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Simultaneous determination of amoxicillin and clavulanic acid in human plasma by HPLC with UV detection

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Abstract

A simple and accurate high-performance liquid chromatographic method with ultraviolet detection at 220 nm has been validated for the simultaneous determination of amoxicillin and clavulanic acid in human plasma. Plasma samples were pretreated by direct deproteinization with methanol. A good chromatographic separation between both compounds was achieved using a reversed phase C8 column and a mobile phase, consisting of acetonitrile-phosphate solution-tetramethyl ammonium chloride solution. The calibration curves were linear over the concentration range of 0.625–20 mg l^{-1} for amoxicillin and 0.3125–10 mg l^{-1} for clavulanic acid with determination coefficients >0.998. The method is accurate (bias $<7\%$) and reproducible (intra- and inter-day R.S.D. $<15\%$), with a quantitation limit of 0.625 and 0.3125 mg l^{-1} for amoxicillin and clavulanic acid, respectively. Analytical recoveries from human plasma ranged from 91 to 102% for both components. This fully validated method, which allows the simultaneous measurement of amoxicillin and clavulanic acid in biological samples, is rapid (total run time <10 min) and requires only a 100 μl sample. This assay is suitable for biomedical applications and was successfully applied to a pilot pharmacokinetics study in healthy volunteers after a single-oral administration of amoxicillin/clavulanic acid combination (500/125 mg). © 2002 Published by Elsevier Science B.V.

Keywords: Amoxicillin; Clavulanic acid; Biological samples; Reversed phase HPLC-UV assay

1. Introduction

Amoxicillin combined with the β-lactamase inhibitor, clavulanic acid, is frequently used as an antibacterial formulation for the treatment of infection caused by β-lactamase-producing bacteria that are resistant to amoxicillin alone [1]. Several methods have been reported for the analyses of those two compounds, such as microbiological assay [2], enzymatic assay [3], ultraviolet spectrometry [4] or polarography [5]. Since these methods are generally known to be not specific

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enough, high performance liquid chromatography (HPLC) assays have also been developed. They usually involved pretreatment of amoxicillin and clavulanic acid with imidazole [6], precolumn [7] and postcolumn derivatization [8] or ion-pair HPLC [9]. More recently, very interesting HPLC procedures for simultaneous assay of these two compounds have also been described, including HPLC with reversed phase [10] or β-cyclodextrin stationary phase [11] with ultraviolet (UV) or amperometric detection [12]. Although those methods are accurate and sensitive, they have, to date, only be validated for quantitative analysis of both compounds in pharmaceutical preparations, yet have never been applied to a clinical situation.

The present paper reports on the development of a new, validated, fast and sensitive method by using HPLC with UV detection for the simultaneous measurement of amoxicillin and clavulanic acid in human plasma after a single-step pre-treatment of the sample. This simple assay using only 100 μl of plasma has been successfully applied to the analysis of plasma samples in a preliminary pharmacokinetics study in healthy volunteers.

2. Experimental

2.1. Reagents

Amoxicillin trihydrate, lithium clavulanate and Augmentin® tablets (labeled to contain 500 mg amoxicillin and 125 mg clavulanic acid per tablet) were provided from GlaxoSmithkline (Worthing, UK). Potassium dihydrogenphosphate, dibasic sodium hydrogen phosphate and tretramethylammonium chloride were obtained from Merck (Darmstadt, Germany). HPLC grade acetonitrile and methanol were purchased from SDS (Peypin, France) and *o*-phosphoric acid from Prolabo (Paris, France). Water was obtained from a Milli-Q system Waters purification system (Millipore, Saint-Quentin-Yvelines, France).

2.2. Instrumentation and chromatographic conditions

The liquid chromatograph consisted of a Varian Model 9012 HPLC pump and a manual Rheodyne 7125 injection valve equipped with a 20-μl loop. The chromatographic separations were carried out using a 5-μm particle size Lichrospher column 100 RP8 (250 × 4 mm I.D., Merck, Nogent Sur Marne, France). The separated components were detected using a Spectroflow 783 UV detector (Eurosep Instruments, Cergy-Pontoise, France) and detector signals were recorded on a Chromjet® integrator (ThermoFinnigan, Les Ulis, France).

