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Determination of amoxicillin in human serum and plasma by high-performance liquid chromatography and on-line postcolumn derivatisation

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Abstract

A highly selective, sensitive and fast HPLC method was developed for the determination of amoxicillin in human serum and plasma. After protein precipitation with perchloric acid, an aliquot of the supernatant was neutralized by mixing it with sodium acetate solution and injected into a C_{18} HPLC column. Detection was done by a fluorescence detector after on-line postcolumn derivatisation with fluorescamine. The practical limit of quantification was 0.1 μg/ml using 0.3 ml of plasma. Linearity was given in the tested range of 0.1 to 15 μg/ml plasma. Inter-day precision (relative standard deviation) over 7 days for 0.42 μg/ml was ±7.27%; for 4.54 μg/ml, ±5.24% and for 13.18 μg/ml, ±5.25%. Stability over 50 days in serum and plasma occurs at −70°C but not at −20°C (−25 to −35% reduction). This method was used for thousands of human serum and plasma samples.

Keywords: Amoxicillin; Antibiotics; Lactams; Penicillins

1. Introduction

Amoxicillin is an orally absorbed, acid stable, broad-spectrum antibiotic. Determination of this drug in pharmaceutical preparations is mainly by HPLC with UV detection around 230 nm [1,2]. The determination in body fluids, however, especially in human serum and plasma down to low levels of 0.1 μg/ml, needs special sample clean up and often special detection for enhancing sensitivity and selectivity. UV detection with column switching [3] or precolumn enrichment [4] or only protein precipitation [5–7] was used. In order to enhance selectivity and sensitivity, some methods were developed with precolumn reactions [8–10] and fluorescence detection. Also postcolumn reactions and UV detection [11] or fluorescence detection [12–15] were developed.

The present paper deals with HPLC and a postcolumn derivatisation method with fluorescamine and fluorescence detection.

The authors developed another method with postcolumn reaction [13], but changed to the following described method because of more precise results.

2. Experimental

2.1. Materials

All chemicals and reagents used were of HPLC grade or analytical grade. Amoxicillin was kindly supplied by Biochemie Kundl (Austria). Fluram was purchased from Sigma (St. Louis, MO, USA).

Other chemicals of reagent grade and solvents of

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analytical and HPLC grade were purchased from E. Merck (Darmstadt, Germany) and J.T. Baker (Phillipsburg, USA).

2.2. Chromatography

The HPLC system consisted of a HPLC pump M480, a GINA 160 automatic sample injector (Gynkotek, Germany) and a fluorescence detector FP 920 (Jasco, Japan); the detection wavelength being 395 nm for excitation and 485 nm for emission. The column oven was from W.O. electronics (Austria).

For postcolumn reaction, two pumps M300 (Gynkotek) were used.

System control and integration was performed using the GynkoSoft data system (Rel. 5.21) from Softron (Germany).

The analytical column, (80×4 mm I.D.), was packed with Nucleosil 120 3 C_{18}, 3-μm particle size (SRD-Pannosch, Vienna, Austria). For analysis of samples the composition of the mobile phase was 0.02 *M* methanesulfonic acid–acetonitrile (92:8, v/v). The flow-rate of the mobile phase was 1.1 ml/min. The column was thermostated at 30°C.

The postcolumn reaction was made by using two different pumps: first for delivering the 0.2 *M* buffer (pH 8; 27.2 g of KH_2PO_4 and 7.45 g of NaOH per 500 ml of water) and second for the reagent (0.01% Fluram in acetonitrile).

After adding the buffer and the reagent through a mixing tee, a knitted PTFE tube (10 m, 0.25-mm I.D.) was used for reaction before detection.

2.3. Calibration

A stock solution of amoxicillin trihydrate in 50% aqueous methanol was used for spiking pooled plasma or serum at different concentrations.

The concentration range was 0.1 to 14 μg amoxicillin per ml of plasma or serum. The calibration was expanded to 30 μg/ml, for use in some of the pharmacokinetic studies.

