DEVELOPMENT AND VALIDATION OF Q-ABSORBANCE RATIO METHOD FOR SIMULTANEOUS ESTIMATION OF AMOXICILLIN AND PROBENECID IN PHARMACEUTICAL DOSAGE FORM

P. Himani*, S. Dhruvi, P. Bhagirath, B. Ankita

Sat Kaival College of Pharmacy, Paramguru Pathsala Complex, Sarsa Cross Road, At & Po-Sarsa, Ta & Dist-Anand, Gujarat-388365, India

ABSTRACT

New spectrophotometric Q-Absorbance Ratio method has been developed for the simultaneous determination of Amoxicillin and Probenecid in oral dosage form. The various parameters, such as linearity, precision, accuracy, limit of detection and limit of quantitation were studied according to International Conference on Harmonization (ICH) guidelines. The Iso-absorptive point was found to be 233 nm. Calibration curves were linear over a concentration range of 5-20 µg/ml for Amoxicillin and 5-20 µg/ml for Probenecid respectively. Accuracy of method was determined through recovery studies which were found 99 - 100.89 % for Amoxicillin and 98.67 - 100.44 % for Probenecid. Method was found to be reproducible with relative standard deviation (RSD) for intra and interday precision to be < 1.5% over the said concentration range. The proposed method is found to be highly simple, sensitive, precise and accurate.

Keywords: Amoxicillin, Probenecid, Q-Absorbance Ratio method, Analytical method Validation, Methanol.

1 INTRODUCTION

Amoxicillin (AMX), an acid stable, semi-synthetic drug belongs to a class of antibiotics called the Penicillins. Chemically AMX is (2S,5R,6R) 6[(2R) 2 amino 2(4hydroxyphenyl)acetyl] amino]3, 3 dimethy l7oxo 4thia 1 azabic yclo [3.2.0] heptanes 2 carboxylicacid. It is
listed in a number of Pharmacopoeias. AMX monograph is available in United States, British and Indian pharmacopoeia\textsuperscript{[1-4]}.

![Chemical structure of Amoxicillin](image1)

**Chemical structure of Amoxicillin**

Probenecid (PRB) is a uricosuric agent used in gout therapy. Chemically PRB is 4-[(Dipropyl-amino) Sulphonyl] benzoic acid. Probenecid is soluble in alcohol (1 in 25), acetone (1 in 12) and insoluble in water\textsuperscript{[5]}.

![Chemical structure of Probenecid](image2)

**Chemical structure of Probenecid**

Amoxicillin and Probenecid tablet is used for treatment for uncomplicated gonorrhea\textsuperscript{[6]}.

Literature review reveals that there is no such reported method has been found for estimation of AMX and PRB in bulk and combined dosage form. The present study aim at the development of simple, rapid, accurate and sensitive method for simultaneous estimation of AMX and PRB in combined dosage form elations by Q-Absorbance Ratio method.

2 MATERIALS AND METHODS

2.1 Apparatus and Instrument

- Double beam UV-visible spectrophotometer (Shimadzu, model 1800) having two matched
quartz cells with 1 cm light path.

- Electronic analytical balance, Shimadzu BL-220H
- pH meter, LI-610
- Volumetric flask – 10, 25, 50, 100 ml (Durasil)
- Pipettes – 1, 2, 5, 10 ml

All instruments and glasswares were calibrated.

2.2 Reagents and Material

- AMX (Gita laboratory)
- PRB (Gita laboratory)
- Methanol AR (Merck Pvt. Ltd)
- Combined tablet formulation (Moxylong tablet) was procured from local market.

2.3 Preparation of standard stock solution

10 mg of each AMX and PRB was added in 100 ml volumetric flask separately and dissolved with methanol. Volume was made with methanol to get final concentration of 100 µg/ml of each.

2.4 Preparation of working standard solution of Amoxicillin and Probenecid

Suitable aliquots of above solution were diluted up to the mark with methanol to get the concentration range of 5, 7.5, 10, 15, 20 µg/ml for AMX and PRB.

