Simple, Economical, and Reproducible LC–MS Method for the Determination of Amoxicillin in Human Plasma and its Application to a Pharmacokinetic Study

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Abstract

A simple, economical, and reproducible high-performance liquid chromatography mass spectrometric (MS) method is developed and validated for the determination of amoxicillin in human plasma. The present method has been successfully used to determine bioequivalence between a test and innovator formulation of amoxicillin 500 mg capsules. The method is validated in terms of selectivity, precision/accuracy, recovery, dilution integrity, matrix effect, effect of anti-coagulant, and stability studies. Sample preparation is carried out by solid-phase extraction (HLB Oasis cartridges). The processed sample is chromatographed on Hypersil Gold (4.6 × 50 mm); 3 µm C18 column, using 10mM ammonium formate buffer (pH 5.0) and acetonitrile, (10:90, v/v) as mobile phase. Amoxicillin is detected by MS–MS detection with turbo-ion spray in positive ion mode. The weighed ($1/X^2$) calibration curves were linear over the range of 0.17 to 17.0 µg/mL. The intra day precision is from 1.3% to 8.8% and intra day accuracy is 94.1% to 108.5%. The inter day precision is from 1.8% to 6.2% and inter-day accuracy is 95.1% to 105.9%. Mean recovery of 66.3% is observed for amoxicillin and 71.6% for internal standard (ampicillin). The stability of amoxicillin is studied at –15°C and –50°C using human plasma with different anti-coagulants (citrate, monobasic sodium phosphate, dextrose, and adenine–citrate, monobasic sodium phosphate, dextrose, and adenine and ethylene diamine tetraacetic acid–ethylene diamine tetraacetic acid). No significant degradation is observed for 60 days.

Introduction

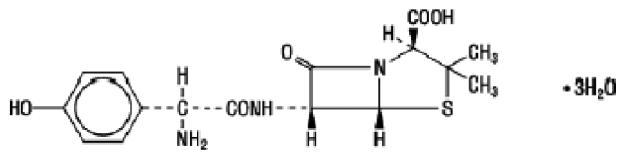
Chemically, amoxicillin (20) is (2S, 5R, 6R)-6-[(R)-(-)-2-Amino-2-) p-hydroxyphenyl) acetamidol] 3,3-dimethyl-7-oxo-4-thia-1-azabicyclo [3.2.0] heptane-2-carboxylic acid trihydrate. Its molecular formula is $C_{16}O_5N_3H_{19}S.3H_2O$ and is having 419.45 as molecular weight (see Figure 1 for structures of amoxicillin and ampicillin).

Amoxicillin is an aminopenicillin and is commercially available as trihydrate. It occurs practically odorless, white crystalline powder that is sparingly soluble in water.

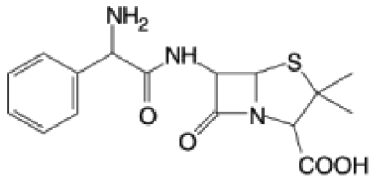
Amoxicillin, semi-synthetic penicillin of the aminopenicillin group, is bactericidal against sensitive organisms. It acts through the inhibition of mucopeptide synthesis in the bacterial cell wall.

Orally administered doses of 250 mg and 500 mg amoxicillin capsules result in average peak blood levels 1–2 h after administration in the range of 3.5 to 5.0 µg/mL and 5.5 to 7.5 µg/mL, respectively. Mean amoxicillin pharmacokinetic parameters from an open, two-part, single-dose, cross-over bioequivalence study in 27 adults comparing 875 mg of Amoxil (amoxicillin) with 875 mg of Augmentin (amoxicillin/clavulanate potassium) showed that the 875 mg tablet of Amoxil produces an AUC_{0-inf} of 35.4 ± 8.1 µg/h/mL and a C_{max} of 13.8 ± 4.1 µg/mL.

In a pharmaceutical industry which develops generic versions of molecules, the timelines for conducting bioequivalence studies (1,2) are very short. Mass spectrometry (MS) is preferred because of short run times and specificity. Hundreds of samples are analyzed everyday with thousands lying for analysis. Also the bioequivalence studies, which are submitted to various regulatory agencies, are to be conducted as per the regulatory requirements. The method used for analysis of plasma samples for BA/BE (bioavailability/bioequivalence) studies should not only



Amoxicillin trihydrate



Ampicillin (IS)

Figure 1. Chemical structures of amoxicillin and internal standard (ampicillin).

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be precise and accurate, but should be also completely validated (3,4,5) with respect to current GLP (good laboratory practices) requirements.

