

# Improved high-performance liquid chromatographic determination of amoxicillin in human plasma by means of column switching

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## Abstract

A highly sensitive and selective HPLC method was developed for the determination of amoxicillin in human plasma. After addition of buffer and internal standard, the sample was ultrafiltered and injected on to a precolumn to remove polar plasma interferences. Detection was effected with a UV detector set at 230 nm. The limit of quantification for amoxicillin was 50.1 ng/ml with an imprecision of 4.2% using 0.25 ml of plasma. Linearity was confirmed over the whole calibration range (25.4–0.0501  $\mu\text{g/ml}$ ) and the inter-day variation ranged from 2.0 to 4.5%. The method was validated according to GLP guidelines and its suitability was demonstrated by the analysis of several hundred samples in a bioequivalence study. The method can be used to determine pharmacokinetic parameters of amoxicillin in humans after a single oral dose of 500 mg.

**Keywords:** Column switching; Amoxicillin; Antibiotics

## 1. Introduction

Amoxicillin ( $\alpha$ -amino-4-hydroxybenzylpenicillin) is an orally absorbed, acid-stable, broad-spectrum antibiotic. Several HPLC methods for the determination of amoxicillin in body fluids have been published, many of which use direct UV absorbance at 230 nm for detection [1–5]. The limit of quantification for these assays is around 0.5  $\mu\text{g/ml}$  in plasma [1–4] and thus they do not meet the sensitivity requirements for a single-dose pharmacokinetic study. Owing to the UV maximum of amoxicillin at this low wave-

length, one would need laborious sample pre-treatment steps to obtain plasma samples free of interferences. The method of Chulavatnatol and Charles [5] was described only for the measurement of amoxicillin in urine. Other approaches have been made featuring precolumn derivatization [6] or postcolumn reactions [7–11] in order to enhance the sensitivity. The assays published by Miyazaki et al. [6], Carlqvist and Westerlund [9] and Mascher and Kikuta [11] have satisfactory limits of quantification but the first needs complicated and time-consuming sample pre-treatment and additionally was not used for a pharmacokinetic study in humans where a large number of samples were analysed, and the other

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two do not utilize an internal standard, which is required for an HPLC method meeting GLP guidelines [12,13]. We decided to use direct UV detection in order to avoid potential imprecisions caused by pre- or postcolumn reactions. While the high selectivity required for injecting large sample amounts was achieved by column switching, the sample preparation could be simplified to a single ultrafiltration step. Careful testing of the system before each sequence of samples demonstrated its reliability for routine measurement.

## 2. Experimental

### 2.1. Reagents and chemicals

Amoxicillin and cefadroxil were obtained from Sigma Chemie (Deisenhofen, Germany). Methanol (Promochem, Wesel, Germany) was of ChromAR grade and potassium dihydrogenphosphate, sodium dihydrogenphosphate monohydrate, 0.1 M sodium hydroxide solution and orthophosphoric acid (Merck, Darmstadt, Germany) were all of analytical-reagent grade. Sodium heptanesulphonate monohydrate (Fluka, Buchs, Switzerland) was used as an ion-pairing additive in the mobile phases. Water was purified with a Milli-Q system (Millipore, Eschborn, Germany).

### 2.2. Instrumentation and chromatographic conditions

The HPLC system consisted of two pumps, a Hitachi 655A-12 (Merck) and a Model 510 (Waters, Eschborn, Germany) for delivering the mobile phases for the precolumn (mobile phase B) and the analytical column (mobile phase A), respectively. A Waters Model 717 autosampler (Millipore–Waters, Eschborn, Germany) equipped with a cooling device to keep the samples at 4°C was used for sample injection. The detector was an LCD 502 variable-wavelength detector (Gamma Analysen Technik, Bremerhaven, Germany) set at 230 nm. The switching valve was a Rheodyne Model 7010 controlled

by a Labtimer (Bischoff Chromatography, Leonberg, Germany). Data acquisition was performed with Maxima 820 software (Millipore–Waters). The analytical column was 250 × 4.6 mm I.D. stainless steel packed with Spherisorb ODS II (5 μm). The precolumn (40 × 4.6 mm I.D.) was filled with Nucleosil 100 C<sub>18</sub> (10 μm) (M. Grom, Herrenberg, Germany).