2.5 Preparation of sample solution (Tablet)

**Formulation:** Label claim for content drug is as follow

- Amoxicillin – 250 mg
- Probenecid – 250 mg

Twenty tablets were finely powdered. A quantity of powder equivalent to 10 mg was weighed and transferred to 100 ml volumetric flask. 60 ml methanol was added to the same flask and sonicated for 15 min. The volume was made up to 100 ml with methanol. The solution was first filtered using whatmann filter paper No. 41 and then through 0.45 µ filter paper in order to remove the excipients. After filtration, aliquots solutions were prepared by taking 1ml sample stock solution. Volume was made up to 10 ml with methanol to produce of 10 µg/ml AMX and 10 µg/ml of PRB in final solution.
2.6 Procedure for determination of wavelength for measurement
1.0 ml of working standard stock solution of AMX (100 µg/ml) and 1.0 ml of working standard stock solution of PRB (100 µg/ml) were pipette out into two separate 10 ml volumetric flask and volume was adjusted to the mark with methanol to get 10 µg/ml of AMX and 10 µg/ml of PRB. Each solution was scanned between 200 - 400 nm against methanol as a reagent blank. Isoabsorptive point was selected from the overlay spectra of AMX and PRB.

2.7 Calibration curve for Amoxicillin and Probenecid
Calibration curve for AMX and PRB consists of different concentrations of standard AMX and PRB solutions ranging from 5 - 20 µg/ml. The solutions were prepared by pipetting out 0.5, 7.5, 1.0, 1.5 and 2.0 ml of the working standard solution of AMX and PRB (100 µg/ml) into series of 10 ml volumetric flasks and the volume was adjusted to mark with methanol. The absorbance of the solutions was measured at 233 nm (λ1) and 278 nm (λ2) against methanol as a reagent blank. Calibration curve was plotted at both wavelengths.

2.8 Method Validation
Limit of Detection and Limit of Quantitation
Calibration curve was repeated for 6 times and the standard deviation (SD) of the intercepts was calculated then LOD and LOQ was calculated as follow from the formula.

\[ \text{LOD} = \frac{3.3 \times SD}{\text{Slope}} \]
\[ \text{LOQ} = \frac{10 \times SD}{\text{Slope}} \]

Where, SD = the standard deviation of Y- intercept of 6 calibration curves.
Slope = the mean slope of the 6 calibration curves.

(Results are shown in section 7.1.3.1)

Simultaneous estimation of AMX and PRB in marketed formulation
The synthetic mixture of AMX and PRB was prepared in ratio of 10:10. Accurately weighed 10 mg of AMX and 10 mg of PRB were transferred to 100 ml volumetric flask, and 100 ml of methanol was added. This solution was filtered through the whatmann filter paper No. 41 and residues were washed with methanol. The filtrate and washing were combined and volume of solution was made up to 100 ml with methanol. 0.1 ml from above stock solution is transferred to 10 ml volumetric flask and dilute to 10 ml with methanol. 0.1 ml of this solution was further diluted up to 10 ml in 10 ml volumetric flask to get final concentration as
10 ppm of AMX and 10 ppm of PRB. Absorbance of the resulting solution was measured at 278 nm and 214 nm against methanol.

The concentration of AMX and PRB can be calculated as,

For AMX

\[ C_x = \frac{Q_m - Q_y}{Q_x - Q_y} \times \frac{A_1}{a_1} \]

For PRB

\[ C_y = \frac{Q_m - Q_x}{Q_y - Q_x} \times \frac{A_1}{a_2} \]

Where,

\[ Q_0 = \frac{\text{Absorbance of sample at 214nm}}{\text{Absorbance of sample at 278 nm}} \]

\[ A_x = \frac{\text{Absorptivity of AMX at 214 nm}}{\text{Absorptivity of PRB at 278nm}} \]

\[ A_y = \frac{\text{Absorptivity of AMX at 214 nm}}{\text{Absorptivity of PRB at 278nm}} \]

\[ A_1 = \text{Absorbance of sample at isoabsorptive point, } a_1 \text{ and } a_2 = \text{Absorptivities of AMX and PRB respectively at isoabsorptive point.} \]

3 RESULTS AND DISCUSSION

Method development by Q-Absorbance Ratio

Determination of wavelength for AMX and PRB

![Fig. 1 Q- Absorbance Ratio spectrum overlay of AMX and PRB](image-url)
Method Validation
Linearity
Aliquots of 0.5, 0.75, 1.0, 1.5 and 2.0 ml of working standard solutions of AMX and PRB (100 µg/ml) were transferred to a series of 10 ml volumetric flask. The volume was adjusted up to mark with methanol to get 5, 7.5, 10, 15 and 20 µg/ml solution of AMX and PRB. The calibration curves of the area under curve Vs concentration were recorded for AMX and PRB. Result is shown in (Table 1-2).