A number of high-performance liquid chromatography (HPLC) methods (10–17) for the estimation of amoxicillin in human plasma are available in the literature; run times vary from 6.0 to 15.0 min. Methods using MS (7,8,9,19) as detection, are also available in the literature. M. Marques et al. (7) have used protein precipitation as sample processing technique. F. Bruno et al. (8) has developed the method in bovine milk. RF Straub et al. (19) has developed the combination method with other β-lactum antibiotics. Because HPLC methods have the disadvantage of longer run times, MS as an instrument, has become the method of choice. Many people have used precipitation as the sample processing technique while using mass spectrometry. Even though precipitation methods are economical in nature, in case of MS it may lead to increase in maintenance time. The other drawback when protein precipitation is used as the sample processing technique and MS is used as an analytical instrument, chances of matrix effect are more (24,26).

Based on the current regulatory requirements, a mass spectrometry method for the estimation of amoxicillin in human plasma was developed. Keeping in mind the number of plasma samples from a BA/BE study, solid-phase extraction (SPE) was used as the sample processing technique. The advantage with SPE is that the sample to be injected is clean, which results in decreasing the maintenance time of the instrument. The other advantages with this method are: (*i*) Short run time of 2.0–2.5 min, meaning that 400–500 samples can be run in a single day; (*ii*) Less sample processing volume (200 µL) is required, which can help in reducing the amount of blood collection during while conducting the study; (*iii*) Complete validation is done as per current regulatory requirements and all parameters meet the acceptance criteria. The method has been successfully used to prove the bioequivalence between a test and a reference formulation of amoxicillin in 500 mg capsules. The PK (pharmacokinetic) values obtained from the study matches with the literature values (11,18); (*iv*) With clean final sample and 10-µL injection volume, the column is able to take a larger number of samples. The method was validated in terms of precision, accuracy, linearity, recovery, dilution integrity, effect of anti-coagulant, ruggedness, and stability studies. Stability studies include freeze-and-thaw, bench top, in-injector, long term, and stock solution. Before the initiation of unknown plasma samples of a biostudy, validation was complete and all validation parameters meet acceptance criteria.

The method was successfully used for the bioequivalence study of amoxicillin 500 capsule.

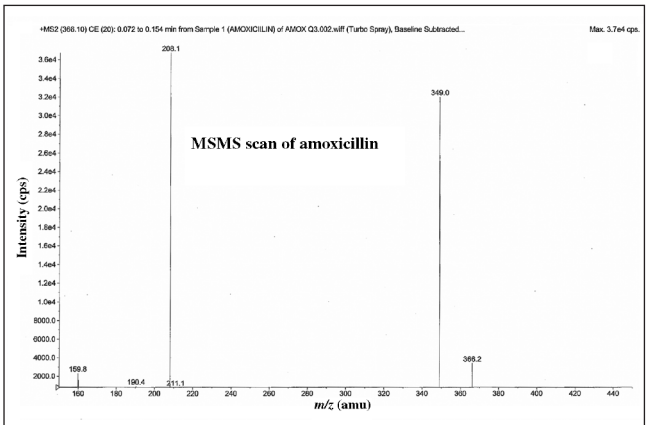

Figure 2. Product ion mass spectra of amoxicillin (precursor to product ion transition of *m/z* 366.2/208.1 was used)

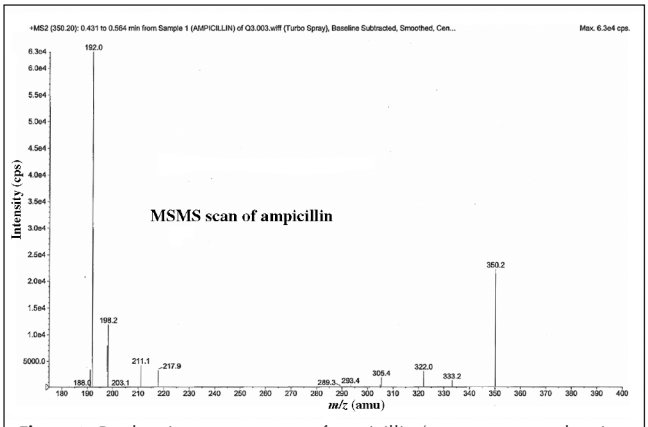

Figure 3. Product ion mass spectra of ampicillin (precursor to product ion transition of *m/z* 350.2/192.0)

Experimental

Chemicals, reagents, and standards

All solvents and reagents used were of analytical or HPLC grade. Acetonitrile and methanol were purchased from S.D. Fine Chem (Mumbai, India). Water was prepared using a Milli-Q system (Millipore, Mosheim Cedex, France). Ammonium formate and formic acid were supplied by Qualigens (Mumbai, India). Working standards of amoxicillin and ampicillin were procured from Sigma Aldrich (Bangalore, India).

HLB Oasis cartridges were purchased from Waters Corp. Bangalore, India (A subsidiary of Waters Corp., Milford, MA).

Standard stock solutions of amoxicillin and internal standard were prepared in water and were stored in refrigerator between 1–10°C. Further dilutions for spiking were also prepared in water.

