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Determination of amoxycillin in human plasma by direct injection and coupled-column high-performance liquid chromatography

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Abstract

This work reports the use of multidimensional HPLC by coupling a restricted access medium (RAM) bovine serum albumin (BSA) octadecyl column (100×4.6 mm I.D., 10 μm particle size and 120 Å pore size) to an octadecyl Hypersil column (150×4.6 mm I.D., 5 μm particle size and 120 Å pore size) to the analysis of amoxycillin in human plasma by direct injection. Ion pairing was necessary to extract amoxycillin with good recovery from the plasma proteins. To prepare the spiked samples, aliquots (60 μl) of the appropriated standard solutions were added to each culture tube containing an 180 μl of plasma and a solution of 0.30 m*M* tetrabuthylammonium phosphate (60 μl). They were vortexed for 15 s and then 290 μl were transferred to autosampler vials. Aliquots (250 μl) of the spiked plasma samples were injected to a column-switching HPLC system. An analysis time of 25 min with no time spent on sample preparation was achieved. The developed method showed good selectivity, sensitivity, accuracy and precision for direct analysis of this polar low wavelength ultraviolet absorption antibiotic using only 180 μl of human plasma. The validated method proved to be reliable and sensitive for the determination of amoxycillin in plasma samples of five healthy volunteers to whom test and reference formulations were administered as an oral dose (500 mg).

Keywords: Restricted access media; Immobilized proteins; Column-switching; Albumin; Amoxycillin; Lactams; Antibiotics

1. Introduction

Amoxycillin (Fig. 1) is an α-amino-substituted β-lactam antibiotic of broad spectrum and it is clinically widely used [1,2]. Although a diverse number of high-performance liquid chromatographic (HPLC) methods have been described for the analysis of this compound in biological fluids [1–8] the assay of this amphoteric, polar substance of low wavelength ultraviolet absorption (230 nm) is still a challenge.

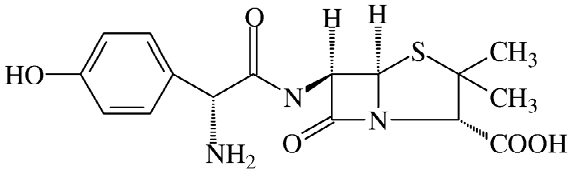

Fig. 1. Chemical structure of amoxycillin.

Multidimensional HPLC has been used with success for the analysis of amoxycillin in human serum. Ion-pairing agent in the mobile-phase and a semipermeable surface column for the sample cleaning-up coupled to an analytical octadecyl silica column were used in this method [8].

The use of a wide variety of restricted access media (RAM) columns has been reported [9–12].

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The unique feature of these columns is that they exclude the macromolecules of the matrix while selectively retaining the hydrophobic micromolecules. To permit direct injection of biological samples, the RAM column must be capable of removing the macromolecules with high efficiency. Recently, we have used RAM bovine serum albumin (BSA) octyl column for the online sample extractions of pantoprazole enantiomers [13], metyrapone and metyrapol enantiomers [14] from human plasma. A recovery of 98.6% of human plasma protein was achieved in 5 min with 100% water as mobile phase at flow-rate of 1.0 ml/min [14].

In the present work this type of RAM column was explored for the analysis of amoxycillin by direct injection of plasma samples.

2. Experimental

2.1. General

Solvents were HPLC grade from J.T. Baker (Philipsburg, PA, USA). Water was purified with a Milli-Q system (Millipore, São Paulo, Brazil). The mobile phase was prepared daily and never recirculated.

Reagents and other chemical were obtained from the following sources: bovine serum albumin from Sigma (Fraction V powder minimum 98%; St. Louis, MO. USA), glutaraldehyde and sodium borohydride were from Merck (Darmstadt, Germany). Tetrabuthylammonium phosphate from Acros Organics (Pittsburgh, PA, USA). Amoxycillin was USP standard.

Pooled control human plasma was supplied by the São Francisco University Hospital, Bragança Paulista, Brazil and stored at −20 °C until use. For analysis, the plasma samples were thawed at room temperature and centrifuged for 10 min at 5000 *g*.

