Rapid Determination of Amoxicillin Levels in Human Plasma by High Performance Liquid Chromatography

Reem Alswayeh, Syed N. Alvi, and Muhammad M. Hammami*

Clinical Studies and Empirical Ethics Department,
King Faisal Specialist Hospital & Research Center,
MBC-03, P.O. Box 3354, Riyadh 11211, Kingdom of Saudi Arabia.

Abstract

A simple, precise, and rapid high performance liquid chromatography (HPLC) method for the determination of amoxicillin level in human plasma using nizatidine as an internal standard (IS) was developed and validated. Plasma samples containing amoxicillin were spiked with IS, vortexed for 30 second, and filtered using Millipore centrifree filters. The compounds of interest were efficiently separated on Atlantis 4.6 x 150 mm, dc18, 5-µm column and detected with a photodiode array detector set at 229 nm. The mobile phase, composed of 0.01 M dibasic sodium phosphate buffer (pH 3.5 adjusted with phosphoric acid) and acetonitrile (95:5, v:v), was delivered at a flow rate of 1 ml/min. No interference in blank plasma or of commonly used drugs was observed, and the detection limit of amoxicillin was 0.3 µg/ml. The relationship between amoxicillin concentration in plasma and peak high ratio of amoxicillin /IS was linear (R² ≥ 0.9986) in the range of 1.0 – 30 µg/ml, intra- and inter-day coefficient of variation and bias were ≤ 3.7% and ≤ 12.0% and ± 9.2% and ± 3.5%, respectively. Mean extraction recovery of amoxicillin and the IS from plasma samples was ≥ 85% and 100%, respectively. The method was used to assess the stability of amoxicillin in human plasma under various clinical laboratory conditions. Further, it was successfully employed to measure amoxicillin level in plasma samples from healthy a volunteer.

Keywords: Amoxicillin, Nizatidine, Human plasma, HPLC.
INTRODUCTION
Amoxicillin (CAS: 26787-78-0) (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 is one of the oldest β-lactam broad spectrum antibiotic that is still prescribed for bacterial infections caused by gram-positive and gram-negative bacteria in children and adults.[1] Its absolute bioavailability is about 76% with mean peak plasma concentration in the range of 5.5 - 7.5 µg/ml, 1 - 2 hours after the ingestion of a 500 mg therapeutic dosage.[2, 3] Figure 1 depicts the structure of amoxicillin and nizatidine, the internal standard (IS) used in the study.

![Fig. 1 Chemical structures of amoxicillin and the internal standard, nizatidine.](image)

Various analytical methods have been reported for the determination of amoxicillin level in different matrixes, including, high performance liquid chromatography-electrospray ionization (HPLC-ESI) mass spectrometry,[4] liquid chromatography tandem mass spectrometry (LC-MS/MS),[5,6,19] HPLC column-switching system,[7] and reversed phase HPLC using ultraviolet[8-16] or fluorescence[17-18] detection. However, some of these methods suffered from long processing time due to liquid-liquid extraction,[5,18] solid phase extraction,[16,19] or derivatization steps,[17] long chromatography run time,[15] limited information on stability for amoxicillin,[5,6,8,12-15,19] or not being validated in human plasma.[18, 20]

This paper describes an HPLC method for amoxicillin determination in human plasma that is simple, precise, and accurate. The method was fully validated and used to determine amoxicillin level in plasma samples from a healthy volunteer and the stability of amoxicillin under various laboratory conditions.
MATERIAL AND METHODS

Apparatus
Chromatography was performed on a Waters Alliance HPLC 2695 (Waters Associates Inc., Milford, MA, USA) consisting of a quaternary pump, autosampler, column thermostat, and photodiode array detector. Atlantis (4.6 x 150 mm, dc18, and 5-µm) steel column at room temperature (24°C) and a guard pak pre-column module with Nova-Pak C18, 4-µm insert were used for separation. Data were collected with a Pentium IV computer using Empower2 Chromatography Software.

Chemical and reagents
All reagents were of analytical-reagent grade unless stated otherwise. Amoxicillin and nizatidine standards were purchased from Sigma, St. Louis, MO, USA. Acetonitrile, phosphoric acid and dibasic sodium phosphate (all HPLC grade) were purchased from Fisher Scientific, Fairlawn, NJ, USA. HPLC grade water was prepared by reverse osmosis and was further purified by passing through a Synergy Water Purification System (Millipore, Bedford, MA, USA). Drug-free human plasma was obtained from the blood bank of King Faisal Specialist Hospital & Research Centre (KFSHRC) Riyadh, Saudi Arabia. Samples from healthy volunteers were collected after obtaining approval from the Research Ethical Committee of KFSHRC.