Mobile phase B (for the loading step) consisted of 10 mM heptanesulphonate and 30 mM sodium dihydrogenphosphate in a solution of purified water–methanol (92:8, v/v). The mobile phase was adjusted to pH 2.5 with phosphoric acid. Mobile phase A (for elution of the sample to the analytical column and subsequent analysis) consisted of 10 mM heptanesulphonate and 30 mM sodium dihydrogenphosphate in a solution of purified water–methanol (75:25, v/v). Mobile phase A was adjusted to pH 2.5 with phosphoric acid. Both mobile phases were filtered through 0.2-μm membrane filters and degassed under vacuum prior to use. The flow-rate of mobile phase A was 1.5 ml/min, generating a back-pressure of 120–140 bar at the analytical column, and the same flow-rate was chosen for mobile phase B to adjust the start of elution from the precolumn for both compounds to about 20–22 min. This was tested prior to every sequence of the validation experiment or sequence of unknown samples.

The connections to the switching valve were made in such a way that the elution direction through the precolumn did not change on switching the valve (front cut). After injection, the sample was loaded on to the precolumn with mobile phase B for 15 min. Then the valve was switched and the sample was eluted from the precolumn to the analytical column by mobile phase A. After an elution time of 5 min the valve was switched again. While the analysis was proceeding the precolumn was equilibrated with mobile phase B and thus was ready for the next injection at the end of the analytical run.

### 2.3. Stock solutions, standards and controls

A stock solution of 500 μg/ml cefadroxil was prepared by dissolving 25.0 mg in 50.0 ml of

purified water. Aliquots of this solution were stored in dry-ice. For sample preparation, an aliquot of 500  $\mu\text{l}$  was thawed and made up to 50 ml with 0.1 M  $\text{KH}_2\text{PO}_4$  (pH 2.5), leading to a final concentration of 5  $\mu\text{g}/\text{ml}$ . A stock solution of 501  $\mu\text{g}/\text{ml}$  amoxicillin was prepared by dissolving an accurately weighed amount in 25 ml of purified water. The stock solution was validated by comparison with two identically prepared solutions. Aliquots of the stock solution were frozen immediately and stored in dry-ice. Calibration levels of amoxicillin in plasma were prepared by diluting the stock solution with drug-free human plasma (1:19, v/v) to obtain the highest calibration level and successive dilution (1:1, v/v) with blank plasma. A calibration row of eleven levels, including blank, was thus obtained, extending from 25.4 to 0.0501  $\mu\text{g}/\text{ml}$ . Each level was divided into aliquots of 1.1 ml and immediately frozen in dry-ice.

Validation samples were prepared similarly by adding defined amounts of the stock solution or the spiked control of higher concentration to defined amounts of blank plasma, leading to concentrations of 19.9, 2.19 and 0.218  $\mu\text{g}/\text{ml}$ . Validation samples were divided into aliquots of 1.1 ml and immediately frozen in dry-ice.

#### 2.4. Sample preparation

Plasma samples were thawed in cold water and thoroughly vortex mixed for 15 s. After 10 min of centrifugation at 4000 rpm at 4°C, 250  $\mu\text{l}$  of the samples were pipetted into microvials and 250  $\mu\text{l}$  of the buffered (pH 2.5) internal standard solution were added (no internal standard was added to blank samples). The samples were then transferred to Centricon 10 microconcentrator tubes (Amicon, Witten, Germany) and centrifuged for 30 min in a fixed-angle rotor at 5000 rpm (4800 g). A 150- $\mu\text{l}$  volume of the clear supernatant was injected into the HPLC system.

#### 2.5. Validation procedures

Before a sequence of samples was measured, an aqueous test solution containing 5  $\mu\text{g}/\text{ml}$  amoxicillin and cefadroxil was injected. The peak

tailing factors ( $T$ ) and resolution ( $R$ ) were determined according to common equations [14]. Additionally, peak heights and the peak area/peak height ratios of the peaks as a measure of system performance were recorded. Accuracy, precision and linearity were determined intra-day and inter-day on three different days. Recovery of the analytes after sample preparation was determined at different concentration levels. The stability of amoxicillin in plasma was tested under different light and temperature conditions prior and subsequent to sample preparation procedures.