2.4. Sample preparation

Plasma or serum samples (spiked or from volunteers) were thawed at 20°C for about 10 min. A 0.3-ml volume of the sample was transferred into a vial and vortexed with 50 μl of 20% perchloric acid for 15 s (pH: 1.0). After centrifugation, (2 min, >2000*g*), 150 μl of the clear supernatant were transferred into an autosampler glass vial and mixed with 150 μl of 1 *M* sodium acetate solution (pH: 5.0) within 30 min. This solution was injected within 18 h. It is important that the time between adding perchloric acid and mixing the supernatant with 1 *M* sodium acetate solution is kept to a minimum (30–45 min, see Table 1).

3. Results and discussion

3.1. Linearity precision and accuracy

Calibration standards and spiked quality control samples of amoxicillin were prepared by spiking blank human plasma or serum with known amounts of amoxicillin. The linearity of the amoxicillin calibration curve was proven for the range from 0.113 μg/ml to 14.908 μg/ml. The coefficient of correlation for all measured sequences was at least 0.9988. Near the lowest calibration standard of 0.113 μg/ml, the quantification limit for amoxicillin was determined.

The intra-day precision of the amoxicillin calibration standards ranged from ±0.86 to ±6.0%. For the spiked quality control samples of amoxicillin, the intra-day precision ranged from ±1.88 to ±4.76%.

Table 1
Stability of amoxicillin in plasma/perchloric acid (pH: 1.0) at room temperature

Concentration spiked (μg/ml)	Deviation from initial value (relative %)			
	10 min	20 min	30 min	45 min
1.57	+1.87	+9.05	−1.46	+1.20
18.85	+7.41	+10.53	+4.82	+1.02

Table 2
Intra-day precision and accuracy of quality control samples in plasma ($n=4$)

Concentration spiked (μg/ml)	±R.S.D. (%)	Accuracy (%)
0.41	±4.76	+7.57
4.54	±2.08	+4.66
13.18	±1.88	+1.65

The intra-day accuracy obtained for amoxicillin concentrations observed in the back-calculated quality control samples ranged between +1.65% to 7.57% (see Table 2).

3.2. Stability of amoxicillin after sample preparation (in the autosampler) at room temperature

Three sets of quality control samples (Q-A, Q-B and Q-C) were prepared as described in Section 2.4. All samples were stored at the autosampler temperature (approximately+25°C) and were analysed 4, 8 and 18 h after the preparation. No or almost no instability of amoxicillin was observed after sample preparation at room temperature over a time period of 18 h at autosampler temperature (approximately +25°C; see Table 3).

3.3. Stability of amoxicillin in plasma or serum

Spiked samples at two concentrations (approx. 1.8 and 18 μg amoxicillin per ml) were prepared. All stability samples were stored at approximately −20°C and −70°C. Of each group, three of those stability samples were measured 50 days after the start of the stability test.

Amoxicillin in human plasma and serum is stable over a time period of at least 50 days at approx. −70°C (see Table 4). For amoxicillin in human plasma and serum, stability at approx. −20°C could not be demonstrated. Periods shorter than 50 days were not tested.

Amoxicillin was also found to be stable for at least three freeze–thaw cycles.

3.4. Inter-day precision

On seven validation days, the inter-day precision and accuracy of the assay were measured by analysing one set of human serum calibration samples and

Table 3
Stability of amoxicillin after sample preparation at room temperature in the autosampler. Deviation in % from the observed concentration value at 0 h, each $n=2$

	Level spiked (μg/ml)	4 h	8 h	18 h
Q-A	0.41	+3.60	Sample vials lost in freezer	−1.92
Q-B	4.54	+1.85	−5.89	−5.11
Q-C	13.18	+0.64	−7.60	−7.60

Table 4
Stability of amoxicillin in plasma and serum at −20° and −70°C over 50 days