Chromatographic conditions
The mobile phase was composed of 0.01 M dibasic sodium phosphate (pH 3.5, adjusted with phosphoric acid) and acetonitrile (95:5, v:v). Before being delivered into the system, the mobile phase was filtered through 0.45 µm polysulfone membrane and sonicated under vacuum for 5 minutes. The analysis was carried out under isocratic conditions using a flow rate of 1 ml/min, at 24°C and a run time of 10 minutes. A photodiode array detector set at 229 nm was used.

Preparation of standard and quality control samples
Amoxicillin and nizatidine (internal standard, IS) stock solutions (1.0 mg/ml) were prepared in methanol. They were diluted with blank human plasma or mobile phase to produce working solutions of 100 µg/ml and 50 µg/ml, respectively. Eleven calibration standards in the range of 1.0–30 µg/ml were prepared in human plasma. Four quality control (QC) samples concentrations: 1.0, 3.0, 15, and 27 µg/ml were prepared in human plasma. 0.5 ml
aliquots from standard and QC samples were transferred into 1.5 ml eppendorf micro centrifuge tubes and stored at –20 °C until used.

Sample preparation
Aliquots of 0.5 ml blank plasma, calibration curve, or QC samples in 1.5 ml eppendorf micro centrifuge tubes were allowed to equilibrate to room temperature. To each tube, 200 µl IS (50 µg/ ml in mobile phase) was added and vertexed for 30 seconds. Samples were filtered using centrifree filters (Millipore, Waters, USA). A 75 µl of the clear filtrate was injected into the HPLC system.

Method validation
The method was validated according to standard procedures described in the US Food and Drug Administration (FDA) bioanalytical method validation guidance.[21] The validation parameter included: specificity, linearity, accuracy, precision, recovery and stability.

Stability studies
Two QC samples (3.0 and 27 µg/ml) were used. Five aliquots of each QC sample were extracted and immediately analyzed (baseline), five aliquots were allowed to stand on the bench-top for 5 hours at room temperature before being processed and analyzed (counter stability, 5 hours at room temperature), five aliquots were stored at -20ºC for 19 weeks before being processed and analyzed (long term freezer storage stability), five aliquots were processed and stored at room temperature for 10 hours, and five aliquots were processed and stored at 4ºC for 24 hours (autosampler stability) before analysis. In addition, fifteen aliquots of each QC sample were stored at -20ºC for 24 hours. They were then left to completely thaw unassisted at room temperature. Five aliquots of each sample were then extracted and analyzed and the rest returned to -20ºC for another 24 hours. The cycle was repeated three times (freeze-thaw stability).

RESULTS AND DISCUSSION
Optimization of chromatographic conditions
The optimal experimental conditions were a mobile phase composed of composed of 0.01 M dibasic sodium phosphate buffer (pH adjusted to 3.5 with phosphoric acid and acetonitrile (95:5, v:v) and a flow rate of 1 ml/min. Under these conditions amoxicillin, nizatidine and components of plasma exhibited a well-defined separation within a 10-minute run. The retention times of amoxicillin and nizatidine were around 3.5 and 7.0 minutes, respectively.
Specificity

Specificity is defined as the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Potential interfering substances in plasma samples include endogenous components, metabolites, and decomposition products. We screened six batches of blank plasma and eight frequently used medications (ranitidine, acetaminophen, ibuprofen, nicotinic acid, ascorbic acid, caffeine, omeprazole and diclofenac) for potential interference. No interference was found in plasma and none of the drugs co-eluted with amoxicillin or the IS. Figure 2 depicts a representative chromatogram of drug free human plasma used in preparation of standard and QC samples.

![Representative chromatogram of drug-free human plasma. The arrows indicate the retention times of amoxicillin (3.5 min) and the internal standard, nizatidine (7.0 min).](image)

Limit of Detection & Quantification and Linearity

The limit of quantification was defined as the lowest concentration on the calibration curve that can be determined with acceptable precision and accuracy (i.e., coefficient of variation and bias ≤ 20%). The limit of quantification of amoxicillin in human plasma was 1.0 μg/ml. The limit of detection, defined as signal to noise ratio of ≥ 3, was 0.3 μg/ml. Linearity was evaluated by analyzing ten curves of nine standard concentrations over the range (1.0-30 μg/ml) prepared in human plasma. Calibration curves were linear with an $R^2 \geq 0.9986$. Figure 3 shows an overlay of chromatograms of a typical calibration curve. The suitability of the calibration curves was confirmed by back-calculating the concentration of amoxicillin in human plasma from the calibration curves (Table 1). All calculated concentrations were well within the acceptable limits.
Fig. 3 Overlay of chromatograms of extracts of 0.5 ml human plasma spiked with the internal standard (IS) and one of nine concentrations of amoxicillin, 1.0, 2.0, 4.0, 6.0, 8.0, 10, 12, 24, and 30 μg/ml.