### 3. Results and discussion

#### 3.1. System suitability, specificity, linearity and sensitivity

Cefadroxil eluted between 31.8 and 32.8 min, whereas amoxicillin was eluted between 34.8 and 38.8 min, both as virtually symmetrical peaks (tailing factors:  $T_A = 1.59 \pm 0.11$  and  $T_C = 1.66 \pm 0.12$ ,  $n = 24$ ). The resolution was between 1.94 and 3.02 (mean  $2.43 \pm 0.31$ ,  $n = 24$ ), indicating excellent separation. The peak area/peak height ratio of amoxicillin was below 60 and that of cefadroxil below 55 in all cases (mean  $49.1 \pm 8.2$  and  $42.4 \pm 6.8$ , respectively,  $n = 24$ ). The specificity of the method was investigated by screening five different batches of blank human plasma. The plasma samples were cleaned up as described above without addition of internal standard. Specificity for lack of interference with other possible concomitant drugs and their metabolites was not investigated because only plasma samples from healthy volunteers participating in a bioequivalence study were to be measured. The time interval where both compounds eluted was free of interferences in all of the plasmas tested and in all subjects' blanks. Fig. 1 shows chromatograms of (a) drug-free plasma, (b) a plasma standard containing 0.100  $\mu\text{g}/\text{ml}$  amoxicillin and (c) a plasma sample taken from a volunteer 5 h after oral intake of 500 mg amoxicillin (1.06  $\mu\text{g}/\text{ml}$ ).