Table 1 Linearity data for AMX at 214 nm and 278 nm in methanol

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Absorbance (233 nm)</th>
<th>Concentration (µg/ml)</th>
<th>Absorbance (278 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.175</td>
<td>5</td>
<td>0.334</td>
</tr>
<tr>
<td>7.5</td>
<td>0.262</td>
<td>7.5</td>
<td>0.495</td>
</tr>
<tr>
<td>10</td>
<td>0.354</td>
<td>10</td>
<td>0.643</td>
</tr>
<tr>
<td>15</td>
<td>0.524</td>
<td>15</td>
<td>0.951</td>
</tr>
<tr>
<td>20</td>
<td>0.669</td>
<td>20</td>
<td>1.266</td>
</tr>
</tbody>
</table>

Correlation coefficient : 0.9981
Intercept : 0.0158
Slope : 0.0331
Regression Equation : y=0.0331x+0.0158
LOD: 1.574
LOQ: 4.77

Correlation coefficient : 0.9982
Intercept : 0.0088
Slope : 0.034
Regression Equation : y=0.034x+0.0088
LOD: 0.854
LOQ: 2.588

Fig. 2 Calibration curve for AMX at 233nm
Fig. 3 Calibration curve for AMX at 278nm

Table 2 Linearity data for PRB at 233 nm and 278 nm in methanol

<table>
<thead>
<tr>
<th>PRB</th>
<th>Concentration (µg/ml)</th>
<th>Absorbance (233 nm) (1)</th>
<th>Concentration (µg/ml)</th>
<th>Absorbance (278nm) (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>0.172</td>
<td>5</td>
<td>0.220</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>0.267</td>
<td>7.5</td>
<td>0.332</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.348</td>
<td>10</td>
<td>0.432</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.515</td>
<td>15</td>
<td>0.639</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.662</td>
<td>20</td>
<td>0.859</td>
</tr>
</tbody>
</table>

Correlation coefficient : 0.9984  
Correlation coefficient : 0.9997  
Intercept : 0.0183  
Intercept : 0.0101  
Slope : 0.0326  
Slope : 0.0423  
Regression Equation : y = 0.0326x + 0.0183  
Regression Equation : y = 0.0423x + 0.0101  
LOD: 1.852  
LOD: 0.7879  
LOQ: 5.613  
LOQ: 2.387

Discussion: AMX and PRB were given linear response from 5-15 µg/ml and 5-15 µg/ml in Q Absorbance Ratio method.

Fig. 4 Calibration curve for PRB at 233nm
Accuracy

The accuracy of an analytical method is the closeness of the test results to the true value. It has been determined by application of the analytical procedure to recovery studies, where known amount of standard AMX and PRB (80%, 100%, and 120%) was spiked into the pre-analyzed amount of formulation. Result is shown in (Table 3-4).

Table 3 % Recovery of AMX

<table>
<thead>
<tr>
<th>Amount of PAM in Sample (µg/ml)</th>
<th>Amount of Std. AMX added (µg/ml)</th>
<th>Total amount of AMX (µg/ml)</th>
<th>Total amount of AMX recovered (µg/ml)</th>
<th>% Recovery</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4</td>
<td>9</td>
<td>3.96</td>
<td>99.00</td>
<td>1.41</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>10</td>
<td>4.97</td>
<td>99.53</td>
<td>1.34</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>11</td>
<td>6.08</td>
<td>100.89</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Table 4 % Recovery of PRB

<table>
<thead>
<tr>
<th>Amount of PRB in Sample (µg/ml)</th>
<th>Amount of Std. PRB added (µg/ml)</th>
<th>Total amount of PRB (µg/ml)</th>
<th>Total amount of PRB recovered (µg/ml)</th>
<th>% Recovery</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4</td>
<td>9</td>
<td>3.94</td>
<td>98.67</td>
<td>0.64</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>10</td>
<td>5.02</td>
<td>100.40</td>
<td>0.72</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>11</td>
<td>6.02</td>
<td>100.44</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Discussion: Result obtained reveals that % recovery of AMX and PRB was within acceptance criteria given in ICH i.e. 98-102%
Precision

Interday precision

Standard solutions containing 5, 10 and 15 µg/ml AMX and PRB were analyzed on three different days as per the procedure. Chromatogram of each sample was taken. SD and %RSD were calculated. Result is shown in (Table 5-6).

