Validated Stability–Indicating LC Method for Estimation of Amoxicillin Trihydrate in Pharmaceutical Dosage Forms and Time-Dependent Release Formulations

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ABSTRACT
The objective of this study was to establish a validated stability-indicating LC method for routine analysis of amoxicillin trihydrate in bulk drug samples, different pharmaceutical formulations, and degradation kinetics of the drug under different ICH recommended stress conditions. Chromatographic separation was achieved by a Capcell Pak C18 column with 50:50% v/v methanol-0.02 M phosphate buffer as mobile phase having pH 3.5 and flow rate of 1.0 ml/min; with UV absorbance at 229 nm. The method was validated for system suitability, linearity, precision, accuracy, robustness, specificity and sensitivity. The drug was subjected to stress degradation by exposure to acid and alkaline hydrolysis, oxidation, and photodegradation. It was observed that peaks of all degradation products were well resolved from the pure drug with significantly different retention times, which indicated the specificity and stability-indicating properties of the method. When the utility of the method was verified by analysis of the drug in marketed formulations and in-house time-dependent release tablet formulations, the assay was found to be 99.6–100.4%. Statistical analysis proves that the method is repeatable, selective, and accurate for the estimation of amoxicillin trihydrate in bulk drug samples and also in pharmaceutical formulations.

KEYWORDS: Amoxicillin trihydrate; HPLC; forced degradation study; validation; specificity.

Introduction
Amoxicillin trihydrate (AMT) is oral semisynthetic penicillin structurally related to ampicillin. The presence of a benzyl ring in the side chain extends the antibacterial activity to Gram-negative bacteria (Brodgen et al., 1981; Dollery, 1998; Tomas, 1979a, b; Waxman, 1983). AMT is marketed in various forms such as tablets, capsules, dispersible tablets, syrups, and pediatric suspensions. Several chromatographic methods are reported for separation and quantitation of amoxicillin in biological fluids such as whole blood, plasma and serum, which require controlled temperature conditions (de Abreu, 2003; Numan et al., 2009; Nelis et al., 1992; Du et al., 2005; Dousa, 2005; de Pourcq et al., 1985; Farrel, 1982; Hernandez et al., 1999). Also some methods are focused on simultaneous estimation of AMT with other drugs (Injac et al., 2009; Nikam et al., 2009; Ashnagar, 2007; Foroutan, 2007; El-Shafie et al., 1996; Miyazaki et al., 1983; Wise et al., 1978; Wen et al., 2008; Wei et al., 2006). However, there is little information on stability–indicating analysis of AMT in pharmaceutical dosage forms (Raju et al., 2009; Hsu, 1992). These methods are found to be highly complex, use high cost solvents and associated with more number of operational variables which make them less acceptable. Therefore, we made an attempt to develop a new solvent system and a validated LC method with UV detection by the use of cost effective solvent methanol along with 0.02 M phosphate buffer. In the present study, we optimized a highly sensitive, economical, and simple assay for regular analysis of amoxicillin trihydrate in bulk drug samples and also in pharmaceutical formulations.

Materials and Methods
Drugs and Chemicals. AMT was obtained as gift sample from Ranbaxy Laboratories (Gurgaon, Haryana, India). HPLC grade methanol was purchased from E-Merck Ltd. (Mumbai, India). Buffer materials and all other chemicals were of analytical reagent grade. Ultra-purified HPLC grade water was obtained from Milli-Q® system (Millipore, USA) water purification unit.
Marketed products Damoxy® (Dabur, Mumbai, India), Amoxil® (Zydus Cadila, Mumbai, India) and prepared in-house time-dependent release matrix tablet formulations were used for assay studies.

**Instrumentation and Chromatographic Conditions.** Chromatographic separation was performed at an ambient room temperature, with Shimadzu LC equipment comprising quaternary LC-10AVP pumps, a SPD-10AVP programmable UV–visible variable–wavelength detector, a column oven, and a SCL-10AVP system controller. The chromatographic separation was achieved with Capacel Pak (Shiseido, Japan) C18 Type MG-reversed-phase column (250 mm × 4.6 mm i.d. with particle size of 5 µm). The mixture of methanol–0.02 M phosphate buffer (50:50% v/v, pH 3.5) was taken as mobile phase. The binary elution was performed at a flow rate of 1.0 ml/min with UV detection at 229 nm. Samples (20 µl) were injected by means of a Rheodyne injector fitted with a 20 µl loop. The data acquisition was controlled by use of Class-VP 5.032 (Shimadzu) software.

**Preparation of Standard Solution.** A stock solution of AMT was prepared by dissolving 10 mg of AMT reference standard in to a 10 ml volumetric flask and dissolved with mobile phase (methanol: phosphate buffer, 50:50 v/v, pH 3.5) to make a solution of 1000 µg/ml, which was further diluted into lower concentration as per the need.

