ISSN : 0974-7419 Volume 10 Issue 2

Trade Science Inc.

Analytical CHEMISTRY

An Indian Journal

Full Paper

ACAIJ, 10(2) 2011 [122-126]

Ampicillin analysis by fully validated HPLC assay in human plasma

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Received: 18th July, 2010 ; Accepted: 28th July, 2010

ABSTRACT

A simple and rapid HPLC assay for ampicillin measurement in human plasma was developed and validated. 0.25 ml plasma sample was precipitated with 50 µl of perchloric acid, and 100 µl of the supernatant was directly injected into 4.6×150 mm, Symmetry Shield, RP8, 4-µm steel column at room temperature (RT). The mobile phase, 0.05 M dibasic sodium phosphate buffer (pH = 7.3) and acetonitrile (87:13, v:v), was delivered at 1.0 ml/min with a run time of 9 min. Ciprofloxacin (internal standard, IS) and ampicillin were detected at 3.9 and 8.1 min, respectively, using Waters 2998 photodiode array detector set at 210 nm. The response was linear over the range of 0.3–15 µg/ml, and the intra-and inter-run coefficient of variations were ≤ 10.7% and 10.4%, respectively. Extraction recovery and intra- and inter-run bias were ≥ 86% (mean 91%), ≤ 11%, and ≤ 7%, respectively. Ampicillin was stable in plasma for 24 hours at RT (≥ 87%), 5 weeks at –20 ^{0}C (≥ 93%), and after 3 cycles of freeze at –20C and thaw at RT (≥ 89%). In precipitated plasma samples, ampicillin was stable for 24 hours at RT (≥ 93%) and 48 hours at –20°C (≥ 85%). Stock solution of ampicillin (1 mg/ml in water) was stable for 48 hours at RT (93%) and 5 weeks at -20°C (97%).

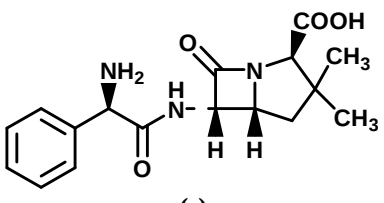
KEYWORDS

Ampicillin trihydrate;
Ciprofloxacin;
HPLC;
Validation;
Stability.

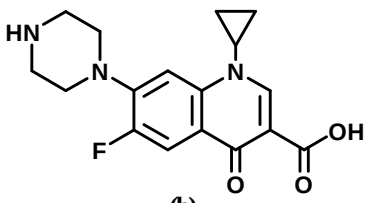
INTRODUCTION

Ampicillin (CAS; 69-53-4) is one of the oldest β-lactam antibiotic that has been used extensively in the treatment of a variety bacterial infections[1]. Its absolute bioavailability is 39-54% with mean peak plasma concentration in range of 4.0-5.4 µg/ml, 1.8-2.6 hours after the ingestion of a 500 mg therapeutic dosage in the form of capsule or suspension[2,3].

Most of the reported methods for the analysis of



(a)



(b)

Figure 1 : Chemical structure of ampicillin (a) and the internal standard ciprofloxacin (b)

ampicillin in pharmaceutical formulation[4-7] or biological fluids[8-22] have various analytical or practical limitations. Further, only sparse information is available on ampicillin stability.

The aims of this study were to 1) establish a simple, fully validated HPLC assay to measure ampicillin level in human plasma with quantitation limits suitable for bioequivalence studies, and 2) determine the stability of ampicillin under various clinical laboratories conditions.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of Waters Alliance 2998 photodiode Separations Module, a 4-μm (particle-size) 4.6×150 mm Symmetry Shield, RP8 steel column, a Nova-Pak C_{18} 4-μm insert in conjunction with Guard Pak pre-column module, and Waters 2998 photodiode array detector (Water Associates, Milford, MA, USA) set at 210nm. Data were collected with a Pentium IV computer using Millennium^{32} Chromatography Manager Software (Water Associates, Milford, MA, USA).

Chemicals and reagents

Ampicillin trihydrate (Figure 1a), was purchased from Boehringer Mannheim, GmbH, Germany, and the internal standard (IS) ciprofloxacin (CAS number; 85721-33-1) (Figure 1b) was purchased from Bayer, Leverkusen, Germany. Acetonitrile and dibasic sodium phosphate were purchased from Fisher Scientific, Fairlawn, NJ, USA, and perchloric acid (69-72%) from Fisher Scientific, Pittsburgh, PA, USA. Water for HPLC was prepared by reverse osmosis and further purified by passing through a Millipore-Synergy UV obtained from Millipore Co., Bedford, MA, USA.

