Original Article

Validated reverse phase-high performance liquid chromatography method of amoxicillin trihydrate for assay and dissolution studies in time-dependent release bilayer tablet formulations

Abstract

Background: Amoxicillin trihydrate is used a broad spectrum antibiotic for the treatment of diverse variety of bacterial infections. **Aim:** The objectives of the present study was an assessment of *in vitro* drug release profile and estimation of the drug content of the prepared *in-house* time-dependent release bilayer tablet formulations of amoxicillin trihydrate (AMT) employing a simple, rapid, sensitive and a cost effective reverse phase liquid chromatography method. **Materials and Methods:** The chromatographic separation was achieved using methanol and phosphate buffer (pH 3.5) at 50:50% v/v as mobile phase at a flow rate of 1 mL/min. **Results:** The method was validated for linearity, accuracy, precision, limit of detection, limit of quantitation and system suitability. The linearity was determined by residual analysis indicated good regression coefficient (r^2) of 0.999 at a concentration ranging between 2 and 16 μg/mL. The assay of both *in-house* formulation and marketed preparation indicated good recovery of 99.95% and 99.60%, respectively. *In vitro* dissolution studies revealed that the prepared *in-house* tablet formulation showed time-dependent biphasic profile of drug release, whereas marketed preparation showed immediate release effect. **Conclusions:** The developed method, therefore, can be used for routine estimation of AMT in bulk and pharmaceutical dosage forms.

Key words:
Amoxicillin, dissolution, linearity, liquid chromatography, mobile phase

Introduction

Amoxicillin trihydrate (AMT), a broad spectrum antibiotic, shows promising antibacterial activity against both Gram-positive as well as Gram-negative bacteria. Chemically, it is classified under semisynthetic penicillins having structural similarity with native penicillin by the presence of the β-lactam ring. The broad spectrum action of AMT is due to the presence of benzyl group in the side chain of the β-lactam ring which increases its sensitivity to inhibit the bacterial cell wall synthesis.[1] Unlike other semisynthetic penicillins, it is administered through peroral route in the form of tablets, capsules, dispersible tablets, syrups and pediatric suspensions with dose ranging between 250 and 500 mg b.i.d/t.i.d. Despite of its advantages for oral drug delivery, the major drawback underlying oral delivery of amoxicillin is its acid labiality and instability in the presence of gastric acid leading to decrease in the therapeutic efficacy.[2]

Several formulation strategies have been employed to improve the oral delivery of amoxicillin in order to prevent its stability in the presence of gastric acid. Few of them include delayed release and/or modified release tablets and capsules, microspheres, etc. However, such formulations have yielded limited fruition and found to be less effective in enhancing the therapeutic efficacy of amoxicillin. Needless to mention, bacterial infections including pharyngitis are

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Sarwar Beg, Kanchan Kohli

Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard, New Delhi, India

Address for correspondence:
Dr. Kanchan Kohli,
Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard,
Hamdard Nagar, New Delhi - 110 062, India.
E-mail: sarwar.beg@gmail.com

the chronobiological diseases whose progression depends on the circadian rhythm of the body. The bacteria has a general reproductive growth cycle consisting of four different phases, viz. lag phase, exponential or log phase, stationary phase and decline phase. The viability of bacterial population is considered to be higher in the log phase than other phases and it is a chronobiology mediated phenomenon specifically happened in the early day time. Hence, the chronomodulated drug delivery systems, which releases the drug at the right site at the right time in the right quantity are highly suitable in enhancing the therapeutic efficacy of drug molecules.[3] These formulations provide better gastric protection of drugs form degradation and offers medications for prolonged periods of time.[4] In routine practice, during development of such dosage forms few quality control techniques are used to ensure the product quality and performance. Among them, dissolution testing is employed as a most useful and reliable technique to characterize the *in vitro* drug release from these dosage forms. Appropriate selection of the dissolution medium, apparatus and dissolution conditions is highly important to produce an effective and reproducible dissolution method.[5]

Further, in order to perform regular, day-to-day routine analysis and quantification of AMT in pharmaceutical formulations, it requires a rapid, sensitive and robust analytical technique. Several liquid chromatography (LC) methods have been reported in the literature for quantitation of AMT in biological fluids, microbiological samples, and simultaneous estimation with other drugs and in pharmaceutical formulations.[6,7] However, majority of these methods requires high cost solvents and controlled conditions such as maintenance of column oven temperature, use of guard columns and organic modifiers, pre-column derivatization, etc. which makes them less meritorious for routine pharmaceutical analysis.

