A Simple Sample Preparation Method for Measuring Amoxicillin in Human Plasma by Hollow Fiber Centrifugal Ultrafiltration

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A simple sample preparation method has been developed for the determination of amoxicillin in human plasma by hollow fiber centrifugal ultrafiltration (HF–CF–UF). A 400-µL plasma sample was placed directly into the HF–CF–UF device, which consisted of a slim glass tube and a U-shaped hollow fiber. After centrifugation at 1.25 × 10^3 g for 10 min, the filtrate was withdrawn from the hollow fiber and 20 µL was directly injected into the high-performance liquid chromatography (HPLC) for analysis. The calibration curve was linear over the range of 0.1–20 µg/mL (r = 0.9996) and the limit of detection was as low as 0.025 µg/mL. The average recovery and absolute recovery were 99.9% and 84.5%, respectively. Both the intra-day and inter-day precisions (relative standard deviation) were less than 3.1% for three concentrations (0.25, 2.5 and 10 µg/mL). The sample preparation process was simplified. Only after a single centrifugal ultrafiltration can the filtrate be injected directly into HPLC. The present method is simple, sensitive and accurate. It could be effective for the analysis of biological samples with high protein contents, especially for the biopharmaceutical analysis of drugs that use traditional isolation techniques for sample preparation such as the protein precipitation method.

Introduction

Sample preparation procedures play a key role in the downstream chromatographic analysis of drugs in complicated biological matrices. To remove protein interference, many tedious and time-consuming manipulations are necessary in conventional sample cleanup methods, but more steps usually introduce more errors into the process. Therefore, it is a significant challenge to develop a sample preparation method with few steps to remove the proteins. Therefore, direct injection techniques (1–8) that require limited or zero sample pretreatment steps are becoming more attractive in the field of biopharmaceutical analysis. Because the errors resulting from multistep manipulation procedures are largely reduced, direct injection techniques are generally successful, with excellent recovery rates of analytes. Furthermore, these techniques have better detection capabilities, are easier to perform and consume less time. The status and problems of these direct injection techniques have been discussed (1). Micellar chromatography, column switching method and restricted access media (RAM) columns are the most widely used direct injection approaches. Micellar chromatography (2, 9, 10) involves the addition of a surface active agent to the mobile phase. Although this improves column performance, the method has low resolution and analytical sensitivity. Column switching (3, 5, 6, 11, 12) is a more sensitive technique, but extra columns, pumps and switching valves are required. The RAM columns (4, 7, 8, 13) are constructed so that specially designed stationary phases restrict the interaction of plasma or serum proteins while partitioning the analyte. Methods using RAM columns are simpler and useful for direct injection of biofluids without pre-extracting the drug. However, loss of column efficiency and increasing backpressure over time are the two major disadvantages associated with the technique. According to the literature (3–13), when using the previously mentioned direct injection techniques, some sample preparation procedures are still inevitable, namely, the addition of extra solutions (3–5, 7, 9, 12), centrifugation (3, 4, 9, 11–13) and filtration through a 0.22-µm (5, 6) or 0.45-µm (8, 9) microbore filter unit. The primary goal for the development of a direct injection assay is to seek easier and faster analyses of drugs in biofluids.

Amoxicillin is an orally absorbed, acid stable, beta-lactam antibiotic for the treatment of a wide range of bacterial infections (14). Amoxicillin is currently one of the most commonly used antibiotics (17, 19). Quantitative analysis of this drug in biofluids is primarily conducted by high-performance liquid chromatography (HPLC) with protein precipitation (PPT) for sample preparation (15–19), in which several-fold precipitation reagents are generally added to biological samples to remove proteins. As a result, the detection capability is greatly reduced. Furthermore, the reproducibility and accuracy are often low due to the co-precipitation of the analyte with the precipitated proteins. Meanwhile, the separation performance of the column is damaged, which likely contributes to proteins remaining in the supernatant (20). Recently, commercial ultrafiltration (UF) devices have become available for the quantitative analyses of drugs in plasma (21). UF devices separate proteins and biofluids by allowing only small molecules that are below a certain molecular weight cutoff value to pass through the membrane. However, intense concentration polarization usually occurs because the centrifugal force is perpendicular to the membrane. In this way, the filtering speed may slow down rapidly and the membrane can even be blocked, which has a conspicuous influence on the recovery and volume of the filtrate. Therefore, an optimized buffer (22) and a high centrifugal force are usually required. Unfortunately, the performance of the membrane is affected when the centrifugal force exceeds the limit, and additional buffer may lead to a poor detection capability. Additionally, the high cost of analysis also discourages the wide use of this method.