**Method Development.** Previous literature reported about the use of different combinations of acetonitrile–phosphate buffer as an ideal mobile phase composition for chromatographic separation. There was no method found using the mobile phase composition containing methanol along with phosphate buffer for chromatographic elution. Apart from these, use of methanol in place of acetonitrile is a quite cost-effective approach. Hence several combinations of methanol-phosphate buffer (0.02 M), i.e. (25/75–75/25) were tried with pH ranging from 3.0–6.0 and flow rate ranging from 0.8–1.2 ml/min. The suitability of mobile phase was decided on the basis of the sensitivity of assay, suitability for stability studies, time required for analysis, peak parameter, and lower retention time, ease of preparation and use of available cost-effective solvents. Out of several tried combinations of mobile phase, binary combination of methanol-phosphate buffer (0.02 M) with composition 50:50% v/v, pH 3.5 provided higher elution of the AMT due to better chromatographic separation. Hence this was selected as final mobile phase combination for further studies.

**Validation Studies.** The linearity of the method was determined by diluting the standard stock solution to 2–16 µg/ml, chromatographing the solutions, and plotting peak area response against concentration. Precision was assessed by measurement of repeatability (intraday precision) by assay of three different concentrations of the drug (2, 8, and 16 µg/ml) at different time intervals in the same day and intermediate (interday) precision by repetition for three days as per ICH guidelines. The accuracy of the method was determined as recovery from 10 µg/ml standard solution spiked with 50%, 100%, and 150% additional AMT. Robustness was assessed by deliberately changing mobile phase flow rate and proportion of methanol to 1.0±0.2 ml/min and 50±2% (v/v), respectively. Detection limit (DL) and quantification limit (QL) were determined by the standard deviation (S₀$m$) method. Blank samples were injected in triplicate and peak area was recorded. DL and QL were determined from the slope (S) of the linearity plot and the standard deviation of the response to the blank sample, S₀$m$, by the use of formula DL = 3.3 x S₀$m$x/S and QL = 10 x S₀$m$x/S. 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. The acceptance criterion was ±2% for %RSD for peak area and retention time. Specificity of the method was determined by subjecting 10 µg/mL concentration of sample solution from reference standard, marketed formulations, inhouse preparation and accelerated degradation conditions in order to verify that none of the degradation products interfere with reference standard by using retention time, peak area, USP theoretical plate count and tailing factor as parameters. Selectivity of the proposed method was assessed by analyzing the mean % recovery from five different replicates having concentrations of 50 µg/ml.

**Stress Degradation Studies.** Stress degradation studies of the drug helps to identify the degradation products, stability of the molecule and specificity of the analytical procedures as per ICH-Q1A stability guidelines (ICH (Q2R1) 1995; ICH (Q1AR2) 2003). For stress testing, methanolic stock solution of AMT having concentration 10 µg/ml was prepared using mobile phase. To 15 ml of 10 µg/ml solution, 15 ml of 1 M HCl, 0.05 M aqueous NaOH, and 20% (v/v) H₂O₂ were added separately and heated under reflux for 2 h at 70 °C for identification of acid, alkaline and oxidative degradation, while separate portions of 15 ml solutions were exposed to artificial light (12,000 lux for 144 h; at 25 °C), and UV light (254 nm for 3 h) for evaluation of photodegradation. At last all samples from degradation studies were injected and analysed to assess specificity.

**Degradation Kinetics Assessment.** For the assessment of degradation kinetics of AMT under acidic and alkaline condition, 15 ml of 1 M HCl and 1 M NaOH solution were added to 15 ml of 10 µg/ml methanolic solution of AMT. They were heated under reflux at 40°C, 50°C and 60°C (313, 323, and 333 K) for 1 hours, 2 hours and 3 hours. After known time intervals, 20 µl solutions from each temperature condition were analysed for the concentration of drug remained. Data were further processed to identify the degradation kinetic and degradation rate constant at all temperature conditions. The degradation rate constant at room temperature (K₀), half-life and shelf-life of AMT at both acidic and basic conditions were calculated with the help of Arrhenius equation as per ICH guidelines.
Stability Studies of the Pharmaceutical Dosage Forms. An accurately weighed amount equivalent to 10 mg AMT was taken from marketed formulations (Damoxy® and Amoxil®) and also from in house time-dependent release tablet formulation and transferred to a 10 mL volumetric flask and diluted with the mobile phase. Extraction was performed by mixing, sonication, and centrifugation at 2000 rpm for 5 minutes. The samples were filtered, suitably diluted to furnish 10 µg/ml solution, and these were analyzed at 229 nm under developed LC method for assay. For stability studies, commercial tablets and in house time-dependent release tablet formulations were stored in HDPE bottle at 40 ± 20°C/75 ± 5% RH as recommended by ICH in Q1A (R2) (ICH (Q1AR2) 2003). Samples stored under these conditions, designed to accelerate degradation, were analyzed for drug content at four different time points (0, 1, 3, and in 6 months) including initial and final time points.