Drug free EDTA and citrate, monobasic sodium phosphate, dextrose, and adenine (CPDA) human plasma was from Ranbaxy clinical pharmacology unit, Majeedia, New Delhi, India. To ensure the safety during usage, all batches of plasma were screened for Hepatitis B and C, human immunodeficiency virus (HIV) 1 and 2, malaria and syphilis. Human plasma, using CPDA and ethylene diamine tetraacetic acid (EDTA) as anticoagulants, were chromatographically screened for interfering substances prior to use. Human plasma batches, free of significant interference, were used to prepare calibration standards and quality control samples.

Instrumentation and operating conditions

High throughput HPLC System from Shimadzu (Tokyo, Japan), LC–MS (API-3000) from MDS Sciex (Toronto, Canada), and Analyst software Version 1.4 for data processing was used.

The column used was Hypersil Gold (4.6 × 50 mm); 3 µm C18 from Thermo Electron Corporation (Waltham, MA), with mobile phase as 10mM ammonium formate buffer (pH 5.0 ± 0.2)–acetonitrile (10:90, v/v) at a flow rate of 0.4 mL/min.

Initially, ammonium formate (pH 6.8) with acetonitrile in a ratio of 20:80 was used as mobile phase. When the pH was adjusted to 5.0 with formic acid, increase in response was observed. This may be because of enhanced ionization.

Similar response was observed with ammonium acetate and ammonium formate (10mM with pH 5.0). Column oven temperature was kept at 35°C.

The precursor to product ion transition of *m/z* 366.2/208.1

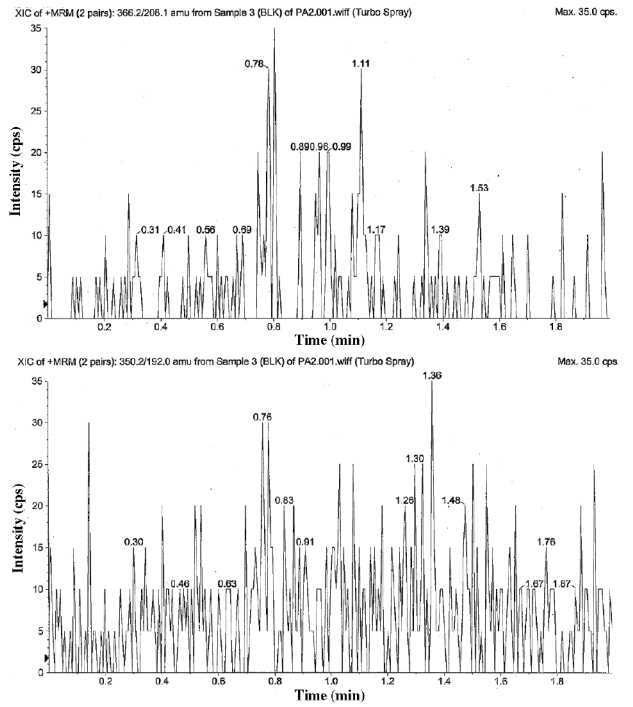

Figure 4. Representative LC–MS–MS chromatogram of extracted blank plasma sample.

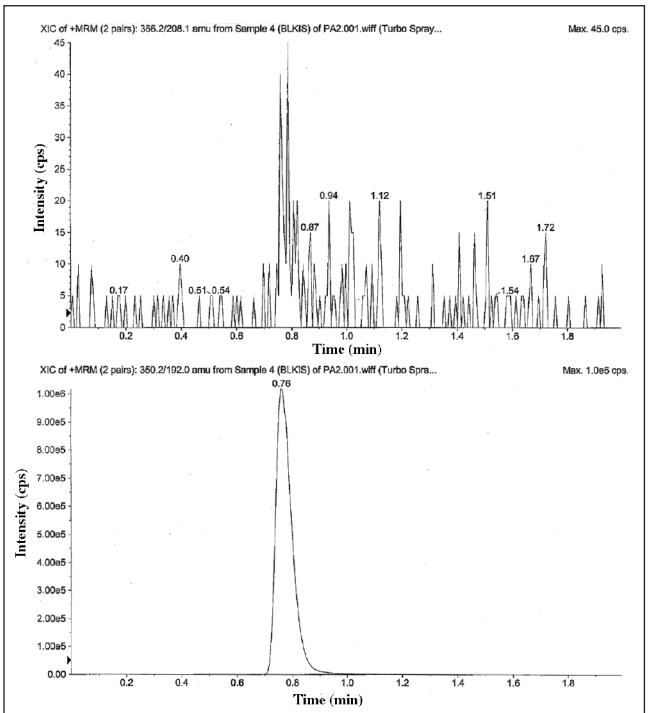

Figure 5. Representative LC–MS–MS chromatogram of extracted blank plasma sample spiked with internal standard.