Therefore, the current study aims at the development of an optimized and validated reverse phase LC (RP-LC) method for estimation and dissolution studies of AMT in the *in-house* time-dependent release bilayer tablet formulations and marketed preparation.

Materials and Methods

AMT was obtained from Ranbaxy Laboratories Ltd. (Gurgaon, Haryana). The high performance LC (HPLC) grade methanol was purchased from SRL Ltd. (New Delhi, India). The ultra-purified HPLC grade deionized water was used throughout the chromatographic procedure, obtained from *in-house* Milli-Q® water purification system, M/s Millipore (Mumbai, India). Dibasic potassium hydrogen phosphate as buffer material and all other chemicals were of analytical grade and purchased from SD Fine Chemicals Ltd. (Mumbai, India).

Instrumentation

The chromatographic separation was obtained in reverse phase LC equipment (Shimadzu, Tokyo, Japan), coupled with quaternary pump (LC-10AVP), Rheodyne injector, ultraviolet (UV)-visible detector (SPD-10AVP), guard column, column oven and SCL-10AVP system controller. Isocratic elution was used throughout the study at a constant flow rate. The column oven temperature was maintained at a constant ambient temperature and the chromatographic separation was facilitated by employing Capacel Pak (C_{18}) Type MG (Shiseido, Tokyo, Japan) reversed phase column with 250 mm × 4.6 mm i.d. and 5 μm particle size. The chromatographic data acquisition was controlled by in built Class-VP 5.032 software (Shimadzu, Tokyo, Japan).

Preparation of time-dependent release bilayer tablets

A diverse variety of chronomodulated drug delivery systems or also referred to as time-dependent release systems are available including coated tablets, microspherical beads, pellets, capsular systems, etc. In the present studies, the time-dependent release bilayer tablets have been prepared owing the low production cost, less tedious unit operations, time economy and ease of scale-up.

The time-dependent release bilayer tablets employed in the present studies were comprised of a delayed release layer and a sustained release layer. The bilayer tablets containing divided dose of drug in the delayed and sustained release layer were prepared by direct compression technique. The delayed release granules were prepared by wet granulation technique. An accurately weighed quantity of AMT was taken in a mortar and granulating fluid containing Eudragit-L100 D55 (entericoated delayed release polymer) dissolved in acetone was used for granulation. The obtained wet mass was passed through the sieve (#BSS18) to obtain the granules. Magnesium stearate was added over dried granules for lubrication. The sustained release layer encompassing the powder blend of drug with HPMC K14 as the rate controlling polymer along with other excipients like Prosolv-HD60, Aerosil-200 and magnesium stearate were prepared by dry blending. Finally, both the layers were compressed into a single bilayer tablet at a fixed compression load of 15 kg/cm^2. Table 1 illustrates the formulation composition of a prototype time-dependent release bilayer tablet. The details about the formulation development of the aspects of the time-dependent release bilayer tablets has been discussed by Beg *et al.*[8]

Preparation of standard solution of AMT

Standard stock solution of AMT was prepared by dissolving 10 mg of AMT reference standard in a 10 mL volumetric flask containing 50:50% v/v mixture of methanol:Phosphate buffer to make a solution of 1000 μg/mL, which was further diluted to obtain the concentration 100 μg/mL.