Samples were eluted with a mobile phase consisting of solvent A (0.01 M aqueous solution of potassium dihydrogenphosphate containing 0.02 M tetramethylammonium chloride and adjusted to pH 2.5 with *o*-phosphoric acid) and solvent B (acetonitrile). The samples were eluted with 7% B for an initial 3 min after injection followed by 1 min linear gradient to 12% B and a 2 min isocratic period at 12% B, then a 1 min linear gradient to 7% B and a 3 min isocratic period.

The flow-rate was 1.0 ml min^{-1} and the wavelength was set at 220 nm. Total analysis time was 10 min. All analyses were performed at room temperature.

2.3. Solutions preparation

Stock solutions of amoxicillin and clavulanic acid were prepared as 1 g l^{-1} in a phosphate buffer (71 ml of 1/15 M potassium dihydrogenphosphate added to 29 ml of 1/15 M dibasic sodium hydrogen phosphate, pH 6.0). The solutions were stored at + 4 °C and remained stable for at least 1 month. Standard solutions were freshly prepared for each run day.

2.4. Standards and quality control

Working solution of amoxicillin (200 mg l^{-1}) and clavulanic acid (100 mg l^{-1}) were prepared by dilution of the stock solutions with phosphate buffer (pH 6.0). Working standards solutions were prepared from this solution by serial dilution with phosphate buffer, to yield final concentrations of 0.625, 1.25, 2.5, 5, 10 and 20 mg l^{-1} for amoxicillin and 0.3125, 0.625, 1.25, 2.5, 5 and 10

mg l^{-1} for clavulanic acid for plasma determination.

Quality controls have been prepared from a pool of blank human plasma spiked with an amount ratio (mm) of amoxicillin to clavulanic acid equivalent to 4:1 (corresponding to final plasma concentrations of 5 and 1.25 mg l^{-1} for amoxicillin and clavulanic acid, respectively). Plasma aliquots were stored at −20 °C until assay.

2.5. Sample preparation

Aliquots (100 μl) of standards, quality controls and subject plasma were transferred into 1.5 ml polypropylene micro-centrifuge tubes. Samples were deproteinized by the addition of 250 μl of methanol, vortexed for 15 s and then centrifuged at $2500 \times g$ for 5 min. Some 20 μl of the clean upper layer was injected directly into the chromatographic system.

2.6. Calculations

Quantitative analysis were performed using the external standard method. Standard curves were obtained by unweighted linear regression of the peak-area amoxicillin and clavulanic acid versus known concentrations of both compounds. Concentrations of quality controls and unknown samples were estimated by applying the linear regression equation of the standard curve to the unknown samples peak-area.

2.7. Recovery of amoxicillin and clavulanic acid

Values of percentage recoveries were determined by comparing the peak areas of plasma blank samples spiked with different amounts of both components and treated as any sample, with the peak areas of the same standards prepared in phosphate buffer. Each point was established from an average of six determinations.

2.8. Method validation

Precision and accuracy of the assay were assessed by replicate analyses of spiked plasma blank samples over three levels of concentrations of amoxicillin and clavulanic acid used for calibration curves. Six and ten separate samples at each concentration level were assayed for intra-day and inter-day evaluations, respectively. Precision is reported as %R.S.D. of the estimated concentrations and accuracy (bias) expressed as [(mean calculated concentration/spiked concentration) × 100 − 100]. Precision was also assessed for the plasmatic pool analyzed with each analytical run.

2.9. Application of the method for the analysis of clinical samples

Three healthy male subjects, enrolled in a clinical study, were each given a single oral dose of amoxicillin/clavulanic acid (500/125 mg), Augmentin®. Plasma samples were obtained from blood samples collected before dosing and at 20, 40, 60, 80, 100, 120, 140, 160, 180, 240, 300, 480 and 720 min after tablet intake. Plasma samples were stored frozen at −20 °C until analysis.

3. Results and discussion

3.1. Chromatography

Representative chromatograms of plasma spiked with drug, patient plasma prior to and after receiving a single oral dose of amoxicillin/clavulanic acid (500/125 mg; Augmentin®) are shown in Fig. 1. The peaks of clavulanic acid and amoxicillin are clearly resolved with short retention times of 5.50 and 9.50 min, respectively. No interfering peaks were observed within the time frame in which amoxicillin and clavulanic acid was detected.