Concentration spiked (μg/ml)	Deviation from the spiked concentration			
	Temp. −20°C		Temp. −70°C	
	Deviation (%)	±R.S.D. (%)	Deviation (%)	±R.S.D. (%)
Serum				
1.80	−24.03	±1.20	+2.03	±2.03
17.70	−29.99	±1.07	+1.25	±1.47
Plasma				
1.80	−31.53	±4.93	−6.27	±4.46
17.70	−34.23	±0.72	+0.91	±0.89

Table 5
Inter-day precision and accuracy of quality control samples in plasma over 7 days

Concentration spiked (μg/ml)	S.D.	S.E.[a]	±R.S.D. (%)	Accuracy (%)
0.413	0.03	0.01	±7.27	+1.37
4.543	0.23	0.05	±5.24	−1.69
13.181	0.66	0.13	±5.25	−4.13

[a] S.E.=Standard error.

at least three sets of spiked quality control plasma samples of amoxicillin.

The calibration curves were evaluated individually for each day by linear regression. The concentrations of the quality control samples were calculated by the calibration curve.

The inter-day precision of the quality control samples ranged between ±5.24 and ±7.27% and the inter-day accuracy ranged between 1.37 and −4.13% (Table 5).

3.5. Recovery

Recovery was measured by comparison of back-calculated concentrations in aqueous solutions mixed with perchloric acid versus precipitated plasma samples. The recovery of amoxicillin was determined as 100.7% (±5.1%) at 0.30 μg/ml, 100.2% (±3.7%) at 4.85 μg/ml and 106.8% (±4.0%) at 19.39 μg/ml (each n=3).