Written consent was obtained from each volunteer prior the study and the protocol was approved by the São Francisco University Medical School Ethics Committee in accordance with the Declaration of Helsinki.

2.2. Equipment

The HPLC system consisted of two Shimadzu LC-10ATVP pumps (Kyoto, Japan), with one of the pumps having a valve FCV-10AL for selecting solvent, an autoinjector model SIL 10AVP, a degasser model DGU-14A, a column oven CTO-10A (at 27 °C), a SPD-6AV UV–Vis detector, a photodiode array model SPD-10AVP and a SCL 10AVP interface. A sample valve HPLC 7000 Nitronic EA (Sulpelco, St. Louis, MO, USA) was used for the automated column switching. Data acquisition was done on a Shimadzu CLASS-VP software.

2.3. Columns

The analytical column used (150×4.6 mm I.D.) (C_{18}-Hypersil, 5 μm particle size and 120 Å pore size) was packed by the ascending slurry method using methanol for the preparation of the slurry (50 ml) and also for the packing. The packing was carried out at a pressure of 7500 p.s.i. (1 p.s.i.= 6894.76 Pa).

The restricted access media BSA columns (100× 4.6 mm I.D.) were prepared as follows: octyl or octadecyl silica columns (Hypersil, 10 μm particle size and 120 Å pore size) were packed as analytical columns. After the columns were conditioned for about 4 h with methanol at a flow-rate of 1.0 ml/min, the immobilization of BSA was done in situ based on the protocol of Menezes and Felix [15] as follows: the columns were first eluted at flow-rate of 1.0 ml/min with 0.05 *M* phosphate buffer (pH 6.0) (50 ml) before passing a 1.0 mg/ml solution of bovine serum albumin prepared in 0.05 *M* phosphate buffer (pH 6.0) (25 ml), and then a 25% (v/v) solution of glutaraldehyde (5 ml). After 5 h, the columns were washed with a 1.0 mg/ml solution of sodium borohydride (10 ml) and then with water (60 ml).

2.4. Sample preparation

A stock solution of amoxycillin (1000 μg/ml) was prepared in 0.01 *M* potassium dihydrogen phosphate buffer (pH 5.0). The buffer was prepared by dissolving 1.69 g of KH_2PO_4 in distilled water (1 l) and the pH was adjusted with a 2 *M* solution of sodium hydroxide. From the stock solution seven calibration standard solutions were prepared in the following concentrations: 6.0, 4.5, 3.0, 1.5, 0.90,

0.30 and 0.15 μg/ml and three quality controls: 0.36, 3.60 and 5.40 μg/ml. Stock solutions were stable for 2 months when stored at −20 °C and no evidence of degradation of the analyte was observed on the chromatograms during this period.

To prepare the samples, aliquots (60 μl) of the appropriated standard solution were added to 180 μl of plasma or mobile phase (for recoveries studies) in a series of culture tubes and then, 60 μl of a 0.3 m*M* solution of tetrabutylammonium phosphate, prepared in 0.01 *M* potassium dihydrogenphosphate buffer (pH 7.2) were added to each tube. The solutions were vortex-mixed for 15 s. Aliquots of 290 μl were transferred to autosampler vials and samples of 250 μl were injected to a column-switching HPLC system.

2.5. Method validation

Using the appropriate standard solution of amoxycillin spiked plasma samples were prepared in triplicate at the following concentrations: 0.05, 0.10, 0.30, 0.50, 1.00 1.50 and 2.00 μg/ml. Plasma calibration curves were constructed by plotting the peak area against the concentration of amoxycillin.

The recoveries of amoxycillin from human plasma were determined by analyzing quality controls samples at three different concentrations: 0.12, 1.20 and 1.80 μg/ml of amoxycillin. The peak-area ratios of five extracted samples at each concentration were compared with those of five injections of standard solutions to derive a percent recovery.

