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Research Article

Development and Validation of Analytical Method for Simultaneous Estimation of Amoxicillin and Probenecid in Bulk and Tablet Dosage form using HPLC

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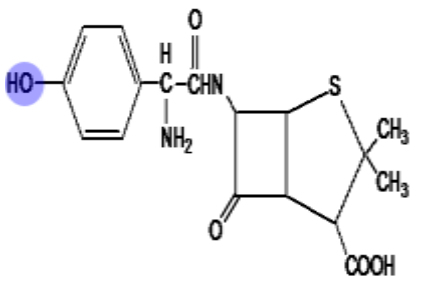
Abstract: A simple Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) method is reported for the simultaneous determination of Amoxicillin and Probenecid in oral dosage forms. Investigated drugs were resolved on BDS hypersil C_{18}, (250mm × 4.6mm, 5µ (particle size) reverse phase column, utilizing a mobile phase of 0.02 M KH_2PO_4 buffrer (pH-3.5) : acetonitrile (65:35v/v). Mobile phase was delivered at the flow rate of 1.0 ml/min. Ultra violet detection was carried out at 228nm. Separation was completed within 10 minutes. Calibration curves were linear with correlation coefficient 0.999 and 0.999 over a concentration range of 5-15 µg/ml for Amoxicillin and 5-15 µg/ml for Probenecid respectively. Recovery was between 99.904 - 99.745% and 99.368 - 99.543% for Amoxicillin and Probenecid respectively. Accuracy of method was determined through recovery studies which were found 99.57-99.90% for AMX and 99.36 – 99.54% for PRB. 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.
Keywords: Amoxicillin, Probenecid, KH_2PO_4 buffrer (pH-3.5), Acetonitrile, Reverse Phase High Performance Liquid Chromatography (RP-HPLC).

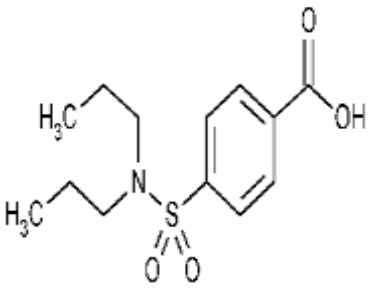
INTRODUCTION

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 azabicyclo [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 [1-3].

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 [4-7].



Amoxicillin



Probenecid

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 aimed at the development of simple, rapid, accurate and sensitive method for simultaneous estimation of AMX and PRB in combined dosage formulations by Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method.

MATERIALS AND METHODS

Reagents

AMX and PRB were kindly supplied as gift samples from Gita laboratory, Ahmedabad. Methanol (HPLC grade) (Merck), Potassium dihydrogen

phosphate (AR grade) (Merck), Orthophosphoric acid (AR grade) (Merck), Acetonitrile (HPLC grade) (Merck), Water (HPLC grade) and Moxylong tablet (Cadila Pharmaceuticals, Ahmedabad).

Equipments

HPLC was performed using a SPD-20AT, Shimadzu consisting of a pump LC20 AT, Rheodyne sample injection port with 20 µl loop, UV detector SPD-20AT and column used was BDS hypersil C_{18} (250mm × 4.6mm, 5µ(particle size), Thermo scientific. Weighing was done on AX200 balance. Delux 101 pH meter was used for checking and adjusting pH. All calibrated glasswares were used for the study.

Preparation of standard stock solution

10 mg of each AMX and PRB was added in 10 ml volumetric flask separately and dissolved with methanol. Volume was made with methanol to get final concentration of 1000µg/ml of each.

Preparation of buffer

Weigh 2.72gm KH_2PO_4 in to a 1000 ml beaker, dissolve and diluted to 1000 ml with HPLC water. pH of the solution was adjusted to 3.5 with orthophosphoric acid (1%).

Preparation of mobile phase

Mix a mixture of above 650 ml of 0.02 M KH_2PO_4 buffrer (pH-3.5) and 350 ml of acetonitrile (65:35 v/v) was prepared, filtered through 0.45µm membrane filter and sonicated on ultra sonic bath.

Preparation of solutions for calibration curve

Standard stock solution of AMX and PRB was diluted as 0.1ml to 10 ml with mobile phase to produce (10 µg/ml). These solutions were further diluted to get solutions of concentrations 5, 7.5, 10, 12.5, 15 µg/ml of each.