Results and Discussion

Method Development. An innovative approach was employed to develop a newer solvent system using combination of methanol-phosphate buffer at different ratios were investigated as the mobile phase. At all the combinations, low theoretical plate value, poor resolution, more than two tailing factor and high retention time were found. The developed method provides better peak parameter with suitable retention time, improves the separation of analyte peak from its impurity and also improves peak parameters such as tailing factor, acceptable retention time, and theoretical plate. pH of the phosphate buffer play crucial role in peak resolution and sensitivity. Optimum pH is highly necessary to retain the β-lactam ring of AMT as increase in hydrogen ion concentration to very high level leads to degradation of β-lactam ring. Therefore, a range of pH values from 3.0 – 6.0 were tried and better sensitivity was found at pH 3.5. Out of several combinations of methanol and phosphate buffer tested, the binary proportion of 50:50 (v/v) of pH 3.5 showed sharp peak, suitable retention time (Rt=3.99 minutes and SD 0.066) and good asymmetry.

Method Validation. Linearity of the method was observed at 2-16 µg/ml with linearity coefficient (R²) was 0.997. The regression equation for the calibration plot was y = (63271x + 27616) (n=6; at 229 nm). There was no significant difference between the slopes of calibration plots prepared on different days (ANOVA, P>0.05). The method was precise with %RSD for repeatability and intermediate precision were in the range of 0.225–1.257% and 0.277–1.314%, respectively. The accuracy, calculated as percentage recovery was between 99.61–100.13%. Low values of the %RSD (0.23–1.08%), SEM (0.042–0.301) and good percentage recovery were indicative of the excellent accuracy of the method.

Statistical validation (student’s t-test) revealed no significant difference (P<0.05) between results from study of accuracy and the theoretical concentration. When the composition of the mobile phase and flow rate was deliberately changed, the peak area and retention time obtained on analysis of 10 µg/ml standard solutions were in the ranges between 452036±2,357.29 to 494279±13,892.72 and 3.992 ± 0.046 to 3.934±0.025 min, respectively. Low RSD values in the range of 0.23–1.08% for these results substantiate the consistency of the method. Hence the proposed method was robust and the optimized values for mobile phase composition, flow rate and pH were 50: 50% v/v, 3.5 and 1 ml/min respectively. D1 and Q1 were 0.38 and 0.26 µg/ml, indicating the method is sensitive. Specificity of the method was determined by comparative study of samples obtained from reference standard, marketed formulations, inhouse preparation and stress degradation study showed that different parameters like Rt (3.717-3.992), peak area (378140–494279), USP plate count (3635.31–4726.80) and tailing factor (0.77–1.33) were in the acceptable limit (Table 1). This confirms that none of the degradation product interferes with drug. Apart from these the method was showed better selectivity with good mean %recovery (99.93%) and RSD (0.260).

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Retention time</th>
<th>Area</th>
<th>USP plate count</th>
<th>Tailing factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMT-Standard</td>
<td>3.992</td>
<td>495380</td>
<td>4726.80</td>
<td>1.33</td>
</tr>
<tr>
<td>M1</td>
<td>3.925</td>
<td>480386</td>
<td>4394.92</td>
<td>0.89</td>
</tr>
<tr>
<td>M2</td>
<td>3.733</td>
<td>420356</td>
<td>4293.11</td>
<td>0.85</td>
</tr>
<tr>
<td>In house</td>
<td>3.825</td>
<td>494279</td>
<td>4722.04</td>
<td>1.17</td>
</tr>
<tr>
<td>AMT-Acid</td>
<td>3.675</td>
<td>392217</td>
<td>4348.65</td>
<td>1.29</td>
</tr>
<tr>
<td>AMT-Base</td>
<td>3.633</td>
<td>424241</td>
<td>3635.31</td>
<td>1.30</td>
</tr>
<tr>
<td>AMT-Oxidation</td>
<td>3.692</td>
<td>460354</td>
<td>3704.19</td>
<td>0.77</td>
</tr>
<tr>
<td>AMT-Artificial light</td>
<td>3.708</td>
<td>387638</td>
<td>3780.14</td>
<td>1.08</td>
</tr>
<tr>
<td>AMT-UV</td>
<td>3.717</td>
<td>378140</td>
<td>3688.95</td>
<td>1.25</td>
</tr>
</tbody>
</table>

M1, M2- Two different marketed tablet formulation

Stress Degradation Studies. The method was capable of separating degradation products in the presence of AMT. Well separated analyte and degradation peaks were obtained after all degradation experiments. Acidic and alkaline stress causes prominently 20–25% and 15–20% degradation, while oxidation and photolysis showed a very little degradation in the range of 5–7% respectively. All parameters related to different degradation conditions are given in Table 1. These parameters confirm that the developed method was sensitive enough to resolve all degradation products from reference standard. The stress degradation study indicated the method enables selective and specific differentiation of the degradation peaks from the analyte peak.