Table 1: Composition of the prototype time-dependent release bilayer tablets of AMT

Ingredients	Quantity
Delayed release layer	
Amoxicillin	145
Eud-L100 D55	23
MCC-PH101	80
Mg. stearate	2
Total	250
Sustained release layer	
Amoxicillin	755
HPMCK4	50
Prosolv-HD60	91
Aerosil	6
Mg. stearate	8
Total	900

AMT – Amoxicillin trihydrate; Mg. stearate – Magnesium stearate; HPMC – Hydroxypropyl methylcellulose; MCC – Microcrystalline cellulose

Method development and validation

Different combinations of mobile phase were tried to achieve maximum chromatographic separation of AMT with efficient resolution. The mixture of methanol and 0.02 M phosphate buffer was used as the mobile phase due to highest solubility of the drug in these solvents. Various rational combinations of mobile phase mixtures in the range of 25:75% and 75:25% v/v with pH 3.5 at a flow rate of 1.0 mL/min and UV detection at 229 nm were used for attaining maximal chromatographic separation based on low tailing, higher peak resolution, higher theoretical plates, low peak asymmetry and higher assay. Further, the response surface methodology was employed using Box-Behnken experimental design for the systematic optimization of mobile phase ratio (X_1), pH (X_2) and flow rate (X_3) chosen as the independent variables (i.e., constraints) against the peak area (Y_1) and retention time (Y_2) as the dependent variables (i.e., response). The optimum mobile phase composition, pH and flow rate were selected based on the higher values for peak area and low retention time.[9] The developed method was validated for linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ) and system suitability. The developed mobile phase composition along with chromatographic conditions was used for quantification and dissolution studies.

Linearity

The linearity of the method was determined by preparing the serial dilutions of the pure drug. The standard solution of 100 µg/mL was prepared using optimized mobile phase composition and dilutions were made from the stock ranging between 1 and 25 µg/mL. The linearity was calculated from the concentrate versus peak area plot by residual analysis method by observing the values of regression coefficient (R^2).[10]

Accuracy

The accuracy of the method was determined by recovery from the test sample of 10 µg/mL concentration spiked with 50%, 100%, and 150% of additional AMT. Based on the percent recovery, the standard deviation (SD), relative SD (RSD) and standard error of mean (SEM) were calculated.

Precision

Precision (inter-day and intra-day) was determining by measuring the three different concentrations of the drug, i.e., 2, 8 and 16 µg/mL. The amount recovered was obtained, from which the SD, RSD and SEM were calculated.

LOD and LOQ

LOD and LOQ were determined by the SD ($S_{y/x}$) method. Blank samples were injected in triplicate and peak area was recorded. LOD and LOQ were determined from the slope (S) of the linearity plot and the SD of the response to the blank sample, $S_{y/x}$, by using the formula LOD = $3.3 \times S_{y/x}/S$ and LOQ = $10 \times S_{y/x}/S$.

System suitability

The system suitability was assessed by six replicate analyses of the drug at a concentration of 10 µg/mL and SD, % RSD, SEM were determined for both peak area and retention time.

In vitro drug release studies

In vitro drug release studies of *in-house* prepared time-dependent release bilayer tablets were performed in USP Type-1 dissolution apparatus (Electrolab, Mumbai, India) using 900 mL simulated gastric fluid for first 2 h followed by phosphate buffer (pH 6.8) (900 mL) for up to 16 h at 50 rpm/37 ± 0.5°C temperature. Aliquot samples (5 mL) were taken at periodic time intervals with replacement by fresh media, and filtered through 0.45 µm nylon filter (Millipore). The accurately measured 20 µL sample from each of the aliquots were injected and peak area was obtained. The unknown concentration of AMT release was calculated from the linear regression equation of the standard calibration curve to obtain the cumulative % drug release which was plotted against time. Similarly, the *in vitro* dissolution study was performed for marketed preparation, i.e., Amoxil® (Dabur, India), containing AMT equivalent to 500 mg. At specific time intervals, aliquots (5 mL) were collected, filtered and analyzed chromatographically to obtain the *in vitro* drug release.

Assay of AMT

Twenty tablets of each from the *in-house* prepared tablet formulations and marketed preparation containing AMT were taken in a mortar-pestle and crushed into fine powder. An accurately weighed amount of powder was transferred into a 50 mL volumetric flask, sonicated for 10 min and volume was adjusted up to 50 mL. The stock was suitably diluted to obtain the concentration 10 µg/mL, which was analyzed chromatographically to obtain assay in terms of percentage recovery.

Results and Discussion

The RP-HPLC method was finally developed for assay and dissolution studies of the AMT in the *in-house* formulations and marketed preparation. A mobile phase containing mixture of methanol and phosphate buffer (50:50% v/v) at a flow rate of 1 mL/min and pH 3.0 was obtained as ideal conditions for chromatographic separation by systematic optimization. Figure 1 depicts a typical chromatogram of standard solution of AMT having concentration 10 µg/mL. The retention time (R_t) of 3.992 min was found to be suitable for efficient time-economy based analysis.