Fig. 1A shows that there were no interferences at

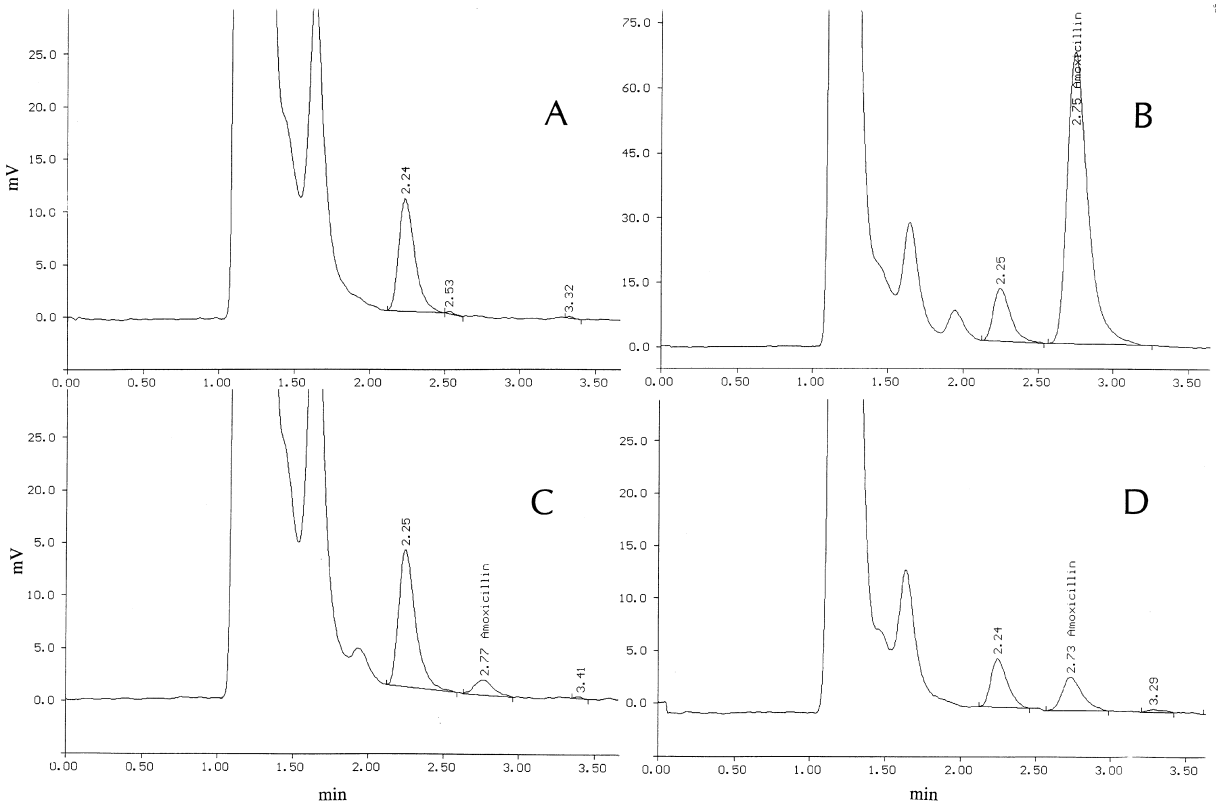

Fig. 1. Chromatograms of (A) blank human plasma, (B) plasma sample of a volunteer 1 h (8.77 μg/ml) and (C) 8 h after the oral intake of 500 mg amoxicillin (0.21 μg/ml) and (D) a calibration sample spiked in plasma at a concentration of 0.41 μg/ml.

the retention time of amoxicillin in human plasma. Representative chromatograms of a calibration sample, a predose sample and two different samples from a volunteer after the oral intake of 500 mg amoxicillin are shown in Fig. 1B–D.

4. Conclusions

As we have analysed amoxicillin over some years, we have developed different methods for determining its concentration in plasma or serum and have also published one of these methods some years ago [13].

If UV detection is used at 230 nm or less, then it is necessary with lower concentrations of amoxicillin, not only to eliminate the plasma proteins, but also to use a cleaning step to eliminate some disturbing low-molecular-mass endogenous substances. In our opinion, precolumn clean up using different C_{18} or C_8 materials, results in a recovery rate of only 70%. A recently published method [3] used heptane sulfonate as an ion pairing reagent to solve this problem, but their total time for one HPLC run, including on-line precolumn clean up, was about 50 min.

We decided to enhance the sensitivity and selectivity and thus a postcolumn reaction on-line with a coulometric detector was used [13]. The method, however, had one drawback and that was, that it took us some time to establish stable conditions when starting to analyse a new block of hundreds of samples. Finding the right potential of the coulometric detector was not easy. After analysing thousands of plasma or serum samples, we decided to adapt a method for determining penicillin V and ampicillin which we developed 4 years after the amoxicillin method. The amoxicillin molecule, however, was too hydrophilic to be completely retained. Although the protein binding is described at about 15–20%, the recovery from plasma was almost exactly 70%. More elution was not helpful, thus about 30% of the amoxicillin at various concentrations was not retained.

Therefore, we developed the method described using postcolumn derivatisation with Fluram. Lee et al. [15] developed, almost 20 years ago, a method for determining amoxicillin in urine with a determination limit of 2.5 μg/ml using the same derivatisation principle.

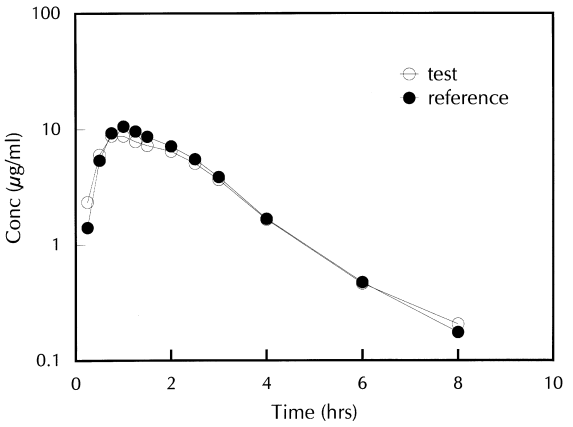

Fig. 2. Semilogarithmic plot of the plasma concentrations from a volunteer after administration of a single oral dose of a test and reference formulation of 500 mg of amoxicillin.

Until now, we have used the described method for analysing thousands of plasma or serum samples in pharmacokinetic and bioavailability studies.

Fig. 2 shows a semilogarithmic plot of the plasma concentrations in a volunteer after administration of a single oral dose of a test and reference formulation of 500 mg of amoxicillin.

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