
<td>18833.799 ± 233.82</td>
<td>3.42</td>
<td>100.06</td>
</tr>
<tr>
<td><strong>Clavulanic acid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LLOQ QC (26.99 ng/mL)</td>
<td>26.457 ± 0.54</td>
<td>2.03</td>
<td>98.03</td>
</tr>
<tr>
<td>LQC (76.9806 ng/mL)</td>
<td>76.026 ± 1.52</td>
<td>2.00</td>
<td>98.76</td>
</tr>
<tr>
<td>MQC (2368.62 ng/mL)</td>
<td>2397.417 ± 41.53</td>
<td>1.73</td>
<td>101.22</td>
</tr>
<tr>
<td>HQC (4737.23 ng/mL)</td>
<td>4608.282 ± 79.72</td>
<td>1.73</td>
<td>97.28</td>
</tr>
</tbody>
</table>

*The inter- and intra-assay data represent the mean ± S.D. of 18 and 12 observations, respectively.
59.71% and AMP (IS) was 77.77%. The recovery of amoxicillin and clavulanic acid was not less than 54% and 47% respectively at three levels.

The precision for dilution integrity standards at 1:2 and 1:4 for AMX were 0.77 and 1.89% and for CLV were 0.89 and 1.40% respectively, which are within the acceptance limit of 15%. The mean accuracy for dilution integrity of 1:2 and 1:5 for AMX were 101.54 and 101.31% while for CLV they were 109.05 and 107.95% respectively. These are both within the acceptance limits of 85.00 to 115.00%.

Bench top stability of AMX and CLV was demonstrated for 6 h 26 min at ambient temperature. Auto sampler stability over 59 h 33 min was established. AMX and CLV in plasma were stable for five freeze–thaw cycles (FTS). The plasma samples were stable for 28 days at −80 °C. The data is tabulated in Tables 5 and 6 for amoxicillin and clavulanic acid respectively. The stock solution short-term stability was established for 22 h 19 min at ambient temperature and the % stability of the solution was found to be 96.34%. The long term stability in solution was established for 9 days 22 h and the % stability was found to be 93.69%.

### 3.3. Pharmacokinetics

Overlay graphs of mean concentration versus time of the two formulations (test and reference) are shown in Fig. 3. The area under the curve from 0 to 12 h was determined with the help of the linear trapezoidal rule. The extrapolation to infinity that is necessary for $\text{AUC}_{0\rightarrow\infty}$ was calculated using a linear regression model from the last three data points in the elimination phase that has been log-transformed. Maximum concentration achieved ($C_{\text{MAX}}$) was obtained directly from measured concentration without interpolation. The parametric point estimates for the mean of test medication/the mean of reference medication were found within the commonly accepted bioequivalence range of 0.8 to 1.25. Therefore, the results indicate that the proposed method is suitable for pharmacokinetic studies to determine the concentration of amoxicillin and clavulanic acid in human plasma. The study was conducted strictly in accordance with guidelines laid down by the International Conference on Harmonization and USFDA. The pharmacokinetic data are tabulated in Tables 7 and 8.

![Fig. 3 – Mean plasma concentration–time profile of (a) Amoxicillin and (b) Clavulanic acid after oral administration of co-amoxiclav test and reference formulation to 24 healthy male volunteers.](image)
The LC–MS–MS method described here has significant advantages over the other techniques already described in the literature. The method has proved to be fast with each sample requiring a run time of 1.5 min only and therefore has a high throughput capability. The assay method is specific due to the inherent selectivity of tandem mass spectrometry. The major advantage of this method is the simple solid phase extraction (SPE) technique without drying and reconstitution steps. The proposed method for simultaneous quantification of amoxicillin and clavulanic acid in human plasma by LC–MS–MS method happens to be first of its kind described so far in the literature. This new method will be helpful for carrying out pharmacokinetic study.

### Conflicts of interest
All authors have none to declare.

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