For validation of the calibration graphs,

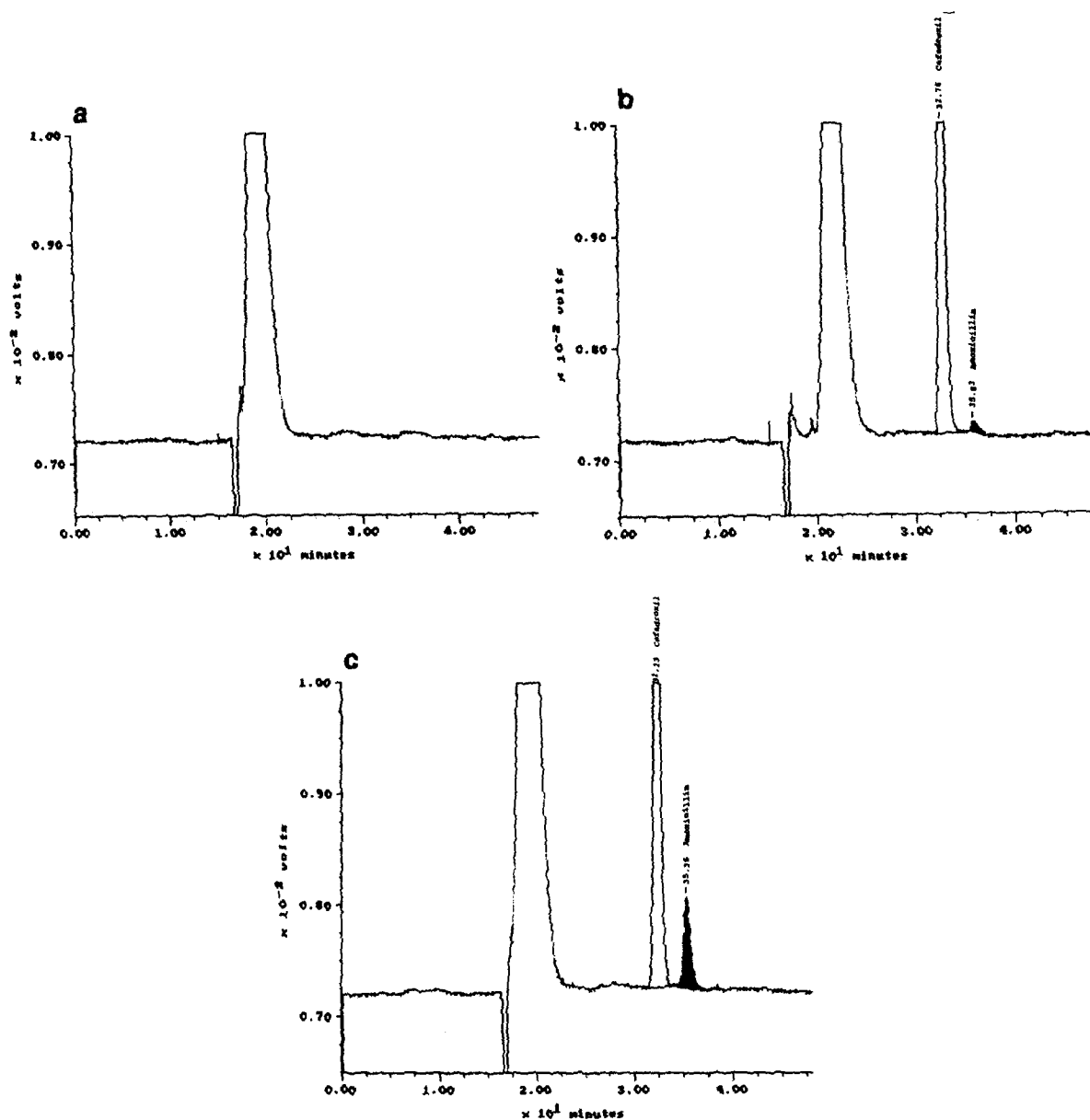


Fig. 1. Chromatograms of (a) blank plasma from a volunteer prior to drug administration, (b) plasma standard containing 0.100  $\mu\text{g/ml}$  amoxicillin and (c) plasma sample from a volunteer 5 h after oral intake of 500 mg of amoxicillin (1.06  $\mu\text{g/ml}$ ).

linearity was tested visually by plotting data points in two ranges (whole calibration range 25.4–0.0501  $\mu\text{g/ml}$  and lower part of the calibration graph 0.787–0.0501  $\mu\text{g/ml}$ ). For each curve, the slope, intercept and correlation coefficient ( $r^2$ ) were calculated by weighted linear regression (weighting factor = 1/concentration)

and the concentrations of the calibration standards were calculated according to the regression equation. The assay showed linearity over the concentration range 25.4–0.0501  $\mu\text{g/ml}$ . The responses versus nominal concentrations fitted well to a straight line, with  $r^2 > 0.999$  for all calibration graphs ( $n = 6$ , Table 1), and the

Table 1  
Calibration parameters of all sequences

Parameter	Day 1		Day 2		Day 3	
	Cal. graph 1	Cal. graph 2	Cal. graph 3	Cal. graph 4	Cal. graph 5	Cal. graph 6
Number of levels used	9	9	10	8	10	10
Slope	0.118	0.115	0.113	0.111	0.113	0.112
Intercept	-0.000610	-0.000151	0.00169	-0.00222	0.00232	-0.000729
Correlation coefficient ( $r^2$ )	0.999 932	0.999 924	0.999 549	0.999 300	0.999 919	0.999 896
Response of lowest level	0.00528	0.00614	0.00775	0.00987	0.00802	0.00638
Intercept expressed as % of the response of the lowest level	11.6	2.5	21.8	22.5	28.9	11.4

individual relative errors of the calibration standards were between -5.17 and 9.37%. Since the R.S.D. of the lowest calibration level was 4.24% and the individual values of the relative error were below 15%, the quantification limit of the assay was set at 0.0501  $\mu\text{g/ml}$ .

### 3.2. Precision and accuracy

For determination of the inter-day precision and accuracy, two replicates of each validation

sample (VS) were measured in one sequence on each of three days. The calibration rows were measured at the beginning and at the end of the sequence with the validation samples in between. The validation samples were analysed individually by using the front and back curves. The inter-day precision determined as the R.S.D. of the mean assayed validation samples ranged between 1.79 and 4.50%. The accuracy calculated as the individual relative error of the validation samples was between -2.7 and 7.6% (Table 2).