Table 5 % Interday Precision data for AMX of 233 nm and 278 nm

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Absorbance at 233 nm □1</th>
<th>Absorbance at 278 nm □2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD %</td>
<td>% RSD</td>
</tr>
<tr>
<td>5</td>
<td>0.173 ± 0.002</td>
<td>1.156</td>
</tr>
<tr>
<td>10</td>
<td>0.352 ± 0.004</td>
<td>1.279</td>
</tr>
<tr>
<td>20</td>
<td>0.665 ± 0.003</td>
<td>0.451</td>
</tr>
</tbody>
</table>

Table 6 % Interday Precision data for PRB of 233 nm and 278 nm

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Absorbance at 214 nm □1</th>
<th>Absorbance at 278 nm □2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD %</td>
<td>% RSD</td>
</tr>
<tr>
<td>5</td>
<td>0.476 ± 0.002</td>
<td>1.504</td>
</tr>
<tr>
<td>10</td>
<td>0.344 ± 0.003</td>
<td>0.872</td>
</tr>
<tr>
<td>20</td>
<td>0.665 ± 0.003</td>
<td>0.527</td>
</tr>
</tbody>
</table>

Intraday precision

Standard solutions containing 5, 10 and 15 µg/ml AMX and PRB were analyzed 3 times on the same day as per the procedure. Chromatogram of each sample was taken. SD and %RSD were calculated. Result is shown in (Table 7-8).

Table 7 % Intraday Precision data for AMX of 233 nm and 278 nm

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Absorbance at 233 nm □1</th>
<th>Absorbance at 278 nm □2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD* %</td>
<td>% RSD</td>
</tr>
<tr>
<td>5</td>
<td>0.175 ± 0.003</td>
<td>1.854</td>
</tr>
<tr>
<td>10</td>
<td>0.355 ± 0.003</td>
<td>0.762</td>
</tr>
<tr>
<td>20</td>
<td>0.653 ± 0.004</td>
<td>0.608</td>
</tr>
</tbody>
</table>

Table 8 % Intraday Precision data for PRB of 233 nm and 278 nm

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Absorbance at 233 nm □1</th>
<th>Absorbance at 278 nm □2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD* %</td>
<td>% RSD</td>
</tr>
<tr>
<td>5</td>
<td>0.173 ± 0.003</td>
<td>1.854</td>
</tr>
<tr>
<td>10</td>
<td>0.347 ± 0.002</td>
<td>0.762</td>
</tr>
<tr>
<td>20</td>
<td>0.664 ± 0.004</td>
<td>0.608</td>
</tr>
</tbody>
</table>

Discussion: Result obtained reveals that SD and % RSD of AMX and PRB was within acceptance criteria given in ICH i.e. less than 1 and less than 2 respectively. So, the proposed method for estimation of AMX and PRB is précised in nature.
Quantitative estimation of Amoxicillin and Probenecid marketed formulation

The proposed method was evaluated in the assay of tablet formulation containing AMX and PRB. Three replicate determinations were carried out on tablets. % assay found was 96.96 % for AMX and that for PRB was 98.28 %. Result is shown in (Table 9).

Table 9 Assay of AMX and PRB

<table>
<thead>
<tr>
<th>Marketed formulation</th>
<th>Label claim(mg)</th>
<th>Mean % Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMX</td>
<td>PRB</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>250</td>
</tr>
</tbody>
</table>

**Discussion:** % Assay of AMX and PRB was found in an acceptance limit so this method could be used for analysis of this combination.

4 CONCLUSIONS

The method described enables the quantification of AMX and PRB in combined tablet dosage form. The validation data demonstrate good precision and accuracy, which prove the reliability of the proposed method. This method was based on the determination of graphical absorbance at two wavelengths, one being Isoabsorptive point for the two drugs (233 nm) and the other being the λmax of Probenecid (278 nm).

Hence, this Q-Absorbance Ratio method can be used routinely for quantitative estimation of both components in solid oral dosage form.

5 ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Gita laboratory (Ahmedabad, India) for providing gift sample of AMX and PRB respectively. The authors are grateful to Sat Kaival College Of Pharmacy, Sarsa for continuous support and guidance.

6 REFERENCES