Degradation Kinetics Assessment. After degradation study, various plots were made to know the degradation kinetics of AMT. The higher value for linearity coefficient obtained by plotting time vs. log % drug remaining at each temperature conditions (313, 323 and 333 K) showed first-order degradation kinetics. The first-order
rate constant (K), half-life (t\(_{1/2}\)), and shelf life (t\(_{90}\)) were determined at each temperature for acid and alkaline degradation (Table 2). Afterward the first-order kinetics data were fitted into the Arrhenius equation and a plot was made between log K vs. reciprocal of temperature (1/T) showed a linear plot. The degradation rate constant (K\(_{25}\)) at room temperature (25\(^\circ\)C) for both acidic and base degradation were 0.102 and 0.095 per hour, respectively. The first-order t\(_{1/2}\) and t\(_{90}\) under acidic conditions at 25\(^\circ\)C were 6.794 and 1.029 hours; for base degradation 7.294 and 1.105 hours, respectively. This revealed that AMT is highly susceptible to acid degradation, with shorter K\(_{25}\), t\(_{1/2}\), t\(_{90}\) than base degradation.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Temp (K)</th>
<th>R(^2)</th>
<th>K (h(^{-1}))</th>
<th>t(_{1/2}) (h)</th>
<th>t(_{90}) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>In 1M HCl</td>
<td>313</td>
<td>0.997</td>
<td>0.278</td>
<td>2.493</td>
<td>0.377</td>
</tr>
<tr>
<td>50</td>
<td>323</td>
<td>0.992</td>
<td>0.351</td>
<td>1.974</td>
<td>0.299</td>
</tr>
<tr>
<td>60</td>
<td>333</td>
<td>0.975</td>
<td>0.485</td>
<td>1.428</td>
<td>0.216</td>
</tr>
<tr>
<td>In 1M NaOH</td>
<td>313</td>
<td>0.996</td>
<td>0.152</td>
<td>4.559</td>
<td>0.690</td>
</tr>
<tr>
<td>50</td>
<td>323</td>
<td>0.991</td>
<td>0.228</td>
<td>3.039</td>
<td>0.461</td>
</tr>
<tr>
<td>60</td>
<td>333</td>
<td>0.989</td>
<td>0.341</td>
<td>2.032</td>
<td>0.307</td>
</tr>
</tbody>
</table>

Stability Studies of Pharmaceutical Dosage Forms. The proposed LC method was found to be sensitive and specific for the quantitative determination of AMT. The mean AMT recovery from assay of initial samples of Damoxy\(^\circ\)®, Amoxil\(^\circ\)® and in-house tablet formulations were found to be 100.41%, 99.60% and 99.95%, respectively. The marketed as well as in-house formulations stored at different accelerated stability temperature conditions showed that the proposed method can be applied for the estimation of recovered drug and degradation products in pharmaceutical dosage forms.

The drug content in all the stability samples was found in the range of 99.41—101.39% during stress degradation studies. High assay values and low SD of AMT in marketed and in-house time-dependent release tablet formulations suggested that the method is well suited for regular analysis of drug. Chromatograms for AMT shown in Figure 1 (a-d) revealed that none of the tablet excipients were found to interfere with the analyte peak. No significant change was observed in Rt (3.733-3.925 min, P>0.05) values for analyte peaks and no other peaks were co-eluting with analyte. In all samples under test, the student’s t-test showed P>0.05 between the drug content of the samples exposed to accelerated storage conditions (40°C/75% RH) as compared to initial drug.
content. The drug content of stability samples are within the limit of ICH recommendation. This indicated that proposed method can be successfully applied for the stability studies and prediction of shelf life of AMT in dosage forms.

In conclusion, a validated stability-indicating LC assay method was developed for AMT, applying the forced degradation protocol recommended by ICH. The developed method is simple, accurate, precise, specific, and could clearly separate drug from degradation products. Hence it can be used for routine analysis of AMT in bulk samples as well as for standard assay and stability studies of pharmaceutical formulations.

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References


ICH draft guidelines on validation of analytical procedures: text and methodology (Q2R1), IFPMA, Switzerland, 1995.


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