Table 2  
Inter-day precision and relative errors (RE) of validation samples

Analysed on day	VS 1		VS 2		VS 3	
	Conc. ( $\mu\text{g/ml}$ )	RE (%)	Conc. ( $\mu\text{g/ml}$ )	RE (%)	Conc. ( $\mu\text{g/ml}$ )	RE (%)
-	19.9 <sup>a</sup>		2.19 <sup>a</sup>		0.218 <sup>a</sup>	
1	20.2	1.5	2.25	2.7	0.212	-2.7
1	20.0	0.5	2.24	2.0	BQL <sup>b</sup>	-
2	21.1	5.9	2.19	-0.2	0.235	7.6
2	20.6	3.2	2.16	-1.4	0.299 <sup>c</sup>	37.4
3	20.2	1.3	2.25	2.8	0.218	-0.1
3	20.3	1.9	2.28	3.8	0.221	1.6
<i>n</i>	6		6		4	
Mean	20.4		2.23		0.222	
S.D.	0.4		0.04		0.010	
R.S.D. (%)	2.0		1.8		4.5	

<sup>a</sup> Nominal amoxicillin concentration ( $\mu\text{g/ml}$ ).

<sup>b</sup> BQL = below quantification limit.

<sup>c</sup> Outlier for unknown reason (not included in statistical evaluation).

On day 2 of the validation experiment, three additional sets of validation samples were measured between the calibration rows for the determination of the intra-day precision and accuracy. The concentrations of all five replicates of each validation sample were calculated using the equation for the front curve. The intra-day precision of the assay determined as the R.S.D. of the mean assayed validation samples ranged from 1.0 to 3.6%. The individual values of the relative errors were between -1.4 and 7.9% (Table 3).

### 3.3. Recovery experiment

The absolute recovery of both compounds (amoxicillin and cefadroxil) was determined at three different concentration levels representing the low, medium and high concentration ranges. The recovery was measured by comparison of peak heights of non-extracted standards in phosphate buffer versus extracted samples of spiked plasma.

The mean absolute recovery of amoxicillin in plasma was found to be 108.2% at 0.500  $\mu\text{g/ml}$ , 104.3% at 5.00  $\mu\text{g/ml}$  and 104.3% at 20.0  $\mu\text{g/ml}$ . The mean recovery of the internal standard

cefadroxil was 98.5% at 0.500  $\mu\text{g/ml}$ , 96.6% at 5.00  $\mu\text{g/ml}$  and 96.4% at 20.0  $\mu\text{g/ml}$ .

### 3.4. Stability experiments

Light stability of amoxicillin in plasma (22.0  $\mu\text{g/ml}$ ) was determined at 4°C under the following conditions: (A) not light protected, stored in direct sunlight near a laboratory window; (B) not light protected, stored under ambient room (fluorescent) light in the laboratory; and (C) light protected.

The results showed a slight decline of the concentration beginning at 6 h storage, being slightly greater for daylight exposure (6.3% loss after 8 h) than for exposure to fluorescent light (4.5% loss after 8 h). However, the samples were stable for at least 4 h (Table 4).

For the determination of the counter stability, high and medium validation samples (VS1 and VS2) frozen in dry-ice were used. Two aliquots of either VS were thawed at certain time points according to a time schedule (24, 6, 5, 4, 3, 2 and 1 h before sample preparation). After thawing, one aliquot was stored at room temperature and the other at 4°C. During storage, the samples were protected from light. At zero time all

Table 3  
Intra-day precision and relative errors (RE) of validation samples

VS 1		VS 2		VS3	
Conc. ( $\mu\text{g/ml}$ )	RE (%)	Conc. ( $\mu\text{g/ml}$ )	RE (%)	Conc. ( $\mu\text{g/ml}$ )	RE (%)
19.9 <sup>a</sup>		2.19 <sup>a</sup>		0.218 <sup>a</sup>	
21.1	5.9	2.19	-0.2	0.235	7.6
20.6	3.2	2.16	-1.4	0.299 <sup>b</sup>	37.4
20.9	4.7	2.21	0.7	0.222	2.0
20.9	5.0	2.31	5.4	0.235	7.9
20.7	4.1	2.33	6.2	0.226	3.8
<i>n</i>	5		5		4
Mean	20.8		2.24		0.230
S.D.	0.2		0.08		0.007
R.S.D. (%)	3.0		3.6		3.0

<sup>a</sup> Nominal amoxicillin concentration ( $\mu\text{g/ml}$ ).