The intra- and inter-day precision and accuracy of the method were evaluated by replicate analysis by the same three quality controls that were used for the recovery experiments. Five samples of each concentration were prepared and extracted on three nonconsecutive days. The accuracy of the method was tested also using blinded unknowns, at two different concentrations, which were prepared by a different analyst.

The acceptance criteria for the limit of quantification were that the precision and accuracy for three-extracted sample were under 20% variability while the limit of detection was calculated taking a signal-to-noise ratio of 3.

Chromatograms of blank pooled plasma were analyzed to evaluate the selectivity of the method.

2.6. Human study

A single 500 mg dose of test and reference formulations of amoxycillin, as a suspension, were administered orally, after an overnight fast, to a group of healthy volunteers participating in a bioequivalence study. Venous blood samples were collected in heparinized vacutainer tubes at 0 (predose) and 0.5, 1, 1.5, 2, 2.5, 3, 4, 6 and 8 h after administration of amoxycillin formulation.

The tubes were centrifuged at 2000 *g* for 10 min, the plasma was collected and stored at −70 °C until analysis. Plasma samples (300 μl) from five volunteers of the group were used.

The volunteers' plasma samples were thawed at room temperature and then after centrifugation at 5000 *g* for 10 min, samples of 180 μl were pipetted into culture tubes and a 60 μl of 0.01 *M* potassium hydrogen phosphate buffer (pH 5.0) were added to each tube followed by addition of 60 μl of a 0.3 m*M* solution of tetrabutylammonium hydrogenphosphate. After vortex-mixing for 15 s, aliquots of 290 μl were transferred to autosampler vials and samples of 250 μl were injected to a column-switching HPLC system. For samples with amoxycillin above the maximum limit of quantification reanalysis were performed using an estimated smaller volume of plasma.

3. Results and discussion

HPLC analysis of micromolecules contained within a matrix with high protein content usually requires a multistep clean-up procedure. To shorten and simplify sample handling various restricted access media columns have been developed and used for direct injection of biological fluids. The immobilized BSA octyl column, as described by Menezes and Felix [15], attracted our attention by its simplicity, as these columns can be prepared. This type of packing has shown excellent efficiency for protein exclusion of human plasma [13,14] and it was then considered for sample clean-up for the analysis of amoxycillin in plasma samples. The low ultraviolet absorbance of this drug associated with its amphotheric nature calls for special sample handling for getting adequate sensitivity and selectivity in the assay method.

Although proteins of human plasma could be

excluded, in 5 min, in high percentage, using a RAM BSA octyl column, using only water as mobile phase at a flow-rate of 1 ml/min [14], amoxycillin showed no retention precision. Thus, at first, the influence of the pH of the mobile phase in the retention time of amoxycillin was investigated. For that, a RAM BSA octyl and octadecyl columns were used. Phosphate buffer (0.01 *M*) was used as mobile phase. The RAM BSA octadecyl column showed higher retention for amoxycillin than the octyl one and was selected for the method development. Fig. 2 shows the retention profile of amoxycillin at different buffer pH values in this column. These results are in accordance with the behavior of amphotheric compounds, which show shorter retention times when the pH of the mobile phase is around the isoelectric point of the compound [16]. Amoxycillin contains three ionizable groups with the following pK_a values: 2.68; 7.49 and 9.63. It is in the ionized form for the entire pH range, with an isoelectric point at pH 4.8 [17,18].

The selection of the appropriated pH of the buffer for using a RAM column must take in consideration the elution, in the minimum time possible, of proteins and others endogenous compounds. Thus taking into account the retention time observed for amoxycillin in varying the pH of the phosphate buffer, the elution of the plasma proteins was at first investigated at pH 3.0 and 3.5. At this pH range the elution of the plasma proteins was very long. Yu and Westerlund [19] point out that the isoelectric point for albumin and most of α- and β-globulins is around 4.7, which means that they can precipitate at around this pH. Lower pH was not considered due to the short retention time of amoxycillin in the RAM column. Varying the buffer pH from 6.0 to 7.2 gave good elution profile for the plasma proteins, but at the pH range of 6.0–6.8 the amoxycillin was co-eluting with an endogenous compound. At pH 7.0 no co-elution with endogenous compound was observed but the retention time of the amoxycillin was too high (40 min).