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The present study employed a hollow fiber centrifugal ultrafiltration (HF–CF–UF) device to measure amoxicillin concentrations in human plasma. The sample preparation process was simplified to a 10-min centrifugation step before the filtrate was injected directly into the HPLC. This method resulted in a satisfactory limit of detection (LOD). Avoiding protein interference in the filtrate, excellent accuracy and high reproducibility were achieved. Moreover, because the direction of centrifugal force is completely parallel to the membrane with a U-shaped hollow fiber (22), the concentration polarization phenomenon has been overcome. As a result, small molecules can pass freely through the ultrafiltration membrane without additional buffer or high centrifugal force. In addition, the proposed method allowed a batch of samples to be simultaneously centrifugal ultrafiltrated. The device consisted of a slim glass tube and a hollow fiber, and thus the cost of analysis was relatively low.

Briefly, the newly developed method was sensitive, accurate, simple, quick and compatible with direct injection techniques. It may be a suitable sample preparation method for the quantitative analyses of analytes in biological matrices.

**Experimental**

**Chemicals and materials**

Amoxicillin standard (No. 0409-9907) was obtained from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Test preparation (No. 95G03/72) was offered by Yamanouchi B. V. (Leiderdrop, The Netherlands) and reference preparation (No. 980502) was purchased from North China Pharmaceutical Company (Shijiazhuang, China). The acetonitrile (HPLC grade) was purchased from Dikma (Lake Forest, CA). Deionized water was prepared using the Milli-Q50 water purification system (Millipore, Bedford, MA). All other chemicals were of analytical grade.

The polysulfone hollow fiber membrane was purchased from Kaijie Membrane Separation Technology (Hangzhou, China). The wall thickness of this fiber was 200 μm. The inner diameter was 1,000 μm and the molecular weight cutoff was 30,000 Da. The slim glass tubes were obtained from YongDa Instrument and Chemical Company (Tianjin, China).

**Apparatus and instruments**

Analysis was performed on an HPLC system consisting of an L-6200A ternary pump (Hitachi, Japan) and a 785A UV detector (Applied Biosystems, Foster City, CA). Data were collected by an HW-2000 chromatograph data workstation (Qianpu Corp, Nanjing, China).

**HPLC conditions**

Separation of the target compound was performed on an Agilent Zorbax SB-C18 column (150 x 4.6 mm, 5 μm). The separations were performed under isocratic elution using a mobile phase containing acetonitrile (25%) and 50 mmol/L potassium dihydrogen phosphate (adjusted to pH 5.0, 97.5%). The chromatogram was monitored at 220 nm. The flow rate was 1 mL/min and the injection volume was 20 μL. The column temperature was set at 28°C.

**Standard solution preparation**

The stock solution of amoxicillin was prepared in deionized water at a concentration of 400 μg/mL and stored at 4°C for a week. A series of working solutions was freshly prepared by diluting the stock solution with deionized water to final concentrations of 200, 100, 50, 20, 10, 5, 2 and 1 μg/mL.

**Sample preparation**

The hollow fiber was cut into 15-cm segments, sonicated in deionized water and then dried naturally until use. Approximately 400 μL of plasma sample was placed into a slim glass tube. The hollow fiber (15 cm) was bent to form a U-shape and inserted into the slim glass tube, as shown in Figure 1. After ultrafiltration for 10 min at 1.25 x 10^3 g, the filtrate in the lumen of the hollow fiber was pushed out from the other end of the hollow fiber using a syringe and 20 μL was injected into HPLC.

