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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%.

Keywords: Amoxicillin; Clavulanic acid; Plasma; HPLC; Monolithic column

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.

2.10. Limit of quantification (LOQ) and recovery

For the concentration to be accepted as LOQ, the percent deviation from the nominal concentration (accuracy) and the relative standard deviation must be ±20% and less than 20%, respectively, considering at least five-times the response compared to the blank response. The relative analytical recovery for plasma at three different concentrations of amoxicillin (1, 5 and 10 μg ml^{-1}) and clavulanic acid (0.5, 2.5 and 5 μg ml^{-1}) was determined. Known amounts of amoxicillin and clavulanic acid were added to drug-free plasma and the internal standard was then added. The relative recovery of amoxicillin and clavulanic acid was calculated by comparing the peak areas for extracted amoxicillin and clavulanic acid from spiked plasma and a standard solution of amoxicillin and clavulanic acid in deionized water containing internal standard with the same initial concentration (six samples for each concentration level).

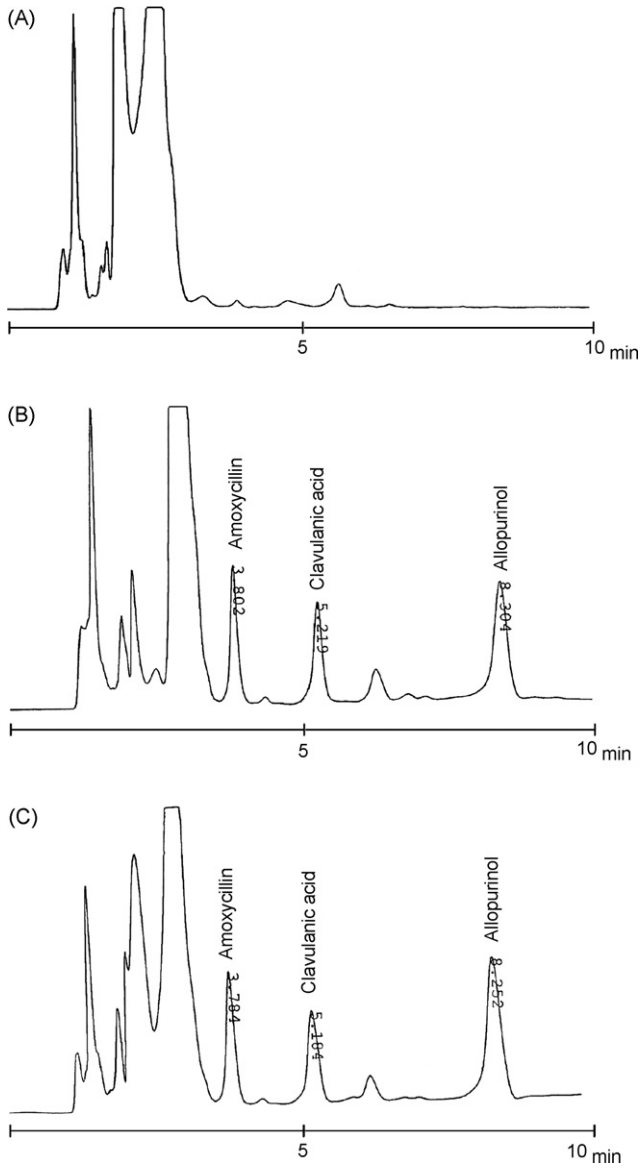

Fig. 1. Chromatograms of (A) blank plasma; (B) blank plasma spiked with 4 μg ml^{-1} of amoxicillin, 2 μg ml^{-1} of clavulanic acid and 4 μg ml^{-1} allopurinol (internal standard); (C) plasma sample from a healthy volunteer 1 h after oral administration 625 mg of coamoxiclav.

Table 1
Relative recovery of amoxicillin from plasma

Amoxicillin spiked concentration (μg ml^{-1})	Amoxicillin concentraion found (n = 3)	Recovery (mean ± S.D.) (%)
1	0.92	91.9 ± 2.1
5	4.64	92.8 ± 4.6
10	9.67	96.7 ± 2.8

3. Results and discussion

Under the chromatographic conditions described, amoxicillin, clavulanic acid and the internal standard peaks were well resolved. Endogenous plasma components did not give any interfering peaks. Fig. 1 shows typical chromatograms of blank plasma in comparison to spiked samples analyzed for a pharmacokinetic study. The average retention times of amoxicillin, clavulanic acid and allopurinol were 3.8, 5.2 and 8.3 min, respectively. The calibration curves were linear over the range of 0.2–12 μg ml^{-1} for amoxicillin and 0.1–6 μg ml^{-1} for clavulanic acid. The linearity of this method was statistically confirmed. For each calibration curve, the intercept was not statistically different from zero. The correlation coefficients (r) for calibration curves were equal to or better than 0.999. The relative standard deviation (R.S.D.) values of the slope were equal to or better than 6%. For each point of calibration standards, the concentrations were recalculated from the equation of the linear regression curves. The relative analytical recovery for plasma at three different concentrations of amoxicillin and clavulanic acid were determined. The average recoveries were 93.8 ± 3.2% (n = 6) and 92.6 ± 1.9% (n = 6) for amoxicillin and clavulanic acid respectively (Tables 1 and 2). The limit of quantification (LOQ), as previously defined, was 15 ng ml^{-1} for amoxicillin and 30 ng ml^{-1} for clavulanic acid. This is sensitive enough for drug monitoring and other purposes such as pharmacokinetic studies. We assessed the precision of the method by repeated analysis of plasma specimens containing known concentrations of amoxicillin and clavulanic acid. As shown in Tables 3 and 4 coefficients of variation were less than 9%, which is acceptable for the routine measurement of amoxicillin and clavulanic acid. Stability was determined for spiked plasma samples under the conditions as previously described. The results showed that the samples were stable during the mentioned conditions. This method is well suited for routine application in the clinical laboratory because of the speed of