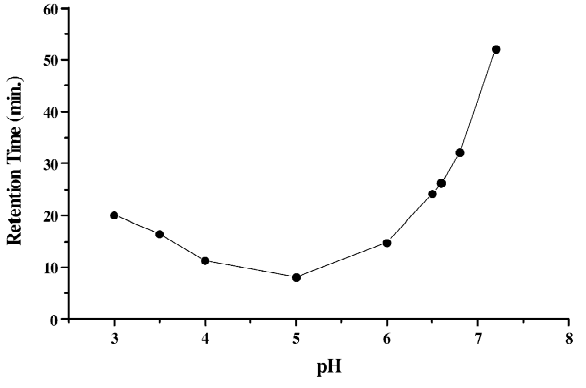

Fig. 2. Influence of the use of 0.01 *M* KH_2PO_4 buffer at various pH values with a flow-rate of 1 ml/min, as mobile phase, in the retention profile of amoxycillin at the RAM BSA octadecyl column.

To circumvent these problems, the use of a small percentage of organic solvent in the mobile phase, for the exclusion of the plasma proteins and endogenous compounds, was investigated. Here, the RAM BSA column was used as an extraction and as an analytical column. Good exclusion profiles of the plasma proteins were obtained with or without an organic modifier; however, the low selectivity of the RAM column did not allow it to be used in the simple mode of analysis for this amphoteric antibiotic. All macromolecules of the sample matrix were excluded before 5 min using 0.01 *M* phosphate buffer, pH of 7.2, at a flow-rate of 1 ml/min. In the plasma pool analyzed, two endogenous compounds were eluted at around 10 and 14 min. This result shows the excellent performance of the RAM BSA octadecyl column for exclusion of plasma proteins. Yu and Westerlund [19] report that a washing volume of at least 20 ml for a SPS (semipermeable surface) C_{18} and of 15 ml for ADS (alkyl diol silica) columns were necessary to flush out most of the proteins and endogenous compounds using a phosphate buffer of 0.05 *M* at pH 7.7.

Due to the low selectivity of the RAM BSA octadecyl column, the coupling to an octadecyl Hypersil column was performed. The employed column-switching system used is illustrated in Fig. 3. To determine the elution profile of the sample matrix, the RAM column was initially connected to an UV–Vis detector. Proteins were eluted with a 0.01 *M* KH_2PO_4 (pH 7.2)–MeCN (98:2, v/v) as mobile phase in the first 9.7 min whereas the analytes were retained on the hydrophobic phase. The mobile phase was delivered by pump 1 (position 1; Fig. 4). At the same time, the analytical column was conditioned with the mobile phase delivered by pump 2. The