Particular attention has been paid to the preparation of the stock solutions. Due to the fact that amoxicillin and clavulanic acid are known to be moderately stable [12], the stock solutions were prepared in an acidic phosphate buffer instead of a neutral buffer or other solvents. Indeed, we found that the use of an acidic buffer allowed the lengthening of the stability of the solutions by comparison with other tested solvents or buffer (i.e. ≈ 1 month vs. < 10 days).

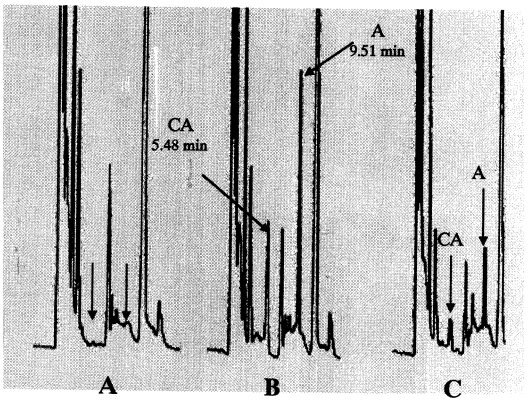

Fig. 1. Representative chromatograms of blank human plasma (panel A), human plasma spiked with amoxicillin and clavulanic acid (2.5 and 1.25 mg l^{-1}, respectively) (panel B) and plasma sample containing both compounds (0.77 and 0.34 mg l^{-1} for amoxicillin and clavulanic acid, respectively) obtained 5 h (panel C) following the oral administration of an amoxicillin/clavulanic acid association (500/125 mg; Augmentin®) in one volunteer. A, amoxicillin; CA, clavulanic acid. Arrows indicate the retention time of both compounds.

The composition of the mobile phase was chosen to provide concomitantly the best peak resolution and retention times. As such, the added tetramethylammonium chloride in mobile phase is an important factor, since it permits the avoidance of the rapid occurrence of worse peak shapes for both components. Besides, the pH of the mobile phase was found to play an important role in peak resolution. It was first fixed at 3.0, but then lowered to 2.5, so that the peaks were not too close.

3.2. Linearity and limit of quantification

The linearity of the method was evaluated with calibration curves made in human plasma ranging from 0.625 to 20 mg l^{-1} for amoxicillin and from 0.3125 to 10 mg l^{-1} for clavulanic acid. Each point was established from an average of ten determinations.

For both compounds, a good linear relationship between detector signal and spiked concentrations was found, as described by the following linear regression equations: $y = 23.7x - 0.13$ ($r^2 = 0.9993$) for amoxicillin and $y = 13.03x - 0.99$ ($r^2 = 0.9989$) for clavulanic acid, where y is the peak area of both compounds and x is the concentration (mg l^{-1}). Values of the coefficients of determination are all satisfactory.

Detection limits were determined as the concentration of components giving a signal to noise ratio $> 3{:}1$. The limits of detection for amoxicillin and clavulanic acid in human plasma were found to be 0.05 and 0.08 mg l^{-1}, respectively. The limits of quantitation (LOQ) of amoxicillin and clavulanic acid in plasma were chosen as the concentrations used for the lowest concentration level on the calibration curves and for which the R.S.D. was $< 15\%$ (i.e. 0.625 and 0.3125 mg l^{-1} for amoxicillin and clavulanic acid, respectively). The LOQ seem to be adequate taking into account the concentrations of amoxicillin and clavulanic acid determined in samples collected in the preliminary clinical investigation conducted in our laboratory and described below.

Table 1
Analytical recovery of amoxicillin and clavulanic acid in human plasma ($n = 6$)

Amoxicillin			Clavulanic acid		
Spiked plasma concentration (mg l^{-1})	Recovery (%)	R.S.D. (%)	Spiked plasma concentration (mg l^{-1})	Recovery (%)	R.S.D. (%)
1.25	95.4 ± 4.8	5.0	0.625	98.3 ± 5.6	5.7
5	96.5 ± 3.4	3.5	2.5	91.3 ± 3.6	3.9
20	98.7 ± 3.2	3.2	10	101.9 ± 1.8	1.7