Table 2
Relative recovery of clavulanic acid from plasma

Clavulanic acid spiked concentration (μg ml^{-1})	Clavulanic acid concentraion found (n = 3)	Recovery (mean ± S.D.) (%)
0.5	0.46	92.0 ± 2.9
2.5	2.33	93.2 ± 1.8
5	4.63	92.6 ± 0.9

Table 3
Reproducibility of the analysis of amoxicillin in human plasma ($n = 6$)

Concentration added ($\mu g\,ml^{-1}$)	Concentration measured (mean ± S.D.)	
	Intra-day	Inter-day
1	1.13 ± 0.07 (6.19)	1.12 ± 0.08 (7.14)
5	5.42 ± 0.28 (5.25)	5.41 ± 0.28 (5.20)
10	10.02 ± 0.54 (5.38)	10.04 ± 0.51 (5.13)

Values in parentheses are coefficients of variation (%).

Table 4
Reproducibility of the analysis of clavulanic acid in human plasma ($n = 6$)

Concentration added ($\mu g\,ml^{-1}$)	Concentration measured (mean ± S.D.)	
	Intra-day	Inter-day
0.5	0.50 ± 0.04 (8.45)	0.49 ± 0.04 (8.16)
2.5	2.50 ± 0.16 (6.21)	2.47 ± 0.18 (7.11)
5	5.00 ± 0.28 (5.65)	4.95 ± 0.31 (6.26)

Values in parentheses are coefficients of variation (%).

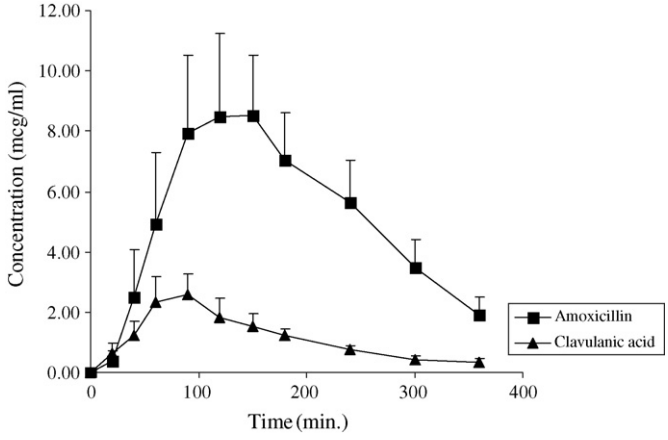

Fig. 2. Mean plasma concentration–time profile of amoxicillin and clavulanic acid in healthy volunteers after a single dose of 625 mg coamoxiclav.

analysis and simple isocratic condition. Accordingly, the chromatographic elution step is undertaken in a short time (less than 10 min) with high resolution. Clavulanic acid is very water soluble and therefore difficult to extract from plasma. Sample preparation was accomplished by deproteinization of the plasma with acetonirile and removal of acetonitrile by extraction with dichloromethane. Also, the use of internal standard provides an advantage as compared with some previous methods that did not used internal standard. Over 5003 plasma samples were analyzed by this method without any significant loss of resolution. No change in the column efficiency and backpressure was also observed over the entire study time, thus proving its suitability. In this study plasma concentrations were determined in twelve healthy volunteers, who received coamoxiclav tablet in a dose of 500/125 mg (amoxicillin/clavulanic acid). Fig. 2. Shows the mean plasma concentration–time curve of amoxicillin and clavulanic acid. Plasma concentration of amoxicillin reached a maximum 132.5 ± 27.1 min after dosing with a level of 8.53 ± 1.97 $\mu g\,ml^{-1}$. Also plasma concentration of clavulanic acid reached a maximum 90.8 ± 24.6 min after dosing with a level of 2.58 ± 0.67 $\mu g\,ml^{-1}$. These pharmacokinetic parameters are in good agreement with that found previously [12,13].

4. Conclusions

A rapid and simple HPLC method has been described for simultaneous analysis of amoxicillin and clavulanic acid in human plasma. Using monolithic column, the chromatographic elution step is undertaken in a short time with high resolution. In addition, the use of a simple sample preparation instead of more complex extraction procedures makes this method suitable for pharmacokinetic and bioequivalence studies of amoxicillin and clavulanic acid simultaneously in humans.

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