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# Development of an Analytical Method for the Determination of Amoxicillin in Commercial Drugs and Wastewater Samples, and Assessing its Stability in Simulated Gastric Digestion

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## Abstract

A highly sensitive analytical HPLC–UV method was developed for the determination of amoxicillin in drugs and wastewater samples at a single wavelength (230 nm). In order to substantially predict the *in vivo* behavior of amoxicillin, drug samples were subjected to simulated gastric conditions. The calibration plot of the method was linear from 0.050 to 500 mg $L^{-1}$ with a correlation coefficient of 0.9999. The limit of detection and limit of quantitation were found to be 16 and 54 μg $L^{-1}$, respectively. The percentage recovery of amoxicillin in wastewater was found to be 97.0 ± 1.6%. The method was successfully applied for the qualitative and quantitative determination of amoxicillin in drug samples including tablets and suspensions.

## Introduction

Amoxicillin trihydrate chemically known as [2S-[2a,5a,6aˆ(S_)]]-6-[[Amino(4-hydroxyphenyl)acetyl]amino]-3,3 dimethyl-7-oxo-4-thia-1-azabicyclo is the most widely used β-lactam antibiotic for the treatment of bacterial infections such as ear, nose, throat, skin and lower respiratory tract caused by susceptible microorganisms (1–3). It has higher absorption ability than other β-lactam antibiotics (4). Available formulations of amoxicillin on markets are capsules, tablets, suspensions and injectable solutions (5). Amoxicillin combination drugs increases the antibacterial effect and bacterial resistance (6). This chemical is also freely soluble in water and methanol, slightly soluble in ethanol and insoluble in acetonitrile, ethyl acetate, benzene and hexane (7). It was reported that pKa values of amoxicillin were 2.67, 7.11 and 9.55 at 37°C and its solubility is the lowest at a pH range of 4–6. Under physiological temperature conditions, pH-apparent solubility profile of amphoteric amoxicillin was determined and U-shaped pH-solubility curve with the minimum solubility at the pH near the isoelectric point was observed (8).

The quality and reliability of drugs depend on their dosage, quantity and the mode of usage. In literature, several analytical methods have been used in the quantitative determination of amoxicillin such as microbiological methods (9), enzymatic quantification (10), spectrofluorometry (11, 12), UV spectrophotometry (13, 14), capillary electrophoresis (15), high performance liquid chromatography (HPLC) (6, 16) and liquid chromatography–tandem mass spectrometry (LC–MS-MS) (17).

HPLC is the most widely used method for the amoxicillin determination due to its simplicity and low handling price. It is applicable to diverse analyte types. However, HPLC requires large amounts of organic solvents, stabilization time and optimization for some parameters as mobile phase and flow rate (18).

Several HPLC studies have been performed for the determination of amoxicillin in different matrices to obtain lower detection limits (19–22). Ashnagar *et al.* performed HPLC studies on the various dosage forms of these drugs manufactured by several pharmaceutical companies in 2007. Zheng *et al.* investigated the distribution of

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amoxicillin in gastric juice and gastric mucosa of rats with HPLC. It was reported that the volume of the gastric juice was reduced by rabeprazole whiles its presence lead to an increase in the concentration and pH value of amoxicillin (23). Teixeira *et al.* found 105 and 101% recovery of amoxicillin from wastewater in 2008.

The aim of this study was not only to develop an analytical method based on HPLC–UV system for the sensitive determination of amoxicillin in commercial drugs and wastewater samples with high recovery values, but also to figure out its behavior in simulated gastric juice. This is the first study in literature for the determination of amoxicillin in wastewater matrices with very high accuracy and precision using HPLC with UV detector. There is no study in literature to predict the *in vivo* behavior of amoxicillin using HPLC system.

## Experimental

### Apparatus

Thermo HPLC (P1500 pump, UV2000 detector, AS3000 auto sampler) coupled with UV detector were employed for the determination of amoxicillin. Inertsil (ODS-3V 5 µ 4.6 × 250 $mm^2$) column was used for commercial drugs, simulated gastric juice and wastewater analysis. Distilled water was obtained from A Milli-Q® Reference System in the central laboratory of Yildiz Technical University. IKA (RTC basic) brand magnetic stirrer, Sartorius Analytic (A 120 S) brand electronic analytical balance and Shimadzu brand (LIBROR AEG-220G) electronic analytical balance were used for sample preparations and extraction studies. Heating simulated gastric juice were made with IKA (ETS-D5) electronic contact thermometer. Preparation of buffer solutions were performed with Hanna brand pH meter. Optimum parameters of HPLC–UV system are presented in Table I.