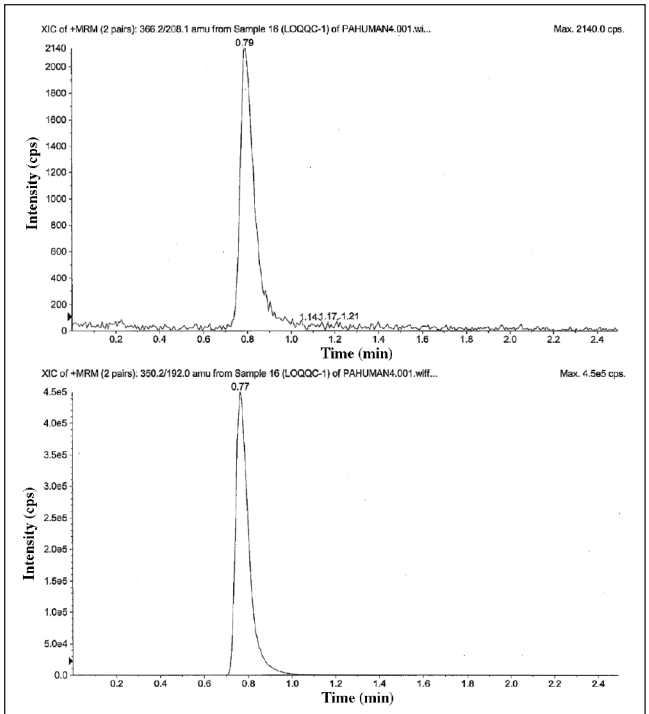

Figure 6. Representative LC–MS–MS chromatogram of extracted plasma sample spiked with amoxicillin at LOQ level with internal standard.

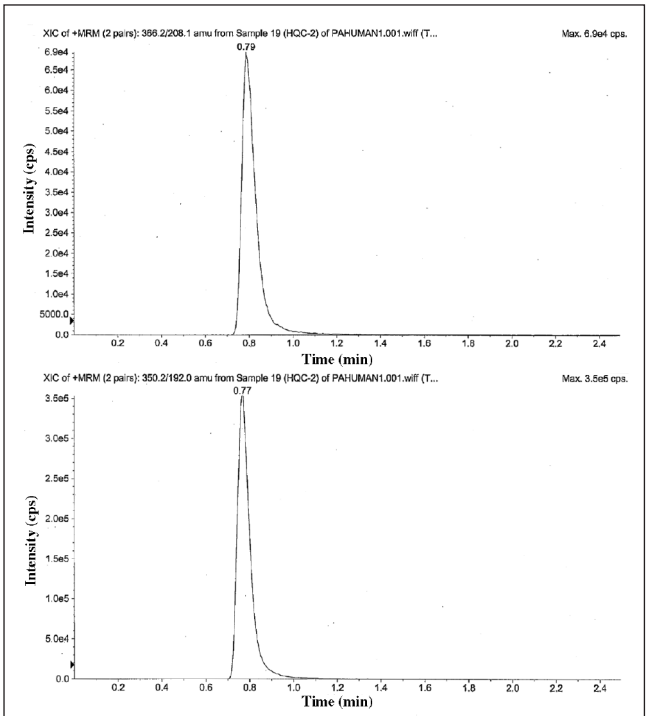

Figure 7. Representative LC–MS–MS chromatogram of extracted plasma sample spiked with amoxicillin at high concentration with internal standard.

and 350.2/192.0 were used to measure analyte and internal standard, respectively. The samples were analyzed by mass spectrometry in the multiple reaction monitoring mode. MS–MS (product ion) scan of amoxicillin and ampicillin has been illustrated in Figures 2 and 3, respectively.

Following mass parameters were optimized using a continuous infusion of 50 ng/mL of amoxicillin and internal standard: Collision gas (psi), 4; Curtain gas (psi), 6; Ion spray voltage (V), 4000; Ion source temperature (°C), 400; Declustering potential (V), 25; Focusing potential (V), 158; Collision energy (V), 18; Dwell time per transition (ms), 200; Mode of analysis, positive; Ion transition for amoxicillin (*m/z*), 366.2/208.1; Ion transition for ampicillin (*m/z*), 350.2/192.0.

Mass spectrometry was calibrated using polypropylene glycol (PPG) standard supplied by MDS Sciex. The calibration was done for both Q1 and Q3 positive and negative ion mode. With a continuous infusion of PPG solution at a flow rate of 2–3 µL/min, eight ions in a mass range of 59–2000 amu were monitored. Peak intensity, peak width (at 50% of peak height), and mass transfer of each ion was checked. Offset values were adjusted to get the peak width of 0.7 ± 0.1 amu for each ion. Mass shift of less than 0.1 amu was also checked during calibration.