Table 1: Back calculated amoxicillin concentrations from ten calibration curves

<table>
<thead>
<tr>
<th>Nominal level (μg/ml)</th>
<th>Calculated level (μg/ml)</th>
<th>CV (%)</th>
<th>Bias (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>1.05</td>
<td>0.08</td>
<td>7.3</td>
</tr>
<tr>
<td>2.0</td>
<td>1.92</td>
<td>0.07</td>
<td>3.6</td>
</tr>
<tr>
<td>4.0</td>
<td>4.11</td>
<td>0.13</td>
<td>3.1</td>
</tr>
<tr>
<td>6.0</td>
<td>6.08</td>
<td>0.11</td>
<td>1.8</td>
</tr>
<tr>
<td>8.0</td>
<td>8.25</td>
<td>0.11</td>
<td>1.3</td>
</tr>
<tr>
<td>10</td>
<td>9.76</td>
<td>0.18</td>
<td>1.8</td>
</tr>
<tr>
<td>12</td>
<td>11.81</td>
<td>0.25</td>
<td>2.1</td>
</tr>
<tr>
<td>24</td>
<td>23.88</td>
<td>0.21</td>
<td>0.9</td>
</tr>
<tr>
<td>30</td>
<td>30.14</td>
<td>0.19</td>
<td>0.6</td>
</tr>
</tbody>
</table>

SD, standard deviation. CV, standard deviation divided by mean measured level x100

Bias = mean calculated level – nominal level divided by nominal level) × 100.

Precision and bias(inaccuracy)

Intra-day and inter-day precision and bias were evaluated by analyzing four QC samples (1.0, 3.0, 15, and 27 μg/ml). The intra-day precision and bias (n=10) ranged from 2.3% to 3.7% and from -9.2% to +9.1%, respectively. The inter-day precision and bias were determined over three different days. The inter-day precision and bias (n=20) ranged from 6.1% to 12.0% and from -3.5% to +2.5%, respectively. The results are summarized in Table 2.
Table 2: Intra - and inter-day precision and bias of amoxicillin assay

<table>
<thead>
<tr>
<th>Nominal level (µg/ml)</th>
<th>Intra-run (n=10)</th>
<th>Inter-run (n=20)</th>
<th>Bias (%)</th>
<th>CV (%)</th>
<th>Bias (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured level (µg/ml)</td>
<td>Measured level (µg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>1</td>
<td>0.91</td>
<td>0.03</td>
<td>1.03</td>
<td>0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.27</td>
<td>0.12</td>
<td>3.07</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>14.03</td>
<td>0.34</td>
<td>14.59</td>
<td>0.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>24.87</td>
<td>0.56</td>
<td>25.87</td>
<td>1.58</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SD, standard deviation. CV, standard deviation divided by mean measured level x100.

Bias, measured level - nominal level divided by nominal level x 100.

Recovery

The absolute recovery of amoxicillin was assessed by direct comparison of absolute peak areas obtained from plasma and mobile phase samples, using five replicates of 4 QC samples (1.0, 3.0, 15, and 27 µg/ml). Similarly, the recovery of the IS was determined by comparing the peak high of the IS in 5 aliquots of human plasma spiked with 200 µl of IS (50 µg/ml) with the peak high of equivalent samples prepared in mobile phase. The results are presented in Table 3.

Table 3: Recovery of amoxicillin and the internal standard from 0.5 ml human plasma

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Human plasma*</th>
<th>Mobile phase*</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>5740(279)</td>
<td>6617(20)</td>
<td>87</td>
</tr>
<tr>
<td>3.0</td>
<td>17456(784)</td>
<td>21261(41)</td>
<td>82</td>
</tr>
<tr>
<td>15.0</td>
<td>80586(572)</td>
<td>98511(86)</td>
<td>82</td>
</tr>
<tr>
<td>27</td>
<td>139729(765)</td>
<td>153182(53)</td>
<td>91</td>
</tr>
<tr>
<td>Internal standard</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>81404(943)</td>
<td>81695(93)</td>
<td>100</td>
</tr>
</tbody>
</table>

*Data represents mean peak height and standard deviation (SD), n = 5. Recovery is ratio of mean peak height in human plasma divided by mean peak height in mobile x 100.

Robustness

Robustness of the assay was evaluated by slight deliberate changes in chromatographic conditions (mobile phase composition ±0.5%, buffer strength ±00.01M, and pH±0.5). No significant changes were observed in calculated concentration or chromatographic behavior.