### Reagents

All analytical reagents were purchased from Merck. HPLC reagents included acetonitrile (>99%), methanol (>99%) and $KH_2PO_4$ (>99%) were used for the preparation of mobile phase. HCl (36–37%), NaCl and pepsin were used for simulated gastric juice studies. Distilled water was obtained from A Milli-Q® Reference System.

### Sample preparations, separation system and system analytical performance

Four different commercial drugs (tablets and suspensions) purchased from a pharmacy were dissolved in water for the analysis. For each drug, three replicate samples were prepared. Wastewater samples were obtained from the Environmental Engineering Department, Yildiz Technical University.

Separation of amoxicillin from the matrix components was achieved by HPLC instrument. All system parameters in HPLC system including flow rate, pH and component of mobile phase, type of analytical column were optimized in order to get high separation efficiency. Under the optimum conditions, system analytical performance was calculated. The limit of detection (LOD) and limit of quantitation (LOQ) values were calculated based on the equations given below:

$$\text{LOD} = 3 \times \text{Standard Deviation (lowest concentration)/Slope}$$
$$\text{LOQ} = 10 \times \text{Standard Deviation (lowest concentration)/Slope}$$

Amoxicillin working standards were prepared by diluting appropriate aliquots from a 1,000 mg $L^{-1}$ stock solution prepared from amoxicillin trihydrate (Sanovel Pharmaceutical Ltd.).

**Table I.** Optimum Parameters of the HPLC–UV System

| Parameter | Optimized value |
|---|---|
| Mobile phase | 5.0% ACN–14.25% $CH_3OH$–80.75% buffer solution |
| Buffer solution | 0.2 M NaOH + 0.2 M $KH_2PO_4$ |
| pH | 8.5 |
| Flow rate (mL $min^{-1}$) | 1.0 |
| Wavelength (nm) | 230 |

### Extraction studies in commercial drugs

In order to perform the quantitative determination of amoxicillin in tablets, an efficient extraction procedure is needed to extract the all analytes from the matrix. In the experimental procedure, tablet samples were dissolved with 500 mL distilled water and mixed for 1 h on magnetic stirrer. The 2.5 mL of tablet solutions were put into 100 mL volumetric flask after filtering through a 0.45 µm filter. The final volume was completed to 100 mL with distilled water. Suspension samples were prepared according to the prospectuses. And 10 mL of suspension solutions were put into 100 mL volumetric flask and diluted with distilled water to 100 mL and mixed one hour on magnetic stirrer. In addition, 0.50 mL 500 mg $L^{-1}$ standard amoxicillin sample was added into 5.0 mL drug samples in flask for spiking experiments and analyzed on HPLC. Samples were analyzed in HPLC–UV system with Inertsil column under optimum conditions.

### Recovery test and stability of amoxicillin in simulated gastric juice

Accuracy of the developed method was checked by recovery experiments. For this purpose, three replicates of 10 mg $L^{-1}$ spiked wastewater sample were prepared and analyzed under the optimum conditions.

Simulated gastric juice was prepared using 0.70 mL HCl, 0.20 g NaCl and 0.32 g pepsin, and the final volume completed to 100 ml with distilled water. The resulting solution with a pH value of ~1.2 was maintained at 37°C and stirred continuously. One random tablet from Brand D drug sample was put into the simulated gastric juice and when it was dissolved, 0.10 mL was taken from solution and diluted to 100 mL with distilled water. The obtained sample was immediately analyzed by the HPLC–UV system and recorded as 0.0 min. Samples were then taken and analyzed after every half-hour period until 240 min to collect the data regarding the stability of the analyte. In addition to this, a spiking experiment was performed at the 120 min period to ensure that there was no matrix effect on amoxicillin peak changing the retention time. A 0.10 mL 500 mg $L^{-1}$ amoxicillin standard was added to 2.0 mL simulated gastric juice containing the dissolved drug sample. Samples including calibration standards were analyzed with Inertsil column under the optimum HPLC–UV conditions.

### Determination of amoxicillin in wastewater

Developed analytical method was also applied to wastewater sample having the high matrix content for the quantitative determination of amoxicillin. In order to figure out the effect of the matrix on analyte

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signal, spiking experiments were also performed. For this purpose, 1.0 mL 100 mg $L^{-1}$ amoxicillin standard was taken and diluted to 10 mL with wastewater sample. The same study was performed with deionized water in place of amoxicillin as a blank study. Prior to analysis, samples were filtered using 0.45 µm filter to eliminate any contamination and to protect the column from unwanted particles. All samples were analyzed under optimum HPLC conditions.