The frequency of calibration of mass spectrometry is done once every three months. LC was calibrated in terms of pump flow, injection volume reproducibility, auto-injector position check, column oven temperature check.

Sample preparation

Sample processing was done using HLB Oasis cartridges. Cartridges were conditioned with methanol and water. After sample (200 µL plasma sample + 50 µL internal standard) was loaded, washing of the cartridge was done with 1 mL of 1% acetic acid in water. The sample was eluted with 400 µL of mobile phase and 10 µL was injected onto the HPLC column for analysis.

Validation parameters

The validation of this procedure was performed in order to evaluate the method in terms of selectivity, linearity of response, sensitivity, accuracy, precision, recovery, stability (in-injector, freeze-thaw, long term, bench top, and stock solution), dilution integrity, matrix effect, ruggedness, and effect of anticoagulants. The linearity, sensitivity, precision, and accuracy evaluations were performed on four batches of spiked samples.

Pre-defined acceptance criteria of ± 15% for precision and 85–115% for accuracy was followed throughout the validation. Stability studies were performed using six replicates of low and high quality control samples and analyzed against freshly prepared calibration curve. Freeze thaw stability (three cycles), bench top stability (6 h), in-injector stability (27 h), and long term stability (60 days) was determined for amoxicillin in human plasma.

Recovery was determined at low, middle, and high concentrations (extracted plasma samples) against un-extracted (neat aqueous) samples of same concentration. Matrix effect was determined using six different lots of drug free plasma at low and high concentrations. Dilution integrity was performed using concentration approximately 1.5 times of upper limit of quantitation (ULOQ) and diluted two times and four times. The two times and four times samples were run against calibration curve, and the precision and accuracy were determined. One precision and accuracy batch was processed and run by a different analyst to check the ruggedness of the method. Two anti-coagulants (CPDA and EDTA) were used to determine the effect of anti-coagulant on the precision and accuracy of analyte. Details of each experiment are covered under the "Results and Discussions" section.

Figures 4–7 show the representative chromatograms of blank plasma sample, blank plasma sample with internal standard, LOQ quality control (QC) sample with internal standard, and high concentration QC sample with internal standard.

Standardization and calculation

The chromatographic data were acquired and processed using computer-based Analyst software Version 1.4. The best fit lines using weighted (1/concentration^2) linear least square regression analysis were obtained by peak area ratio of amoxicillin to internal standard. The concentration of amoxicillin in plasma samples were calculated using linear regression parameters by corresponding calibration curve.

Results and Discussion

Selectivity

Six lots of plasma were used to evaluate the selectivity of the method. Aliquot from each was processed along with six aliquots of

Table I. Back Calculated Calibration Curve Concentrations for Amoxicillin

	Nominal concentration (µg/mL)										
CC-ID	**0.177**	**0.369**	**0.923**	**1.846**	**3.692**	**7.383**	**14.766**	**17.579**	**Slope**	**Intercept**	***r***
1	0.174	0.374	0.943	1.939	3.978	7.284	13.787	16.433	0.1360	0.00176	0.9983
2	0.176	0.365	0.955	1.939	3.917	7.472	13.794	16.289	0.1440	0.000043	0.9984
3	0.174	0.374	0.945	1.931	3.921	7.288	14.080	16.364	0.1510	0.001280	0.9987
4	0.172	0.382	0.952	2.014	3.864	7.034	14.135	16.144	0.1610	0.002450	0.9977
Mean	0.174	0.374	0.949	1.956	3.920	7.270	13.949	16.308			
S.D (±)	0.002	0.007	0.006	0.039	0.047	0.180	0.184	0.124			
C.V. (%)	0.9	1.9	0.6	2.0	1.2	2.5	1.3	0.8			
% Nominal	98.3	101.3	102.8	105.9	106.2	98.5	94.5	92.8			

lowest standard sample. None of the blanks showed significant interfering peaks at the retention time of amoxicillin and ampicillin.

Linearity and sensitivity

The linearity of the amoxicillin was determined by weighted least square regression analysis of standard plot associated with eight point standard curve. The calibration was shown to be linear from 0.177 to 17.579 µg/mL for amoxicillin. Best fit calibration lines of chromatographic response versus concentrations were determined by weighted least square regression analysis with weighting factor of 1/concentration^2. The *r* values were consistently 0.99 or greater during the course of validation for amoxicillin. Table I shows the back-calculated calibration curve concentrations for amoxicillin. Figure 8 shows the representative calibration curve.

The limit of quantitation was 0.177 µg/mL for amoxicillin. The inter-batch precision and accuracy at LOQ QC concentration for amoxicillin using internal standard ratio method was 6.2% and 100.4%, respectively.