Chromatographic conditions

The mobile phase consisted of 0.05 M dibasic sodium phosphate buffer (pH = 7.3) and acetonitrile (87:13, v:v) and was delivered at a flow rate of 1.0 ml/min at room temperature. It was filtered through a 0.45 μm size membrane filter (Millipore Co., Bedford, MA, USA) and degassed before use. The autosampler was programmed to inject 100 μl into the chromatograph with a run time of 9 minutes.

Preparation of stock and working solutions

Ampicillin trihydrate (1 mg/ml) stock solution was prepared in water and used for stability studies and to prepare a working solution (15 μg/ml) in plasma. The working solution was prepared weekly to construct calibration curve and quality control (QC) samples. Ciprofloxacin (IS) working solution (50 μg/ml) was prepared weekly in the mobile phase from a stock solution in methanol (1 mg/ml).

Calibration standard/ Quality control samples

Calibration standards were prepared by mixing nine different volumes of ampicillin working solutions in blank human plasma to produce final concentrations of blank, zero (blank plasma spiked with IS only), 0.3, 0.6, 1.2, 2.0, 4.0, 6.0, 8.0, 12.0, and 15.0 μg/ml. QC samples were prepared by mixing four different volumes of ampicillin working solution in blank human plasma to produce final concentrations of 0.3, 0.9, 7.5, and 13.5 μg/ml. Samples were vortexed for 10 seconds, and aliquots of 0.25 ml of calibration standards and QC samples were transferred into 1.5 ml eppendorf microcentrifuge tubes and stored at -20ºC.

Sample preparation

Aliquots of 0.25 ml of calibration standard or QC samples in microcentrifuge tubes were allowed to equilibrate to room temperature. To each tube, 40 μl of the 50 μg/ml IS working solution was added and vortexed for 10 seconds. After the addition of 50 μl of perchloric acid, the mixture was vortexed again for 1 min and then centrifuged for 7 min at 13200 rpm at room temperature. The supernatant organic layer was carefully transferred into the autosampler vials and 100 μl were injected into the HPLC system.

Stability studies

Stability of ampicillin in plasma: Adequate numbers of aliquots of two QC samples (0.9, and 13.5 μg/ml) were prepared. Aliquots were analyzed in 5 replicates immediately (baseline), after being processed and stored at room temperature for 24 h or at –20°C for 48 h (autosampler stability), after being allowed to stand on the bench-top for 8 or 24 h at room temperature before processing (counter stability), after being stored at –20°C for 5 weeks before processing (long term freezer

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TABLE 1 : Specificity of ampicillin assay

Drug name	Retention time
Ampicillin*	8.1
Ciprofloxacin	3.9
Diclofenac	4.3
Acetaminophen	5.0
Ranitidine	5.8
Nicotinic Acid	2.0
Ascorbic Acid	ND
Caffeine	4.8
Ibuprofen	ND
Omeprazole	6.0

1.0 mg/ml solutions in methanol or water* were diluted in mobile phase to 10 µg/ml and 100 µl were injected

TABLE 2: Extraction recovery of ampicillin and ciprofloxacin

Nominal concentration (µg/ml)	Plasma		Mobile phase		** Recovery (%)
	*Mean peak area	SD	*Mean peak area	SD	
Ampicillin 0.3	17919	973	20057	752	89
0.9	61235	1163	70742	3318	86
7.5	760030	14259	806980	6437	94
13.5	1360830	50697	1419426	19656	96
Ciprofloxacin 0.5	865130	20905	969590	16694	89

***Mean peak area of 5 replicates. ** Mean peak area of spiked plasma sample divided by mean peak area of spiked mobile phase sample X 100. SD, Standard Deviation**

stability), or after being stored at –20°C for 24 h and then left to completely thaw unassisted at room temperature before processing (with the cycle repeated three times, freeze- thaw stability).