## Results

### Chromatographic separation of amoxicillin

The first step in the separation study was to select an appropriate mobile phase according to the Inertsil column. For this purpose, a phosphate buffer solution and methanol were employed as mobile phase. The ratio, pH and flow rate of the phosphate buffer/methanol mobile phase were optimized to obtain relatively the best peak shape and retention time for amoxicillin. Under the optimum conditions presented in Table I, a symmetrical peak shape was recorded at a retention time of ~8.1 min with good resolution seen in Figure 1. Tailing factor calculated as about 1.0 shows that there is no tailing in the analytical signal. There is no shift in the analyte retention time to both longer and shorter retention times. %RSD in the retention time of analyte for different days was calculated as lower than 2%.

### Analytical performance of amoxicillin

The method developed was validated concerning to linearity, precision, LOD and LOQ. For the purpose of determining the system's performance values, standard solutions in the range of 50 µg $L^{-1}$–500 mg $L^{-1}$ was injected into the HPLC–UV system under the optimum conditions. Chromatographic examples shown in Figure 1 were obtained for the calibration standards of 5.0–100 mg $L^{-1}$. % RSD value found in the calculation of LOD and LOQ was 8.28% (slope was 27,800). The calibration plot gave a correlation coefficient ($R^2$) value of 0.9999. The limits of detection and quantitation calculated were 16 and 54 µg $L^{-1}$, respectively. Detection limit of the developed method is low enough to perform the quantitative determination of amoxicillin in variety of matrices. Results found compete with literature values. Hoizey *et al.* performed simultaneous determination of amoxicillin and clavulanic acid in human plasma by HPLC–UV system and LOQ value for amoxicillin was found to be 625 µg $L^{-1}$ performed the validation of a chromatographic method based on HPLC for the determination of amoxicillin in wastewaters after its degradation by advanced oxidation process (19, 24). Their method allows the determination of analyte in a concentration range of 10–100 mg $L^{-1}$. They found LOD and LOQ values as 2.32 and 7.04 mg $L^{-1}$, respectively (19). It is clear that LOD and LOQ values found in our study are much lower than these studies. Repeatability expressed by the coefficient of variation (CV) of five repeated measurements for real samples ranged from 0.34 to 1.65% based on peak area and 0.65–1.78% based on peak height. These results are compatible with the literature values. Teixeira *et al.* developed a fast screening procedure for antibiotics in wastewaters by direct HPLC–DAD analysis and they found the repeatability of the method as CV ranged from 1 to 8% for their analytes including amoxicillin (25).

## Discussions

### Determination of amoxicillin in commercial drugs

Quantitative determination is one of the most important studies for the pharmaceutical industry because of the direct effect of active ingredients on human health. Sensitive and accurate analytical method allows the scientist to perform quantitative determination at trace levels without the interference effect. High extraction efficiency of amoxicillin from drugs to extraction solution is also very crucial. It is specified on the label that 80 mL suspensions of Brands A and B drugs contains 400 mg amoxicillin trihydrate. Quantitative determination was made from peak areas of the calibration plot which gave a correlation coefficient ($R^2$) value of 1.000. HPLC chromatograms of Brand A presented in Figure 2, show the small shift in the retention time to the left part as compared with the standards. In order to make sure that the signals belong to amoxicillin, a spiking experiment was performed for Brand A and it was clear in Figure 3 that, signals in the retention time of 8 min are belonging to the amoxicillin. The same result was obtained when the spiking experiment was repeated for Brand B. Analysis results show that Brands A and B suspensions contain 3960.6 ± 13.5 and 3736.6 ± 13.5 mg amoxicillin, respectively.

According to prospectuses, Brands C and D tablets contain 1,000 mg amoxicillin per tablet. Integration of HPLC chromatograms yielded 1016.1 ± 16.7 and 1014.7 ± 10.4 mg amoxicillin for Brands C and D tablets, respectively. Once Student *t*-test was applied for statistical analysis, it was observed that there is no difference in the labeled and found values. Similar observation was obtained in these brands. Spiking experiments were done for Brands C and D drug samples. Amoxicillin had the same retention time in the spiked sample with the analyte in unspiked one. The retention times of the analytes were in conformity to have no matrix effect on analyte signal causing the shift to the lower/higher retention time. It is clear that precision of the results (%RSD) was found to be lower than 1.7% for all samples analyzed.