The linearity range was determined by residual analysis with the help of linear regression. Linear residual plot between the observed responses (peak area) and residuals, which indicated that the concentration ranging between 2 and 16 µg/mL are within the ±5% limit of significance. The linear calibration plot of AMT was constructed and the regression coefficient (r^2) was found to be 0.999, as shown in Figure 2. The accuracy was calculated as percentage recovery, which was found to be ranging between 99.61% and 100.13%, as depicted in Table 2. Further, the values of % RSD (0.23-1.08%) were well within the limits (i.e., not more than 2%), indicated good percentage recovery of the method. Table 3 illustrates the data for inter-day and intra-day precision studies. The method showed good precision with % RSD values for inter-day and intra-day precision were in found to be ranging between 0.225-1.257% and 0.277-1.314%, respectively. LOD and LOQ were found to be 0.38 and 0.26 µg/mL, confirmed that the method is sensitive enough to detect the lowest quantity of analyte as possible. Table 4 shows the data for system suitability studies which revealed that the % RSD and SEM were within the limit.

The *in vitro* dissolution studies were performed in triplicate (n = 3), in two different dissolution media, i.e., initially for 3 h in 0.1 N HCl followed by phosphate buffer (pH 6.8) up to 16 h. As the prepared *in-house* formulation was found to contain a bilayer tablet containing a delayed release layer and a sustained release layer, hence dissolution was performed in two different media. However, the dissolution studies of the marketed preparation was carried out in phosphate buffer (pH 6.8) as the drug release was independent of pH of the dissolution medium, due to its immediate release profile of drug dissolution.

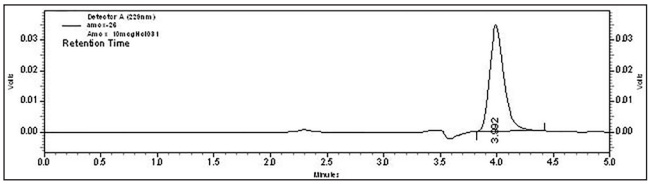

Figure 1: Chromatogram of pure drug amoxicillin trihydrate in the selected mobile phase composition

Table 2: Accuracy study

Sample	Level (%)	Concentration (µg/mL)	Amount recovered (µg/mL)	% recovery	% RSD
AMT	50	25	24.91	99.61	1.08
	100	50	50.03	100.06	0.37
	150	75	75.10	100.13	0.23

AMT – Amoxicillin trihydrate; RSD – Relative standard deviation

Table 3: Intra-day and inter-day precision study

Days	Nominal concentrations		
	2 µg/mL	8 µg/mL	16 µg/mL
Day 1			
Amount recovered	1.988	7.981	15.993
SD	0.025	0.032	0.036
% RSD	1.257	0.401	0.225
SEM	0.011	0.014	0.016
Day 2			
Amount recovered	1.974	8.014	15.994
SD	0.025	0.55	0.060
% RSD	1.266	0.686	0.375
SEM	0.011	0.024	0.027
Day 3			
Amount recovered	1.972	7.954	15.976
SD	0.028	0.020	0.037
% RSD	1.419	0.251	0.231
SEM	0.012	0.009	0.016

SD – Standard deviation; RSD – Relative standard deviation; SEM – Standard error of mean

Table 4: System suitability study

Parameters?	Amoxicillin (10 µg/mL)	
	Retention time (min)	Peak area
Mean (n=6)	3.925	304,517
SD	0.066	729.42
% RSD	1.69	0.239
SEM	0.027	297.78

SD – Standard deviation; RSD – Relative standard deviation; SEM – Standard error of mean