Stock solutions stability: Five aliquots of the stock solutions of ampicillin and the IS were analyzed (after dilution to 10 µg/ml in mobile phase) at baseline, after storage for 48 h at room temperature, or after storage at –20°C for 5 weeks. Stability of the working solutions of ampicillin and the IS, were evaluated up to 2 weeks at –20°C.

Assay validation method

The procedures used for validation were as described in the US Food and Drug Administration (FDA) bioanalytical method validation guidance[22].

RESULTS

Optimization of chromatographic conditions

In order to improve specificity and minimize interference from plasma or solvent system that may occur at lower wavelengths, we optimized the absorbance wavelength based on photodiode array extracted spectra. We performed the analysis at 210 nm. Different ratios of the components of the mobile phase were investigated, and a ratio of 87:13(v:v) was found best to achieve separation of ampicillin from IS and minimize background absorbance. Under the described conditions, the IS and ampicillin were well resolved within a run time of 9 minutes, and their retention time were 3.9 and 8.1 minutes, respectively.

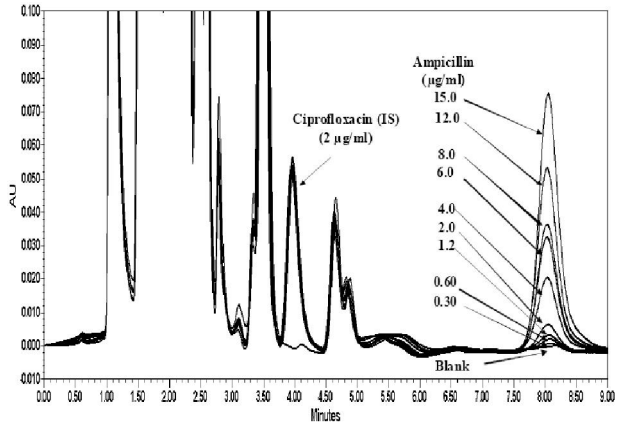

Figure 2 : Overlay of ten chromatograms of blank human plasma spiked with 0 (B), 0.3, 0.6, 1.2, 2.0, 4.0, 6.0, 8.0, 12.0 or 15.0 µg/ml of ampicillin as well as 2 µg/ml of ciprofloxacin (IS)

Linearity

Linearity was determined in the range of 0.3-15.0 µg/ml using ten calibration curves. The data were analyzed by linear regression using the formula: Conc. = a + b (PAR), where Conc. is the concentration of ampicillin, a is the intercept, b is the slope, and PAR is the peak area of ampicillin divided by the peak area of the IS. The concentrations of the calibration standards of the ten curves were back-calculated using the individual regression lines. Linearity studies (n=10) showed mean (SD) for R^2 of 0.9971 (0.0010) a slope of 0.2768 (0.2199), and an intercept of 0.0364 (0.0655). Figure 2 depicts an overlay of chromatograms of a representative standard curve.

Limit of detection

The limit of detection (LOD), defined as three times the baseline noise, was 0.2 µg/ml.

Specificity

To evaluate specificity of the assay, we screened

TABLE 3 : Intra-run and inter-run accuracy and precision of ampicillin assay

Nominal concentration (µg/ml)	Intra-run (n=10)				Inter-run (n=20)			
	Mean measured concentration (µg/ml)	SD	Precision (CV*,%)	** Bias(%)	Mean measured concentration (µg/ml)	SD	Precision (CV*,%)	** Bias(%)
0.3	0.3	0.03	9.1	0.1	0.3	0.03	10.41	4
0.9	0.8	0.07	8.6	11	0.8	0.08	9.1	7
7.5	6.9	0.54	7.9	8	7.3	0.60	8.2	3
13.5	12.5	1.33	10.7	7	13.1	0.64	4.9	3

***Coefficient of variation (CV) = Standard Deviation (SD) divided by mean measured concentration X 100. **Bias = Absolute value of 1 minus mean measured concentration divided by nominal concentration X100**

TABLE 4 : Stability of ampicillin in plasma samples and stock solution

Nominal concentration (µg/ml)	*Plasma Samples								**Stock solution	
	Unextracted			Extracted		Freeze-thaw				
	8 h RT	24 h RT	5 wks –20°C	24 h RT	48 h –20°C	One cycle	Two cycles	Three cycles	48 h RT	5 wks –20°C
0.9	89	87	93	98	85	106	110	100	93	*97*
13.5	100	99	100	93	94	106	106	89		