Procedure for Sample Preparation from marketed formulation

Label claim: AMX - 250mg
PRB - 250mg
Mfg By: Zydus, Cadila Pharmaceuticals , Ahmedabad,

Twenty tablets, each containing 250 mg AMX and 250 mg PRB were finely powdered. A quantity of powder equivalent to 10 mg AMX was weighed and transferred to 100 ml volumetric flask. 60 ml mobile phase was added to the same flask and sonicated for 15 minutes. The volume was made up to 100 ml with mobile phase. The solution was first filtered using whatmann filter paper No. 41 and then through 0.45µ 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 mobile phase to produce of 10 µg/ml AMX and 10 µg/ml of PRB in final solution.

Dilutions for precision studies

Precision of the method was checked by system precision and repeatability (Intraday and Interday studies). In system precision 6 replicates of mixed standard (containing AMX 10µg/ml and PRB 10µg/ml) were used. Repeatability was done by using 3 replicate readings at 3 concentration levels. For Intraday variability trials were taken in a day and for Inter day variability studies were done on 3 consecutive days. Concentration levels used for AMX and PRB were 5, 10, 15 µg/ml.

Dilutions for Recovery study

To study accuracy of the method, recovery study was carried out by addition of standard drug solution to sample at 3 different levels, 80%, 100% and 120% of the test concentration (test concentration is 10µg/ml for AMX and 10 µg/ml for PRB).

Robustness study

Robustness of the method was determined by small, deliberate changes in flow rate, mobile phase ratio and pH of mobile phase. Flow rate was changed to 1 ± 0.2 ml/min. The mobile phase ratio was changed to 65:35 ± 2, pH of mobile phase was changed to 3.5 ± 0.2.

LOD and LOQ determination

Limit of detection can be calculated by using following formula

$$\mathbf{LOD = 3.3\ \sigma/S}$$

Limit of quantitation can be calculated based on standard deviation of the response and the slope.

$$\mathbf{LOQ = 10\ \sigma/S}$$

Where σ = Standard deviation of the response
S = Slope of the calibration curve

RESULTS AND DISCUSSION

Method Development

The solutions of Amoxicillin (AMX) and Probenecid (PRB) working standards were injected into the HPLC system and run in different solvent systems as mobile phases. Different mobile phases containing water, methanol, acetonitrile, Buffers (phosphate) in different proportions were tried. Finally 0.02 M KH_2PO_4 buffrer (pH-3.5): acetonitrile (65:35 v/v) was selected as an appropriate mobile phase which gave good resolution and acceptable peak parameters for both AMX and PRB. Representative chromatogram of mixed standard of AMX and PRB is shown in Fig. 1.

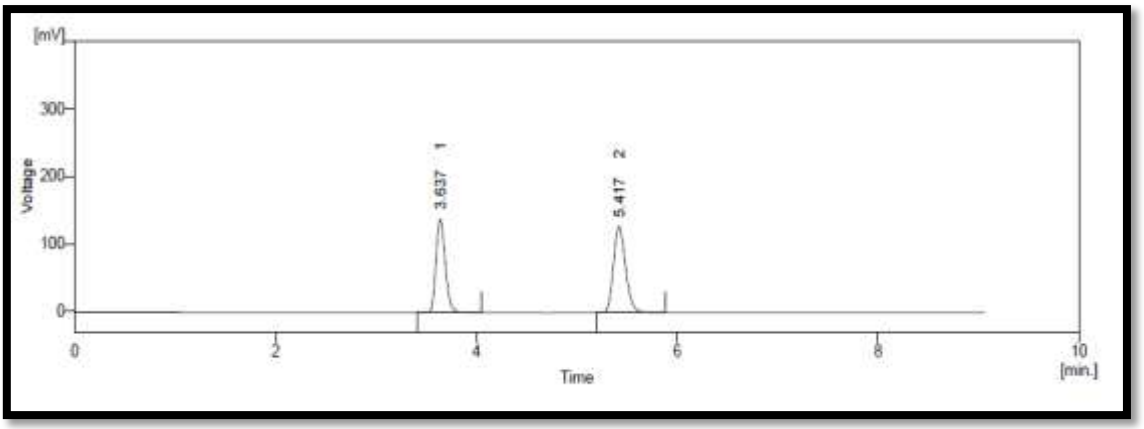


Fig-1: Chromatogram of working standard mixture of AMX and PRB

From the standard stock solution further dilutions (AMX 10 µg/ml and PRB 10 µg/ml) were done using mobile phase and scanned over the range of 190-390 nm and the spectra were overlain. As in marketed formulations content of AMX is far greater (10 mg) than PRB (10 mg), a wavelength at which AMX shows comparatively low absorbance than PRB was of concern. It was observed that at 228 nm both AMX and PRB showed considerable absorbance. Overlain spectra of both drugs are shown in Fig.2.