Table 2
Intra- and inter-day precision and accuracy of amoxicillin and clavulanic acid in human plasma

Amoxicillin				Clavulanic acid			
Intra-day ($n=6$)	Mean ± S.D. (mg l^{-1})	R.S.D. (%)	Bias[a] (%)	Intra-day ($n=6$)	Mean ± S.D. (mg l^{-1})	R.S.D. (%)	Bias[a] (%)
1.25	1.24 ± 0.11	8.9	−0.8	0.625	0.60 ± 0.06	10.0	−4.0
5	4.92 ± 0.43	8.7	−6.9	2.5	2.39 ± 0.16	6.7	−4.4
20	21.2 ± 1.12	5.3	1.6	10	10.1 ± 0.49	4.8	1.0
Inter-day ($n=10$)				Inter-day ($n=10$)			
1.25	1.26 ± 0.17	13.5	0.8	0.625	0.64 ± 0.07	10.9	2.4
5	5.06 ± 0.48	9.5	1.2	2.5	2.42 ± 0.19	7.8	−3.2
20	19.2 ± 1.38	7.2	−4.0	10	9.65 ± 0.59	6.1	−3.5

[a] Expressed as [(mean calculated concentration/spiked concentration) × 100 − 100].

3.3. Analytical recovery

The percentage recoveries (mean value ± S.D., $n=6$) of amoxicillin and clavulanic acid obtained in spiked human plasma over the entire concentration range are given in Table 1. These results indicate that the simple protein precipitation procedure with methanol is sufficient to ensure extraction recovery from plasma $>90\%$.

3.4. Intra-day and inter-day precision and accuracy

Within-assay and between-assay precision and accuracy determined in human plasma for three levels of concentrations of amoxicillin and clavulanic acid are reported in Table 2. All results were within the ranges acceptable for bio-analytical purposes.

The quality control constituted by a plasmatic pool of patients spiked with both amoxicillin and clavulanic acid (5 and 1.25 mg l^{-1}, respectively) has been assessed over 20 analyses. The results (Table 3) show that the R.S.D. values remain satisfactory.

3.5. Applicability in human plasma samples

The concentration-time profile for amoxicillin and clavulanic acid in plasma obtained from three healthy volunteers participating in an ongoing pharmacokinetics study are shown in Fig. 2. Detailed pharmacokinetics data for all subjects ($n=12$) enrolled in the clinical study will be reported in a separate article.

In conclusion, we developed a HPLC-UV method to quantify both amoxicillin and clavulanic acid in human plasma using a single chromatographic run for application to pharmacokinetics studies. This simple assay demonstrates good reproducibility, has a short analysis duration and has been successfully applied to the analysis of plasma samples from healthy volunteers administered a single oral dose of a formulation of amoxicillin/clavulanic acid. Since this paper demonstrates the applicability of the assay for biomedical applications, consequently, it is reasonable to assume that it will also be suited for bioequivalence studies. Finally, the proposed method may represent an alternate rapid assay for analysis of these two components in pharmaceutical dosage forms.

Table 3
Precision of the plasmatic quality control (5 mg l^{-1} of amoxicillin and 1.25 mg l^{-1} clavulanic acid; $n=20$)

	Mean ± S.D. (mg l^{-1})	R.S.D. (%)
Amoxicillin	5.12 ± 0.28	5.5
Clavulanic acid	1.23 ± 0.13	11.4

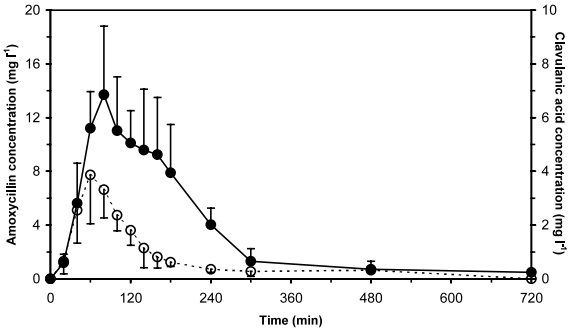

Fig. 2. Mean plasma concentration-time profiles (mean ± S.D.) of amoxicillin (∼) and clavulanic acid (□) following a single oral administration of amoxicillin/clavulanic acid (500/125 mg; Augmentin®) in three healthy volunteers.

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