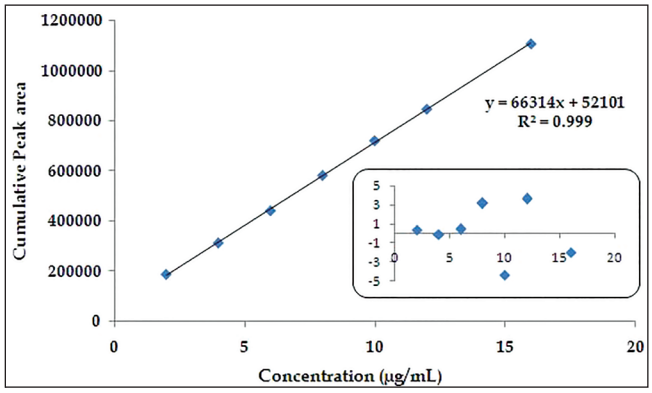

Figure 2: Linear calibration plot and residual plot of amoxicillin trihydrate

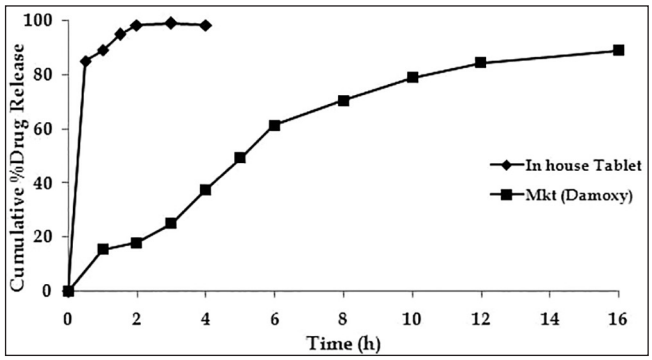

Figure 3: *In vitro* drug release profiles form the *in-house* tablet formulation and marketed preparation estimated using the developed chromatographic method

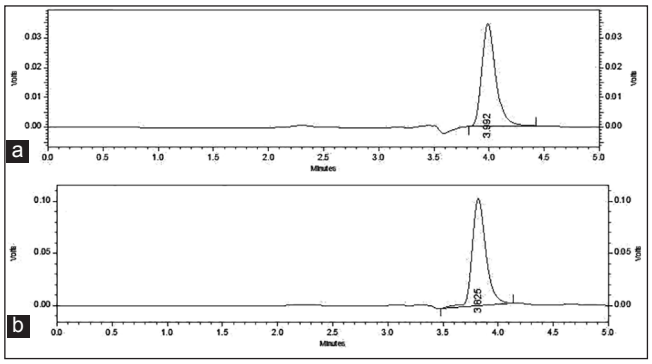

Figure 4: Chromatogram of amoxicillin trihydrate in the prepared *in-house* tablet formulation and marketed preparation, (a) *in-house* bilayer tablet formulation, (b) marketed preparation

Aliquots of the dissolution were collected and analyzed by the developed RP-HPLC method.

Figure 3 portrays the cumulative % drug release versus time (h) of the *in-house* time-dependent release bilayer tablet formulation and marketed preparation. The *in vitro* dissolution profile revealed a biphasic release of AMT, i.e., a sustained release profile of drug delivery with delayed burst release after a lag-time of 3 h. The marketed preparation, however, showed an immediate release effect of drug dissolution, i.e., more than 90% drug release was observed in 45 min. In case of *in-house* prepared tablet formulations, biphasic drug release was observed with 25% release in initial 3 h followed by 49% release in 5 h due to burst release from the delayed release layer and finally more than 85% drug release was observed at the end of 16 h. This confirmed the time-dependent drug release profile of the prepared *in-house* tablet formulations of AMT.

The assay of AMT in the prepared *in-house* tablet formulations and marketed preparation was obtained as mean AMT recovery from assay of *in-house* tablet formulations and marketed preparation were found to be 99.95% and 99.60%, respectively. Figure 4a and b depicts the chromatogram of the aliquots collected after dissolution of *in-house* and marketed formulations at different time points of dissolution.

Conclusions

The present studies successfully stated the suitability of the developed RP-LC method for quantification of analyte in the pharmaceutical formulations. The developed optimized mobile phase composition provides good linearity range rationally determined by residual analysis method. Further, the developed method had good accuracy, precision and low values of LOQ and LOD. The method was found to be suitable for routine pharmaceutical analysis of AMT. Conclusively the developed method could be used as a quality control tool in product development along with *in vitro* dissolution studies to control the variability among the development batches.

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