Stability (%) = mean measured concentration (n=5) at the indicated time divided by mean measured concentration (n=5) at baseline×100. *Spiked plasma samples were analyzed immediately (baseline, data not shown), after storing for 8 or 24 hours at room temperature (8 h RT and 24 h RT) or 6 weeks at – 20°C (5 wks –20°C); analyzed after storing the extract for 24 hours at room temperature (24 h RT) or 48 hours at –20°C (48 h -20°C); or analyzed after 1 to 3 cycles of freezing plasma at –20°C and thawing at room temperature (freeze-thaw). **Ampicillin, 1 mg/ml in water

eight frequently used medications (10 µg/ml in mobile phase) and six different batches of human plasma. All batches of blank plasma were free from interfering components. None of eight commonly used drugs co-eluted with ampicillin or the IS (TABLE 1).

Recovery

The extraction recovery of ampicillin was determined by dividing mean peak areas of five replicates of four QC samples (0.3, 0.9, 7.5, and 13.5 µg/ml) prepared in plasma (as described under sample preparation), by mean peak areas of five replicates of equivalent concentrations prepared in mobile phase. The recovery of the IS was determined similarly at a concentration of 0.5 µg/ml. The results of the extraction recovery studies of ampicillin and the internal standard are presented in TABLE 2. Recovery was ≥86% (mean 91%) for ampicillin and 89% for the IS.

Precision and bias

Precision was calculated as coefficient of variation (standard deviation divided by mean measured concentration×100) and bias (inaccuracy) as the absolute value of (1 minus mean measured concentration divided by nominal concentration)×100. The intra-run and inter-run precision and bias were determined by analyzing four QC samples: 0.3, 0.9, 7.5, and 13.5 µg/ml over three different days (TABLE 3). Intra-run precision and bias (n = 10) ranged from 7.9% to 10.7% and from 0.1 % to 11 %, respectively. The inter-run precision and bias (n = 20) ranged from 4.9% to 10.4% and from 3% to 7%, respectively.

Stability

The stability of ampicillin in plasma, in precipitated plasma samples, and in stock and working solutions, under usual storage conditions was investigated. The results are presented in TABLE 4. The data indicate that: 1) ampicillin in plasma is stable for at least 24 hours at room temperature and 5 weeks at –20°C, 2) in precipitated samples, ampicillin is stable for at least 24 hours at room temperature or 48 hours at –20°C, 3) ampicillin in plasma is stable after at least three cycles of freeze at –20°C and thaw at room temperature, and 4) ampicillin in water (1 mg/ml), was stable for at least 5 weeks at –20°C.

Further, the working solutions of ampicillin and the IS (15 µg/ml in plasma and 50 µg/ml in mobile phase, respectively) were stable for at least 2 weeks at –20°C (92% and 97%, respectively) and the IS in methanol (1

mg/ml) was stable for at least 5 weeks at –20°C (95%).

Robustness

The robustness of the proposed method was evaluated by slightly altering the pH of the phosphate buffer and the proportion of acetonitrile in the mobile phase. No significant effects were observed. Further, chromatographic resolution and peak response were stable over about 700 injections of processed plasma samples using one column.

DISCUSSION

We describe a rapid, simple, accurate, and precise HPLC assay for the determination of therapeutic levels of ampicillin in human plasma. Simplicity, rapidity, and smaller sample volume are the main advantages of the current assay.

The described assay involves a simple precipitation step in sample preparation, avoiding pre-column dervatization with mercury chloride[8] or formaldehyde[13], or post-column dervatization with fluorescamine[12], or solid–phase extraction[9], and column switching[10]. A short run time of 9 minutes and a small plasma volume of 250 µl favorably compare to more than 20 minutes[9,11] and from 0.50 to 1 ml[7,9,11-13] in previously reported assays. Further, the recovery of ampicillin from plasma was ≥ 86% (mean 91%) compared to previously reported recovery as low as 75%[13]. Furthermore, some of the previously reported assays were not validated to measure ampicillin level in human plasma[15-21], or did not examine ampicillin stability[9-11].

CONCLUSION

In summary, the results of this study expand the information on ampicillin stability and indicate several advantages of the described assay over previously reported assays, especially for therapeutic drug monitoring and bioequivalence studies.

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