Precision and accuracy

Four precision and accuracy batches were run to check intra- and inter-day precision and accuracy. Each batch of spiked plasma samples included one complete calibration curve (consisting of two blank plasma, two blank plasma with internal standard, and eight different non-zero concentrations) and six replicate quality control samples [LOQ QC, low quality control (LQC), middle quality control (MQC), and higher quality control (HQC)] made up of concentration corresponding to LOQ QC (0.182 µg/mL), LQC (0.507 µg/mL), MQC (6.755 µg/mL), and HQC (13.510 µg/mL) for amoxicillin (Table II).

The intra-day accuracy using internal standard area ratio method ranged from 94.1% to 108.5% and the inter day accuracy ranged from 95.1 to 105.9% for amoxicillin.

The intra-day batch precision, using internal standard area ratio method, ranged from 1.3% to 8.8% for amoxicillin, and the total precision ranged from 1.8% to 6.2% for amoxicillin.

Recovery

The percentage recovery of amoxicillin was determined by measuring the peak area response of extracted quality control samples at low, middle and high levels against the peak area response of un-extracted quality control samples of equivalent concentrations. Mean percent recovery of amoxicillin at low, middle, and high levels was 66.3%. The recovery of internal standard using the same method was 71.6%.

Stability studies

Freeze-and-thaw stability

The freeze-and-thaw stability of the spiked plasma samples was determined during third freeze thaw cycle by using replicate number of quality control samples at low and high levels and the degradation was determined against freshly spiked calibration curve. The thawing was performed at room temperature and six replicates at each level were used. Percent stability observed after third freeze thaw ranges between 99.8–110.8%. The precision varies from 1.6–1.9% (Table III).

Bench top stability

For bench top stability, evaluation involved analysis of repli-

Table II. Intra-day Precision and Accuracy for Amoxicillin

	LOQ QC	LQC	MQC	HQC
Actual conc. (µg/mL)	0.182	0.507	6.755	13.510
Mean observed conc. (µg/mL) PA1	0.182	0.541	7.018	12.885
C.V. (%)	2.7	2.6	3.8	1.7
% Nominal	99.9	106.7	103.9	95.4
Mean observed conc. (µg/mL) PA2	0.194	0.550	7.074	12.898
C.V. (%)	1.8	3.0	3.8	1.3
% Nominal	106.7	108.5	104.7	95.5
Mean observed conc. (µg/mL) PA3	0.184	0.532	6.995	12.806
C.V. (%)	2.0	2.5	3.4	2.1
% Nominal	100.9	104.9	103.6	94.8
Mean observed conc. (µg/mL) PA4	0.171	0.524	6.675	12.783
C.V. (%)	8.8	1.7	2.2	2.2
% Nominal	94.1	103.4	98.8	94.6
Inter-day precision and accuracy for amoxicillin				
Actual conc. (µg/mL)	0.182	0.507	6.755	13.510
Mean observed conc. (µg/mL)	0.1828	0.5370	6.9405	12.8429
C.V. (%)	6.2	3.0	3.9	1.8
% Nominal	100.4	105.9	102.7	95.1

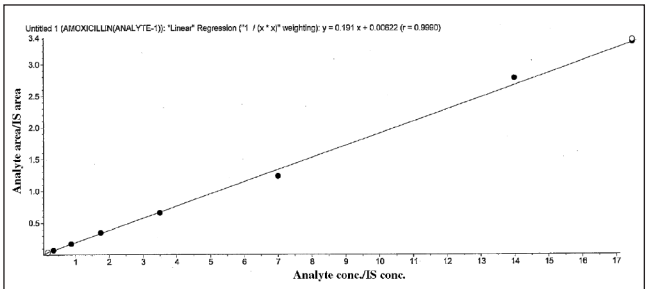

Figure 8. Representative calibration curve (analyte concentration vs. analyte by internal standard area ratio).

Table III Stability studies

Activity	% Stability	Precision (%)
Freeze-thaw stability (three cycles)	99.8–110.8	1.6–1.9
Bench top stability (6 h)	97.8–99.4	2.1–2.6
In-injector stability (27 h)	98.3–99.1	1.2–2.3
Long term stability (60 days)		
–15°C (CPDA*)	100.1–107.1	1.6–7.6
–50°C (CPDA)	99.1–100.1	2.4–7.6
–15°C (EDTA†)	99.4–108.4	2.3–8.2
–50°C (EDTA)	101.1–101.4	2.1–7.0

* CPDA—citrate, monobasic sodium phosphate, dextrose, and adenine.
† EDTA—Ethylene diamine tetraacetic acid.

cates of LQC and HQC stability samples, which had been kept at room temperature for a designated time (6.0 h). These samples were processed and run against the freshly spiked calibration curve. The comparative stability ranged from 97.8 to 99.4% for amoxicillin. The precision varies from 2.1–2.6% (Table III).