Stability

Stability of analytes in biological matrices is an important pre-analytical variable. It is necessary to perform stability studies of the analyte and IS to determine the range of
appropriate conditions and time of storage. Amoxicillin and IS stability in processed and unprocessed plasma samples was investigated (Table 4). No significant reduction in concentration or change in chromatographic behavior of amoxicillin or the IS was observed. Amoxicillin in processed samples (3.0 and 27 μg/ml) was found to be stable for 10 hours at room temperature (≥ 96%) and 24 hours at 4°C (≥ 94%). Amoxicillin in unprocessed plasma samples (3.0 and 27 μg/ml) was stable for at least 5 hours at room temperature (≥ 92%), 19 weeks at −20°C (89%), and after three freeze-and-thaw cycles (≥ 92%).

Table 4: Stability of amoxicillin under various clinical laboratory conditions

<table>
<thead>
<tr>
<th>Nominal level (μg/ml)</th>
<th>Unprocessed (5 hrs RT)</th>
<th>(19 wks, −20°C)</th>
<th>Processed (10 hrs RT)</th>
<th>(24 hrs 4°C)</th>
<th>Freeze-Thaw Cycle (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(5 hrs RT)</td>
<td>(19 wks, -20°C)</td>
<td>(10 hrs RT)</td>
<td>(24 hrs 4°C)</td>
<td>1</td>
</tr>
<tr>
<td>3.0</td>
<td>101</td>
<td>89</td>
<td>97</td>
<td>96</td>
<td>102</td>
</tr>
<tr>
<td>27</td>
<td>92</td>
<td>89</td>
<td>96</td>
<td>94</td>
<td>98</td>
</tr>
</tbody>
</table>

Stability (%) = mean measured level (n=5) at the indicated time divided by mean measured level (n=5) at baseline x 100

Spiked plasma samples were processed and analyzed immediately (baseline, data not shown), after 5 hours at room temperature (5 hrs, RT), after freezing at −20 °C for 19 weeks (19 wks, −20 °C), or processed and then analyzed after storing for 10 hours at room temperature (10 hrs, RT) or 24 hours at 4 °C (24 hrs, 4 °C); or after 1 to 3 cycles of freezing at −20°C and thawing at RT (Freeze -Thaw).

Application to a volunteer sample

Figure 4 depicts an overlay chromatogram of samples collected from a volunteer before and 2.0 hours after ingestion of a single oral dose of 500 mg amoxicillin. Measured concentrations were zero and 9.36 μg/ml, respectively.

![Chromatogram of plasma samples](image)

Fig. 4 An overlay of chromatograms of plasma samples obtained from a healthy volunteer before (A) and 2 hours after (B) a single oral dose of 500 mg amoxicillin dose. The calculated concentrations were zero and 9.36 μg/ml, respectively.
CONCLUSION
The described HPLC assay is simple, precise, and rapid. It requires only 0.5 ml plasma and utilizes a convenient method for sample preparation. The assay was applied to monitor stability of amoxicillin under various conditions generally encountered in the clinical laboratories. Further, it was successfully applied to determine levels of amoxicillin in samples obtained from a healthy volunteer.

ACKNOWLEDGEMENT
This work was funded by a grant to Dr. Muhammad M Hammami, from the King Abdul-Aziz City for Science and Technology, Riyadh, Saudi Arabia (National Comprehensive Plan for Science and Technology # 10-BIO 961-20).

REFERENCES
7. Zijtveld JV, van Hoogdalem EJ, Application of a semipermeable surface column for the
determination of amoxicillin and clavulanic acid in human plasma by isocratic reversed-
phase HPLC using UV detection. Journal of Pharmaceutical and Biomedical Analysis.
2007; 45: 531-534.
RP-HPLC method in bulk drug and pharmaceutical dosage forms. International Journal
determination of amoxicillin trihydrate and bromhexine hydrochloride in oral dosage
forms. International Journal of Pharmacy and Pharmaceutical Science. 2010; 2(1): 129-
133.
11. Tavakoli N, Varshosaz J, Dorkoosh F, Zargarzadeh MR, Development and validation of a
simple HPLC method for simultaneous I vitro determination of amoxicillin and
metronidazole at single wavelength. Journal of Pharmaceutical and Biomedical Analysis.
12. De Abreu LRP, Ortiz RAM, De Castro SC, Pedrezzoli Jr JP. HPLC determination of
amoxicillin comparative bioavailability in healthy volunteers after a single dose
Simultaneous determination of amoxicillin and clavulanic acid in human plasma by
HPLC with UV detection. Journal of Pharmaceutical and Biomedical Analysis. 2002; 30:
661-666.
Validation and application of reversed-phase HPLC method for the determination of
5(12): 2219-2224.
15. Menelaou A, Somogyi AA, Barclay ML, Bochner F, Simultaneous quantification of
amoxicillin and metronidazole in plasma using high-performance liquid chromatography