**Results and Discussions**

**Optimization of sample pretreatment procedure**

**Optimization of hollow fiber**

The thickness and inner diameter of the hollow fiber influences the volume of ultrafiltrate, and different thicknesses associated with different microbores of the wall of the hollow fiber.
influence the yield of ultrafiltration. Combinations of different wall thicknesses and inner diameters of hollow fibers were evaluated (50 and 1,000 μm, 200 and 1,000 μm, 800 and 1,000 μm, 50 and 500 μm, 200 and 500 μm, 800 and 500 μm).

The results indicated that for the types of hollow fiber used in the current HF–CF–UF device, a larger inner diameter resulted in a larger volume of filtrate. The microbore of the wall of the hollow fiber decreased with an increase in the thickness of the hollow fiber. Thus, a thicker wall led to a better ultrafiltration. However, when an 800-μm hollow fiber was inserted into the 2.5-mm glass tube, a smaller volume of filtrate was obtained. Therefore, hollow fibers with a wall thickness of 200 μm and an inner diameter of 1,000 μm were chosen for subsequent experiments. These hollow fibers had molecular weight cutoffs of 10,000 or 30,000 Da. Both fibers were tested in the study and the results showed no significant differences in ultrafiltration efficiency and chromatogram, so the hollow fibers with the molecular weight cutoff of 10,000 or 30,000 Da were accepted for use in these experiments.

**Optimization of centrifugation**

The efficiency of ultrafiltration is correlated with the centrifugal force and centrifugal time, which were optimized to obtain the most efficient filtration. Samples were prepared by mixing 450 μL of blank human blood with 50 μL of a 100-μg/mL amoxicillin standard solution. An arbitrary centrifugation time (10 min) was first set to test sample filtration at different forces of 1.25 × 10^3 g (4.00 × 10^3 rpm), 2.00 × 10^3 g (5.00 × 10^3 rpm), 2.80 × 10^3 g (6.00 × 10^3 rpm), 4.00 × 10^3 g (7.00 × 10^3 rpm), 5.00 × 10^3 g (8.00 × 10^3 rpm), 8.00 × 10^3 g (1.00 × 10^4 rpm) and 1.20 × 10^4 g (1.40 × 10^4 rpm). Because the centrifugal force was parallel to the membrane of the hollow fiber in the HF–CF–UF device, only a weak centrifugal force was necessary to force the analyte to enter the hollow fiber, quickly achieve equilibration and overcome concentration polarization. The results showed that a centrifugal force of 1.25 × 10^3 g (4.00 × 10^3 rpm) is sufficient to achieve equilibration, while an overly strong force would transiently flatten the hollow fiber and obtain little ultrafiltrate. Therefore, 1.25 × 10^3 g was chosen as the optimal centrifugal force for subsequent experiments. Different centrifugal times were tested (3, 5, 7, 10, 15, 18 and 20 min) with the same samples, and it was discovered that the volume of filtrate increased when centrifugation was performed for less than 7 min, after which the volume of filtrate was unchanged. Therefore, a centrifugation time of 10 min was used for subsequent experiments.

**Method validation**

**Selectivity**

The selectivity of the method was tested by comparing the amoxicillin standard solution, filtrate of blank plasma, filtrate of control plasma spiked with amoxicillin and the filtrate of plasma sample. According to the obtained chromatograms (Figures 2A–D), no significant interference was observed from endogenous compounds peak during analysis, implying that the method is sufficiently specific.

**Linearity, limit of detection and limit of quantification**

A calibration curve was generated by plotting the peak area with eight different concentrations of amoxicillin (20, 10, 5.0, 2.5, 1.0, 0.5, 0.25 and 0.10 μg/mL) using blank plasma (450 μL) spiked with 50-μL standard working solutions for two replicates of each concentration and then operated as described previously. The calibration equation was \( A = 3.29 \times 10^3 C + \)
6.06 × 10^{-2}, with a correlation coefficient of 0.9996 by a weighted factor of 1/C^2, indicating a good linear relationship ranging from 0.1 to 20 μg/mL of amoxicillin.

The LOD and limit of quantification (LOQ) were determined by signal-to-noise (S/N) ratios of 3 and 10, respectively. The LOD and LOQ were 0.025 and 0.1 μg/mL, respectively.

