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Short communication

Simultaneous determination of amoxicillin and clavulanic acid in human plasma by isocratic reversed-phase HPLC using UV detection

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Abstract

A simple, rapid and sensitive isocratic reversed phase HPLC method with UV detection using internal standard has been developed and validated for simultaneous determination of amoxicillin and clavulanic acid in human plasma. The assay enables the measurement of amoxicillin and clavulanic acid for therapeutic drug monitoring with a minimum quantification limit of 15 and 30 ng ml^{-1}, respectively. The method involves simple, one-step extraction procedure and analytical recovery was complete. The separation was carried out in reversed-phase conditions using a Chromolith Performance (RP-18e, 100 mm × 4.6 mm) column with an isocratic mobile phase consisting of 0.02 M disodium hydrogen phosphate buffer–methanol (96:4, v/v) adjusted to pH 3.0. The wavelength was set at 228 nm. The coefficients of variation for inter-day and intra-day assay were found to be less than 9.0%.
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1. Introduction

Amoxicillin is presently the most commonly used antibiotic. Clavulanic acid is a powerful inhibitor of β-lactamase enzyme and is most often formulated in combination with antibiotics such as amoxicillin for treatment of infection caused by β-lactamase producing bacteria that are resistant to amoxicillin alone [1]. Various high-performance liquid chromatography methods have been developed and validated for the assay of these two compounds in pharmaceutical preparations and biological fluids using special techniques such as precolumn derivatization, derivatization followed by solid phase extraction, post column derivatization, β-cyclodextrin stationary phase, amperometric detection and ion pair technology [2–10]. Capillary electrophoresis with UV detection was also used for simultaneous determination of amoxicillin and clavulanic acid in pharmaceutical formulations [11]. Bioequivalence study of tablet formulation of amoxicillin–clavulanic acid was reported by Sourgens et al. [12,13]. They used two different HPLC method for determination of amoxicillin and clavulanic acid separately in human plasma. Simultaneous determination of amoxicillin and clavulanic acid in human plasma provides problems due to their amphoteric nature thus causing them to elute among other endogenous, polar substances in plasma. In addition, their high polarity precludes the use of standard liquid extraction steps. There are number of reports on simultaneous determination of amoxicillin and clavulanic acid in human plasma by HPLC–mass spectrometry [14,15]. Although these methods are selective, fast and sensitive but are not suitable for routine clinical analysis because of their specialty requirement and financial reasons. There are only a few report presenting simple, fast and sensitive method on simultaneous determination of amoxicillin and clavulanic acid in human plasma. Mascher and Kikuta [16] described an HPLC method using a post column derivatization procedure with fluorescamine and fluorescence detection. The described method only used for determination of amoxicillin in human plasma. Hoizey et al. [17] reported the development of a new method by using HPLC with UV detection

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for simultaneous measurement of amoxicillin and clavulanic acid in human plasma. This method used a gradient condition for elution of samples without using internal standard. Our study describes the development and validation of a simple, rapid and sensitive reversed phase HPLC method with UV detection to measure amoxicillin and clavulanic acid simultaneously in human plasma, which takes advantage of isocratic condition and also using internal standard in order to obtain more precise results. In our method, separation was performed on a reversed-phase monolithic column, which has lower separation impedance comparing to the particulate packings [18], and therefore it allows easy optimizing chromatographic conditions to obtain desirable resolution in a short time. We also demonstrate the applicability of this method for pharmacokinetic studies in humans.

2. Experimental

2.1. Chemicals

Amoxicillin and clavulanic acid were supplied by Kowsar Pharmaceuticals (Tehran, Iran). Coamoxiclav is available as oral tablet containing 500 mg of amoxicillin, 125 mg clavulanic acid other inactive ingredients. HPLC-grade methanol and all other chemicals were obtained from Merck (Darmstadt, Germany). Water was obtained by double distillation and purified additionally with a Milli-Q system.

2.2. Instruments and chromatographic conditions

The chromatographic apparatus consisted of a model Wellchrom K-1001 pump, a model Rheodyne 7125 injector and a model K 2501 UV detector connected to a model Eurochrom 2000 integrator, all from Knauer (Berlin, Germany). The separation was performed on Chromolith Performance (RP-18e, 100 mm × 4.6 mm) column from Merck (Darmstadt, Germany). The wavelength was set at 228 nm. The mobile phase was a mixture of 0.02 M disodium hydrogen phosphate buffer–methanol (4:96, v/v) adjusted to pH 3.0 at a flow rate of 1.3 ml min^{-1}. The mobile phase was prepared daily and degassed by ultrasonication before use. The mobile phase was not allowed to recirculate during the analysis.

2.3. Standard solutions

Stock solutions of amoxicillin (6 mg ml^{-1}) and clavulanic acid (2 mg ml^{-1}) were prepared in methanol. Then 0.2, 1, 2, 4, 6, 9 and 12 μg ml^{-1} working standards of amoxicillin and 0.1, 0.5, 1, 2, 3, 4 and 6 μg ml^{-1} working standards of clavulanic acid were freshly prepared in plasma from the stock solution before analysis.

2.4. Sample preparation

To 500 μl of plasma in a glass-stoppered 15 ml centrifuge tube were added 20 μl of allopurinol as internal standard (100 μg ml^{-1}) and 700 μl of acetonitrile. After mixing (30 s), the mixture centrifuged for 5 min at 8000 × *g*. Then 750 μl dichloromethane was added to 500 μl of supernatant. After mixing (30 s), the mixture centrifuged for 5 min at 8000 × *g*. Then, a mixture of 20 μl of supernatant and 30 μl of mobile phase was injected into liquid chromatograph.

2.5. Biological samples

Twelve male healthy volunteers were included in this study. The study protocol was approved by the Ethics Committee of Shaheed Beheshti University of Medical Sciences and written informed consent was obtained from the volunteers. Coamoxiclav tablet was administered in a dose of 500/125 mg (amoxicillin/clavulanic acid) to the volunteers after over night fasting. Plasma samples were collected at 0, 20, 40, 60, 90, 120, 150, 180, 240, 300 and 360 min after dosing and then frozen immediately at −20 °C until assayed.

2.6. Stability

The stability of amoxicillin/clavulanic acid was assessed for spiked plasma samples stored at −20 °C for up to 1 month. The stability of stock solutions stored at above mentioned temperatures was determined by injecting appropriate dilutions of stocks in distilled water at different days (1, 15, and 30) and comparing their peak areas with fresh stock prepared on the day of analysis. Samples were considered to be stable, if the assay values were within the acceptable limits of accuracy and precision.

2.7. Plasma standard curve

Blank plasma was prepared from heparinized whole-blood samples collected from healthy volunteers and stored at −20 °C. After thawing, stock solution of amoxicillin and clavulanic acid was added to yield final concentrations ranging from 0.2 to 12 μg ml^{-1} for amoxicillin and 0.1–6 μg ml^{-1} for clavulanic acid. Internal standard solution was added to each of these samples to yield a concentration of 4 μg ml^{-1}. The samples were then prepared for analysis as described above.

2.8. Selectivity

Control human plasma, obtained from twelve healthy volunteers, was assessed by the procedure as described above and compared with respective plasma samples to evaluate selectivity of the method.

2.9. Precision and accuracy

The precision and accuracy of the method were examined by adding known amounts of amoxicillin and clavulanic acid to pool plasma (quality control samples). For intra-day precision and accuracy six replicate quality control samples at each concentration were assayed on the same day. The inter-day precision and accuracy were evaluated on three different days.

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