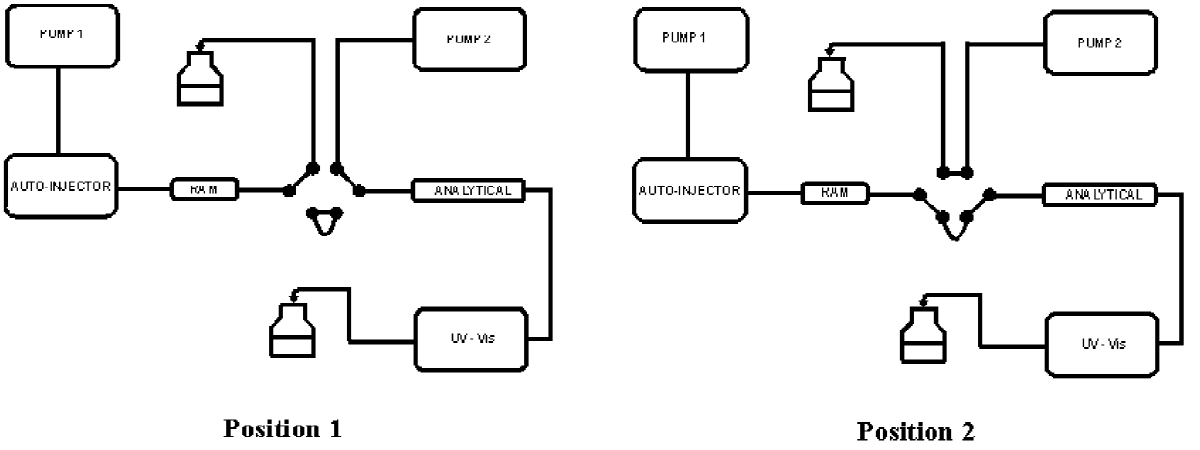

Fig. 3. Schematic diagram of the column-switching system.

switching valve redirected the flow from the waste to the analytical octadecyl column, between 9.7 and 14.5 min, for transferring the analyte fraction from the RAM to the analytical column (position 2; Fig. 3). After this time, the switching valve was back to position 1 for cleaning and conditioning the RAM column while the amoxycillin was analyzed on the octadecyl column. The injection volume was 250 μl to satisfy the desired sensitivity. Table 1 shows the time events used for the analytical procedure.

No significant differences were observed in the elution profile of the proteins or in the retention time and transferring time of amoxycillin when two different BSA RAM octadecyl columns were used. The increase in backpressure of the RAM column during the method development could be reduced by cleaning or replacing the sealings of the RAM column, which can get clogged by proteinaceous compounds [20]. The inversion of the column was sometimes made, to decrease the backpressure, before cleaning the sealings. The chromatographic performance of the RAM and the analytical columns was maintained with over 238 injections of 250 μl each (150 μl of plasma) but the upper limit has not yet been determined [13,14].

The chromatograms in Fig. 4 show the analysis of

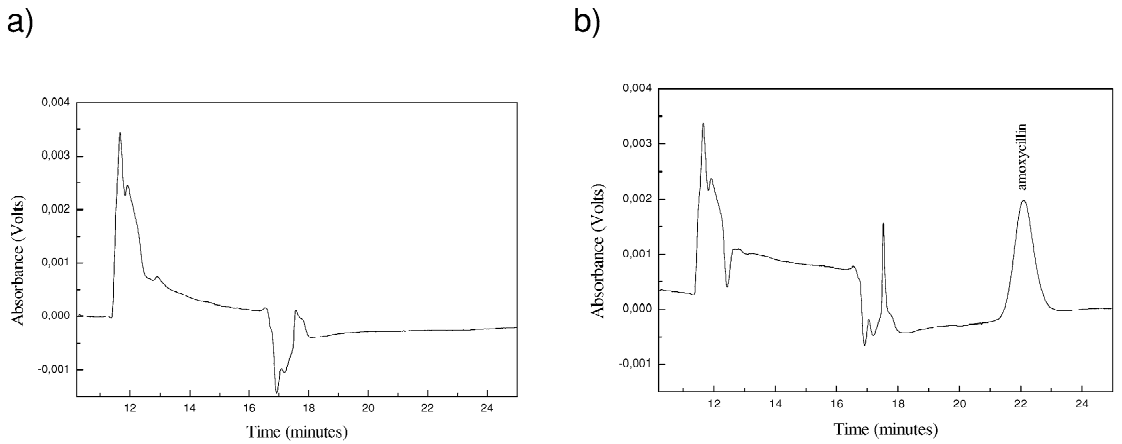

Fig. 4. Typical chromatograms of (a) plasma free drug, (b) spiked plasma with amoxycillin (0.50 μg/ml) obtained under the established analysis conditions.