Accuracy, precision and absolute recovery
The accuracy and precision of the proposed method were estimated by assaying five replicates of the 10, 2.5 and 0.25 μg/mL amoxicillin-spiked plasma. The intra-day precision was evaluated by analyzing five replicates of each plasma concentration on the same day. For the inter-day precision, five replicates of each plasma concentration were analyzed on three consecutive days. Absolute recovery was assessed by comparing the peak areas of the amoxicillin-spiked plasma with those of the amoxicillin standard solution at the same concentrations.

Satisfactory results were obtained and listed in Table I. The average recovery and absolute recovery were 99.9 and 84.5%, respectively, and both the intra-day and inter-day precisions [relative standard deviation (RSD)] were less than 3.1%.

Stability
The freeze-thaw stability of amoxicillin in human plasma was evaluated. Control plasma (450 μL) was spiked with 50 μL of a working solution containing 10, 2.5 or 0.25 μg/mL of amoxicillin. Five replicates of each concentration were subjected to freezing for 24 h at −80°C and thawing at room temperature for three cycles. In addition, for the plasma samples at the same concentrations, stabilities at freezing and room temperature for 2 h, respectively. Appropriate stabilities were also assessed after keeping samples at −80°C for zero, seven, 14 or 21 days, and by placing samples at room temperature for 2 h, respectively. Appropriate stabilities were obtained, and all RSD values for the stability samples were below 3.0%.

Analysis of human plasma samples
Twelve male healthy volunteers aged 18–25 participated in the bioequivalence study, which was conducted at the Second Hospital of Hebei Medical University. The study relied on the reported PPT method for the sample preparation process. After the bioequivalence study was completed, the remaining plasma samples from six volunteers were collected and employed to validate the present assay with the HF–CF–UF method. The study protocol was approved by the Ethics Committee of the Second Hospital of Hebei Medical University, and each volunteer gave written informed consent to participation. The amoxicillin test and reference preparations were administered at single oral dose of 500 mg to the volunteers after overnight fasting. Approximately 3 mL of blood was collected in a centrifuge tube at 0, 20, 40, 60, 90, 120, 150, 180, 240, 300 and 360 min after dosing. The plasmas were obtained after a 10-min centrifugation at 4.0 × 10^3 g and then stored at −80°C immediately until analysis. In the PPT process, a 0.4-mL plasma sample and a 0.8-mL methanol solution were mixed. After centrifugation for 10 min at 8.0 × 10^3 g, 20 μL of supernatant was injected into the HPLC. The results of the amoxicillin plasma concentration–time curve of the six subjects measured by the HF–CF–UF method were similar to the results by the PPT method, as shown in Figure 3. Three replicates of each sample were evaluated, and all RSD values were less than 5.1%. All of the data are presented in Supplementary Table I.

The concentrations of amoxicillin obtained by the PPT method were compared with the corresponding results based on HF–CF–UF for sample preparation. The comparisons were made at the same time with the same patient. Statistical analysis of the two sets of data demonstrated that the P-value was > 0.1 according to a paired-sample t-test, and with a significance level of α = 0.05, there was no difference between the two methods. An adequate correlation was obtained between the results of the HF–CF–UF method and those of the PPT method (r^2 = 0.9864). Therefore, the HF–CF–UF method reported in this work can be used to quantify amoxicillin concentrations in human plasma.

Non-specific adsorption
HF–CF–UF, as a kind of membrane separation technique, may address such concerns as non-specific adsorption, which should first be taken into consideration. Therefore, to evaluate the adsorption phenomenon, five replicates of different concentrations of amoxicillin standard solution were prepared (0.25, 2.5 and 10 μg/mL) and subsequently 400 μL was placed into the HF–CF–UF device and centrifuged at 1.25 × 10^3 g for 10 min. Both intra and extra solutions of the hollow fiber were injected into the HPLC.