In-injector stability

In-injector stability of replicate quality control samples was determined. The LQC and HQC samples kept in auto-injector (10°C) were analyzed after approximately 27 h and the concentration was calculated against the freshly spiked calibration curve. The comparative stability ranged from 98.3 to 99.1% for amoxicillin. The precision varies from 1.2–2.3% (Table III).

Stock solution stability (long-term and short-term)

Stock solution stability (long term) of amoxicillin and ampicillin was determined for 10 and 17 days, respectively. The stocks were kept in a refrigerator (between 1–10°C). After the stability period, fresh stocks of amoxicillin and ampicillin were prepared. A dilution of each with same concentration was prepared and six replicate injections were given. Mean response obtained from the stability stock was compared with the response obtained from fresh stock. The percent stability observed for amoxicillin was 97.8% and for ampicillin percent stability was 97.6%.

To check the stability of amoxicillin and ampicillin dilutions at room temperature (short-term), dilutions of amoxicillin and internal standard were prepared as a mixture and also separate dilution of internal standard was prepared. These dilutions were kept at room temperature for approximately 24 h. After 24 h, the same dilutions were injected along with fresh dilutions of same concentration to check the stability. In case of analyte and internal standard mixture, the area ratio obtained for stability dilution was compared with area ratio obtained from fresh dilutions. The percent stability was 99.9%. Six injections of fresh and stability dilutions were injected. Mean response was compared. The percent stability observed was 94.7%.

Long-term stability

Long-term stability of spiked plasma samples was determined. The LQC and HQC samples were stored at –15°C in cold room and at –50°C in deep freezer with CPDA and EDTA as anticoagulants for 60 days. These samples were analyzed against freshly spiked calibration curve. The percent stability ranged between 99.1–108.4%. There is no impact of any anti-coagulant on the stability of amoxicillin when stored for longer periods (Table III).

Table IV. Dilution Integrity of Amoxicillin

LQC#	27.025* (µg/mL)	HQC#	27.025† (µg/mL)
1	26.541	1	28.578
2	26.505	2	27.585
3	26.534	3	27.284
4	27.805	4	28.130
5	26.707	5	27.880
6	27.310	6	27.328
Mean	26.9003		27.7975
SD (±)	0.53726		0.50116
C.V. (%)	2.0		1.8
% Nominal	99.5		102.9

* 2 times dilution.
† 4 times dilution.

Other validation parameters

Dilution integrity

Dilution integrity is performed to check if any sample, if diluted, gives the accurate and precise results. During the analysis of plasma samples from a biostudy, sample dilution becomes

Table V. Matrix Effect on Amoxicillin

QC-ID	LQC (µg/mL)	HQC (µg/mL)
Matrix ID	0.507*	13.510*
1	0.574	13.301
	0.569	13.380
2	0.577	13.552
	0.570	13.627
3	0.567	13.081
	0.580	13.138
4	0.565	13.349
	0.564	13.500
5	0.574	13.956
	0.577	13.540
6	0.577	13.760
	0.560	13.761
Mean	0.5712	13.4954
SD (±)	0.00628	0.26064
C.V. (%)	1.1	1.9
% Nominal	112.7	99.9

* Nominal concentration.

Table VI. Effect of Anticoagulants in Human Plasma for Amoxicillin

	CPDA*		EDTA†	
	LQC (µg/mL)	HQC (µg/mL)	LQC (µg/mL)	HQC (µg/mL)
S No	0.507‡	13.510‡	0.507‡	13.510‡
1	0.534	13.057	0.515	13.038
2	0.543	12.447	0.502	12.898
3	0.527	13.140	0.512	13.239
4	0.533	13.178	0.504	12.577
5	0.533	12.493	0.506	12.694
6	0.533	12.653	0.512	14.229
Mean	0.5338	12.8280	0.5085	13.1125
SD (±)	0.00515	0.33475	0.00521	0.59599
C.V (%)	1.0	2.6	1.0	4.5
%Nominal	105.3	95.0	100.3	97.1

* CPDA: Citrate, phosphate, dextrose, and adenine.
† EDTA: Ethylene diamine tetraacetic acid.
‡ Nominal concentration.

necessary in case of insufficient sample or if the value obtained is beyond the calibration curve.

To check the dilution integrity, approximately 1.5 times the ULOQ concentration was spiked in plasma and diluted by factor of 2 and 4 with drug free plasma. Six aliquots of each (2 and 4 times) was processed and run against calibration curve. The precision observed for two times dilution samples was 2.0% and accuracy was 99.5%. For four time dilution samples, the precision observed was 1.8% and accuracy was 102.9% (Table IV).