Table 1
Time events for the switching of columns and of mobile phases

Time (min)	Pump	Event	Valve position
0.00–9.70	Pump 1 (eluent A)	Plasma proteins are excluded by RAM column	1
	Pump 2 (eluent C)	Conditioning of the analytical column	
9.70–14.50	Pump 1 (eluent A)	Analytes are transferred to the analytical column	2
14.50–25.00	Pump 2 (eluent C)	Analysis of the amoxycillin	1
19.00–21.50	Pump 1 (eluent B)	Washing of RAM column	1
21.50–25.00	Pump 1 (eluent A)	Conditioning of RAM column	1

Eluents: pump 1: (A) 0.01 *M* phosphate buffer (pH 7.2)–MeCN (98:2, v/v), (B) CH_3CN–H_2O (25:75, v/v); pump 2: (C) 0.01 *M* phosphate buffer (pH 6.5)–MeCN–MeOH (96:3:1, v/v), flow-rate: 1.0 ml/min, λ: 228 nm.

a plasma-free drug and spiked plasma with amoxycillin at the established conditions. The chromatograms show that no endogenous compounds are interfering with the detection of the antibiotic.

The response of the UV detector at 228 nm was linear from 0.05 to 2.00 μg/ml with following regression equation and correlation coefficient $y = 5.56953 \cdot 10^{-6}x - 0.0121235$ ($r = 0.999$).

Ion pairing was necessary to extract the amoxycillin with good recovery and precision from the plasma proteins. The formation of a amoxycillin–tetrabutylammomium ion-pair by addition of the cationic counter-ion, in the sample preparation stage, gave extracts with excellent recoveries at the three quality control levels analyzed (Table 2).

Table 2
Extraction recovery of amoxycillin from human plasma

Concentration (μg/ml)	Recovery ($n=5$) (%)
0.12	90.4
1.20	92.8
1.80	90.8

The intra- and inter-day precision and accuracy of the method were determined by analyzing five replicates of three quality controls on 3 nonconsecutive days. Precision is expressed as relative standard deviation (RSD) and the accuracy was evaluated by backcalculation and expressed as the percent deviation between amount found and amount added at the three concentrations examined. These results are shown in Table 3.

Two blinded samples containing unknown concentrations to the analyst produced accuracies of 93% at the concentration levels of 0.60 and 1.60 μg/ml with RSD values of 0.61 and 1.65% for the duplicates analysis.

The limit of quantification was of 0.050 μg/ml (RSD = 6.0%, $n=3$ and accuracy of 85.3%) while the limit of detection was 0.020 μg/ml.

To evaluate if the selectivity of the method was maintained in the presence of amoxycillin metabo-

Table 3
Accuracy and intra- ($n=5$) and inter-day ($n=3$) precision for the assay of amoxycillin in human plasma

Concentration (μg/ml)	1st day		2nd day		3rd day		Pooled ($n=15$)
	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
0.120	4.63	89.8	4.63	96.4	2.01	89.0	3.76
1.20	1.61	101	3.16	96.6	1.76	94.5	2.18
1.80	1.09	99.0	1.51	94.6	1.81	93.5	1.47

lites, plasma samples collected from two healthy volunteer 2.5 h after an oral dose of amoxycillin (500 mg), were analyzed using a photodiode array detection system and the peak purity of amoxycillin was examined. No interfering metabolites were detected on the samples examined. The ultraviolet spectrum was scanned at the elution of the amoxycillin band, in the following samples: amoxycillin solution, pool of plasma spiked with amoxycillin and plasma from one volunteer, demonstrating the selectivity of the method.

The utility of the assay was demonstrated by analyzing samples collected from five healthy volunteers to whom test and reference formulations of amoxycillin were administered as an oral dose (500 mg). The assay proved adequate for establishing pharmacokinetic parameters in all samples evaluated. The limit of quantification allowed the determination of plasma levels of all 8-h samples evaluated.

4. Conclusions

A column-switching HPLC method with UV detection, which uses only 180 μl of plasma samples, was efficiently developed, validated and used for assaying amoxycillin in human plasma. The developed method is simple and requires a total analysis time of only 25 min per sample, with no time involved for sample pretreatment. It has proved to be a selective and sensitive approach for the direct sample analysis of this polar, low wavelength ultraviolet absorption antibiotic. The RAM BSA column used is easily prepared and robust.

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