Three types of hollow fiber materials were studied—polysulfone, polypropylene and polivinilidene difluoride—which are available and easy to obtain commercially. The ratios of the obtained concentrations of intra and extra solutions of the hollow fiber to the corresponding standard concentrations were both approximately 100% (RSD values were less than 5%), which indicated that for these types of hollow fiber, there was no significant non-specific adsorption in this study. The results are presented in Table II.
Comparison with other methods

Several methods have been reported on the analysis of amoxicillin in human plasma (9, 12, 15, 17, 24–26). The PPT method has conventionally been used for the measurement of amoxicillin in biofluids (15, 17). While straightforward, the method has poor analytical sensitivity. Moreover, co-precipitation of the analyte with occlusion in the protein pellet may occur, which leads to a low extract recovery rate (50–80%). Additionally, the assay is usually associated with a shortened analytical column lifetime, probably due to the proteins remaining in the supernatant. Although post-column reactions and derivation have enhanced the detection capability (23) (the LOQ at 0.1 μg/mL), the additional sample manipulations might introduce additional errors and interferences. To remove the protein interferences, common techniques such as solid-phase extraction (SPE) (24) or liquid–liquid extraction (LLE) have been employed, but large volumes of hazardous solvents and numerous laborious and time-consuming procedures naturally resulted in poor working efficiency. In addition, there have been several reports on the determination of amoxicillin by HPLC–mass spectrometry (MS) (25, 26). Although better selectivity and excellent detection capability (LOQ at 5 ng/mL) are attractive, the procedures are still complicated and costly, and require special conditions. Column switching, a direct injection technique, has been used to measure amoxicillin levels in human plasma (12). The simplified procedure used in the assay ensures higher accuracy and low LOQ (0.05 μg/mL). However, the precolumn purification and re-equilibration may require a longer analysis time and extra buffers, and centrifugation is still needed.

By contrast, the newly developed method in this paper was easy to perform. The sample preparation process was significantly minimized. Approximately 400 μL of the sample without further treatment was added into the hollow fiber centrifugal ultrafiltration device. After a simple 10-min centrifugal ultrafiltration, the filtrate, excluded from the interference of macromolecules, could be directly injected into HPLC. The method was fully validated and proven to be sensitive enough for the analysis of clinical samples (the LOD was 0.025 μg/mL, which was 20 times lower than the result obtained by using the PPT method). Additionally, excellent recovery rates were achieved (the extraction recovery rate was 84.5%, which was higher than that obtained by using the PPT method). This result indicated that the measured amoxicillin practically consisted of the unbound form presented in plasma, and the result was used to evaluate the total concentration of amoxicillin. The plasma protein binding ratio of amoxicillin is approximately 15–20% (24) and is stable in healthy bodies. Compared with LLE, SPE and the newly developed solid phase micro-extraction (SPME) or stir bar sorptive extraction (SBSE) techniques require no additional reagent or complicated and expensive devices. Meanwhile, the short analysis time and simple performance procedure also confirmed the superiority of this method. In addition, neither extra instruments (such as precolumns, pumps and switching valves) nor longer analysis time were demanded, unlike the column-switching technique. The advantages of this method are consistent with the character of the direct injection technique.

Comparison with previously reported HF–CF–UF

The HF–CF–UF method has been used in the determination of cefaclor concentrations in human plasma (22). The hollow fiber tube was 6 cm in length and 5 mm in diameter. Although the differences in the length and diameter of the tube did not directly contribute to the improvement of the performance of the device, the hollow fiber was easier to insert into the glass tube. Moreover, in an experiment described by Li et al. (22), 25 μL of an optimized 2.0M phosphate buffer solution (pH 2) were added to the sample to release the drugs from the drug–protein complex to determine the total drug concentration. In this study, no additional reagents were required, and the sample could be directly loaded into the HF–CF–UF device for the analysis of free drug to substitute the total concentrations of amoxicillin. Therefore, this method is more precise and simpler, which indicates a new free drug analytical method that may be used to determine the unbound level of other drugs.

Conclusions

This study developed a simple, sensitive and accurate sample preparation method without a tedious performance procedure, long analysis time or expensive analysis. No preparation steps except a single 10-min centrifugation were required, and the filtrate can be applied to HPLC. The proposed sample preparation method based on a hollow fiber centrifugal ultrafiltrate device might be very useful for the determination of analytes in biological samples in human pharmacokinetic and bioequivalence studies.

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References