<sup>b</sup> Outlier for unknown reason (not included in statistical evaluation).

Table 4  
Light stability of amoxicillin in plasma at 4°C

Treatment <sup>a</sup>	Time (h)	Mean conc. (µg/ml)	Stability (%)
Initial	0	22.0	100.0
A	2	21.7	98.6
	4	21.9	99.5
	6	21.3	96.8
	8	20.6	93.6
	2	21.7	98.6
B	4	22.1	100.5
	6	21.4	97.3
	8	21.0	95.5
C	2	22.0	100.0
	4	22.3	101.4
	6	22.2	100.9
	8	22.0	100.0

<sup>a</sup> A = not light protected, stored in direct sunlight near a laboratory window; B = not light protected, stored under ambient room (fluorescent) light in the laboratory; C = light protected.

samples were prepared and measured. Stability at 4°C and at room temperature was given over a 24-h period for a concentration of 19.9 µg/ml (VS1). At a concentration of 2.19 µg/ml (VS2) there seemed to be a decline to 95.0% (4°C) and 94.1% (room temperature), but no decline was

observed within 5 h, which is sufficient for sample preparation (Table 5).

Stability was also tested after sample preparation at two different concentrations and storage conditions (21.0 and 2.22 µg/ml at 4°C and in dry-ice). No instability of amoxicillin was observed after 24 and 48 h.

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

For the investigation of the long-term stability, aliquots of two different concentrations were stored in liquid nitrogen (–180°C). No significant change in content was found after 25 and 31 weeks of storage.

#### 4. Conclusion

The assay proved to be reliable and sensitive enough to determine the pharmacokinetics and relative bioavailability of two oral 500-mg amoxicillin formulations. The lower limit of quantification of 0.0501 µg/ml was sufficient to allow the determination of amoxicillin plasma levels in more than 50% of the 10-h samples from healthy volunteers in a bioequivalence study (Fig. 2). These data allowed a reliable estimate of the areas under the curves and terminal half-lives in all subjects examined.

Table 5  
Counter stability of amoxicillin at ambient temperature and at 4°C

Storage (h)	Room temperature		4°C		Room temperature		4°C	
	Conc. (µg/ml)	Stability (%)	Conc. (µg/ml)	Stability (%)	Conc. (µg/ml)	Stability (%)	Conc. (µg/ml)	Stability (%)
–	19.9 <sup>a</sup>	100.0	19.9 <sup>a</sup>	100.0	2.19 <sup>a</sup>	100.0	2.19 <sup>a</sup>	100.0
1	18.8	94.5	19.2	96.5	2.06	91.1	2.25	102.7
2	20.4	102.5	20.4	102.5	2.15	98.2	2.13	97.3
3	20.0	100.5	20.3	102.0	2.16	98.6	2.16	98.6
4	21.6	108.5	20.3	102.0	2.15	98.2	2.16	98.6
5	20.0	100.5	20.5	103.0	2.23	101.8	2.16	98.6
6	19.7	99.0	20.0	100.5	2.09	95.4	2.09	95.4
24	19.7	99.0	20.1	101.0	2.06	94.1	2.08	95.0

<sup>a</sup> Nominal amoxicillin concentration (µg/ml).

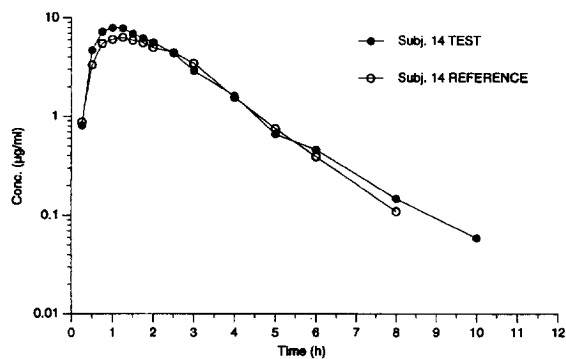


Fig. 2. Semi-logarithmic plot of the plasma levels in a volunteer after administration of a single oral dose of a test and reference formulation of 500 mg of amoxicillin.

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