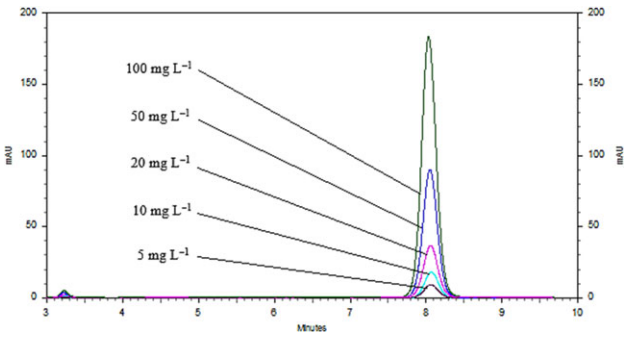



**Figure 1.** System analytical performance chromatograms (0.50–500 mg/L) of amoxicillin.

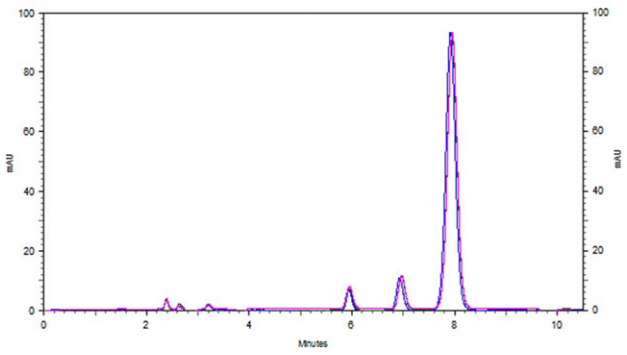



**Figure 2.** Chromatograms of three replicates of Brand A.

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## Stability of amoxicillin in simulated gastric juice

In literature, there are some studies regarding the stability of amoxicillin based on the influence of different environmental conditions. Hirte *et al.* studied new hydrolysis products of amoxicillin, their pH-dependent formation and search in municipal wastewater. They tried to find out the abiotic degradation of amoxicillin and the stability at pH 3, 7 and 11 (26). The purpose of this part of the study was to examine the behavior of the drugs' amoxicillin content in a simulated gastric digestion system. A random tablet from Brand D drug was dissolved in simulated gastric juice, from which a sample was analyzed after every half-hour period up to 240 min. The chromatogram obtained showed an undefined peak which occurred at 3.1 min. The undefined peak occurred throughout the 240 min period, showing an increase in peak area and peak height with increasing time while amoxicillin's peak decreased. This undefined peak can be said to be a result of amoxicillin degradation (Figure 4). Furthermore, spiking experiment (Figure 5) showed that there was no shift in amoxicillin peak and the undefined peak did not belong to amoxicillin. It was observed that there was a clear decrease in the peak areas and peak height of analyte with time in simulated gastric juice.

## Determination of amoxicillin in wastewater

Amoxicillin in a dense wastewater matrix was determined under the optimum HPLC conditions to find out the feasibility of qualitative and quantitative measurements. Due to the lack of the certified/standard reference material, accuracy of the method was checked by spiking experiments. Known concentration of amoxicillin was prepared in wastewater sample and it was analyzed under the optimum conditions. This aim required some systematic experiments. An unspiked wastewater sample was analyzed and showed no signal at the retention time of amoxicillin, indicating the absence of amoxicillin in the wastewater sample. A recovery test was therefore performed on the wastewater sample by spiking to have a concentration of 10 mg $L^{-1}$. The spiked sample was analyzed, and analyte concentration was determined using a calibration plot based on the peak area. A concentration of 9.70 ± 0.16 mg $L^{-1}$ was calculated, which corresponds to a percentage recovery of 97.0 ± 1.6% for amoxicillin in wastewater. This result is the good indicator for the accuracy of the method. If compared with the retention time of amoxicillin in standards, there was about 0.6 min shift to lower retention time. Therefore, another experiment was performed to prove that the 0.6 min shift in the retention time is due to matrix and determine possible matrix-based problems on the retention time of the analyte. For this purpose, 0.10 mL 500 mg $L^{-1}$ amoxicillin standard was added to 0.90 mL of the 10 mg $L^{-1}$ already spiked sample. The addition of a low volume from a high concentration standard minimizes possible effects on the retention time. Obtained chromatograms showed that the shift in the retention time is caused by matrix presented in Figure 6. Recovery experiments showed that matrix of the wastewater did not have a negative effect on the quantitative determination of amoxicillin with the exception of shift in the retention time. To the best of our knowledge, this is the first study in literature for the determination of amoxicillin in waste water with high accuracy and precision (<2% as %RSD) using a simple HPLC instrument.