Matrix effect

In case of mass spectrometry, determination of matrix effect is a must. Matrix effect can be observed across different lots of plasma. To determine the matrix effect, six lots of plasma (free from analyte and internal standard) were chosen. Concentration equivalent to LQC and HQC were spiked in each lot of plasma. At each level, samples in duplicate were processed and run against freshly spiked calibration curve. Precision and accuracy in each plasma at LQC and HQC was checked. Precision of 1.1–1.9% and accuracy between 99.9–112.7% was observed (Table V).

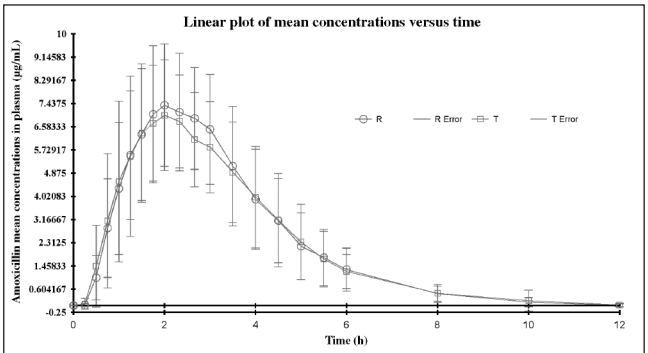

Figure 9. Mean plasma concentration (± SD) time profile of amoxicillin after single oral does of 500 mg capsules (reference and test) to healthy human subjects under fasting conditions (n = 24).

Table VII. Ruggedness–Within Batch Precision and Accuracy for Amoxicillin

		Nominal concentration (µg/mL)					
QC-ID	LOQ QC		LQC		MQC		HQC
LOQQC	0.182*	LQC	0.507*	MQC	6.755*	HQC	13.510*
1	0.172	1	0.531	1	6.638	1	13.159
2	0.188	2	0.546	2	6.926	2	12.961
3	0.170	3	0.534	3	6.678	3	12.278
4	0.197	4	0.526	4	6.416	4	12.400
5	0.168	5	0.537	5	6.880	5	12.645
6	0.180	6	0.539	6	6.819	6	12.954
Mean	0.1792		0.5355		6.7262		12.7328
SD (±)	0.01146		0.00689		0.18895		0.34870
C.V. (%)	6.4		1.3		2.8		2.7
% Nominal	98.4		105.6		99.6		94.2

* Nominal concentration.

Table VIII. Pharmacokinetic Parameters with Amoxicillin 500 mg Capsule in Healthy Human Subjects

	T_{max} (h)		C_{max} (µg/mL)		AUC_{0-t} (µg.h/mL)		AUC_{0-inf} (µg.h/mL)		Half life (h)	
	Reference	Test	Reference	Test	Reference	Test	Reference	Test	Reference	Test
Mean	2.197	2.200	8.596	8.469	26.882	26.323	27.455	26.915	1.342	1.344
SD	0.786	0.763	1.877	1.799	6.681	6.270	6.699	6.241	0.263	0.229

Effect of anti-coagulant

To confirm the effect of anti-coagulant, calibration curve standards and six sets of quality control samples at LQC and HQC levels were prepared in human plasma containing CPDA as anti-coagulant. Also six sets of quality control samples (LQC and HQC) were prepared in human plasma containing EDTA as anti-coagulant. Precision ranged from 1.0 to 4.5 and accuracy ranged from 97.1–105.3% (Table VI).

Ruggedness

The ruggedness of the extraction procedure and chromatographic method was evaluated by analysis of a batch of six sets of quality control samples and a set of calibration standard by another analyst using a different column of same make. The precision ranged between 1.3–6.4% and accuracy ranged between 94.2–105.6 (Table VII).

Conclusion

The above analytical method described is valid for the determination of amoxicillin (over a range of 0.177 to 17.579 µg/mL) using ampicillin as internal standard in human plasma using a Hypersil Gold (4.6 × 50 mm); 3 µm C18 column. The method offers significant advantages in terms of faster run time (2.0 min) and lower sample requirements. The method is fully validated as per the current regulatory requirements and all the parameters of validation passed the acceptable limits. With simple SPE procedure and short run time, the method can be considered suitable for application to pharmacokinetic studies.

Method applications

The method was used in the bioequivalence study of amoxicillin 500 mg capsules. The study design was open label, balanced, randomized, two-treatment, two-sequence, two-period, single-dose, crossover comparative bioavailability study of amoxicillin 500 mg capsules produced by Ranbaxy Research Laboratories, Gurgaon, India and AmoxilÒ 500 mg capsules produced by Smith-Kline Beecham, Brazil in healthy, adult, male, human subjects under fasting condition. The samples were collected at pre-dose, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.33, 2.66, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 8.0, 10, and 12.0 h.

Table VIII shows the PK parameters (T_{max}, C_{max}, AUC_{0-t}, AUC_{0-inf}, and $T_{1/2}$) and Figure 9 for linear plot of mean concentration versus time.

The PK values obtained are matching with the literature values (11,18).

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