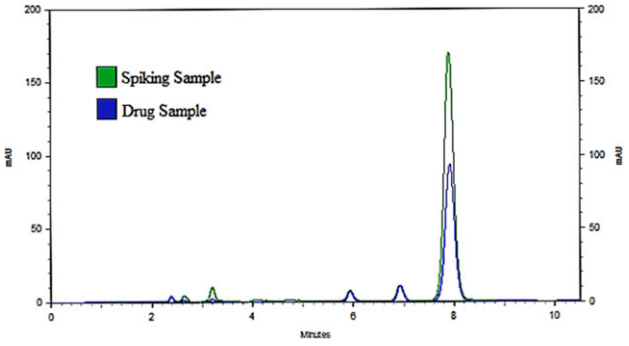



**Figure 3.** Chromatograms of spiking sample and drug sample for Brand A.

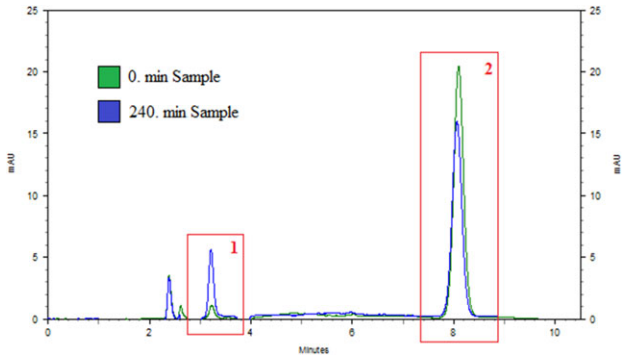



**Figure 4.** Chromatograms of amoxicillin in simulated gastric juice at 0 and 240 min.

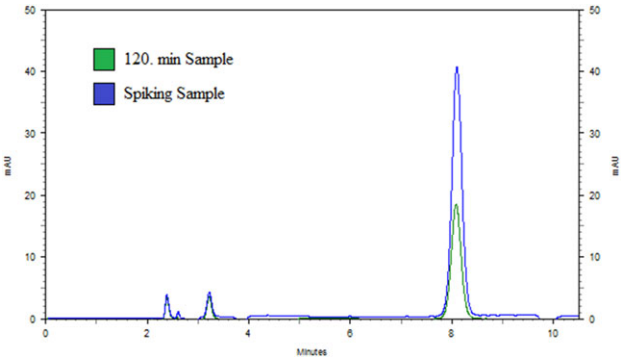



**Figure 5.** Chromatograms of spiking sample and 120 min sample of amoxicillin in simulated gastric juice.

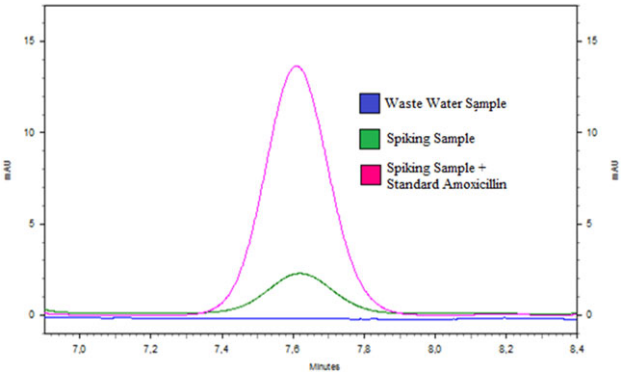



**Figure 6.** Chromatograms of wastewater sample, spiking sample and standard added sample.

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## Conclusion

A sensitive analytical method was developed for the determination of amoxicillin in different matrices. Method is applicable for routine analytical laboratories for accurate and sensitive determination of the analyte. All the system parameters in the extraction and separation studies were optimized to get not only the high recovery but also the good separation of analyte from other chemical in the matrix. Chromatographic determination of amoxicillin is completed in 10 min. There is no tailing in the signal and resolution is high. LOD and LOQ values were calculated as 16 and 54 $\mu g\,L^{-1}$, which allow us to perform sensitive measurement of the analyte by simple HPLC–UV system. Results shows that quantitative determination of amoxicillin in wastewater and commercial drugs can be performed using the analytical method developed. Wastewater analysis usually comes along with a complicated matrix but spiking experiment helped to define the complication. Stability studies of amoxicillin provides being informed on rational drug use. HPLC method developed would be used with the combination of different extraction/preconcentration method to obtain lower detection limits.

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