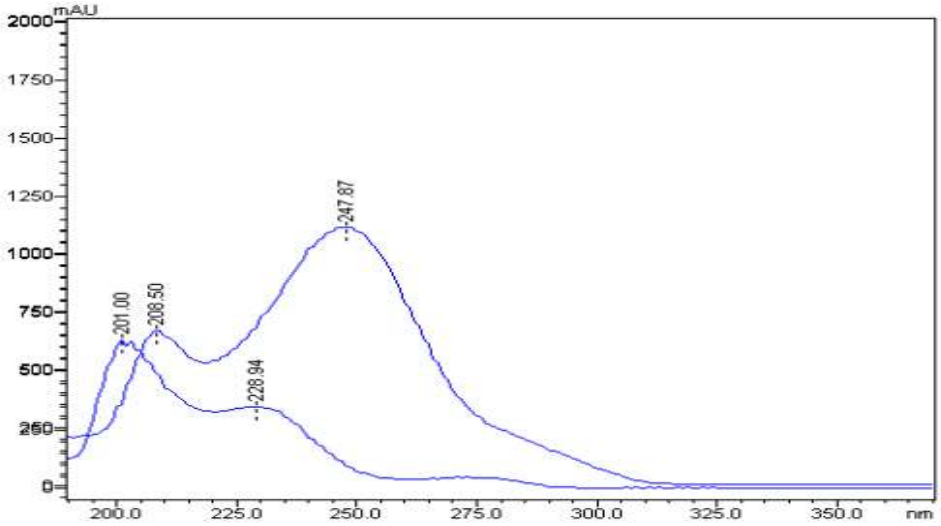


Fig-2: Overlain spectra of AMX and PRB (10 µg/ml) each

Table-1: Summary of chromatographic conditions

Sr. no.	Parameters	Conditions
1	Column	BDS Hypersile C_{18} (250mm X 4.6 mm i.d., 5 µm particle size)
2	Mobile phase	0.02 M KH_2PO_4 buffrer (pH-3.5) : acetonitrile(65:35 v/v)
3	Flow rate	1 ml/min
4	Detection wave length	228 nm
5	Sample injector	20 µl

Validation of developed method

Linearity

From standard stock solution, aliquots of 0.5 ,0.75, 1.0 ,1.25 and 1.5 ml was transferred to 10 ml volumetric flask and the volume was made up to the mark with mobile phase to obtain concentration of AMX 5-15 µg/ml and PRB 5-15 µg/ml. The calibration curves of the area under curve Vs concentration were recorded for both drugs. The results are shown in [Table no. 2 and Fig. 3 and 4].

Precision

Intra-day precision of the method was determined by repeat analysis (three identical injections) at three concentration levels. Inter-day

precision was established by performing the analysis next day on freshly prepared solution [Table no. 3].

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. From this percentage recovery values were calculated [Table no. 4].

Robustness

Robustness of the method reflects the reliability of an analysis with respect to deliberate variations in the method parameters. Here, the flow rate, pH and mobile phase composition were slightly changed to lower and higher sides of the actual values to find if the change in the peak area and retention time were within limits. The results obtained with changes in the parameters are shown in [Table no. 5].

System Suitability Testing

System suitability testing is used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. Parameters such as theoretical plates, tailing factor, resolution were determined and compared against the specification [Table no. 6].

Table - 2: Linearity of AMX and PRB

AMX		PRB	
Concentration (µg/ml)	**Peak Area**	**Concentration (µg/ml)**	**Peak Area**
5	444.579	5	536.272
7.5	656.498	7.5	791.895
10	897.736	10	1082.833
12.5	1105.425	12.5	1330.529
15	1345.057	15	1622.653
Correlation coefficient : 0.9995		Correlation coefficient : 0.9994	
Intercept : 10.094		Intercept :11.722	
Slope : 89.995		Slope : 108.46	
Regression Equation : y=89.995x-10.094		Regression Equation : y=108.46x-11.722	
LOD (µg/ml) : 0.348		LOD (µg/ml) : 0.379	
LOQ (µg/ml) : 1.054		LOQ (µg/ml) : 1.150	

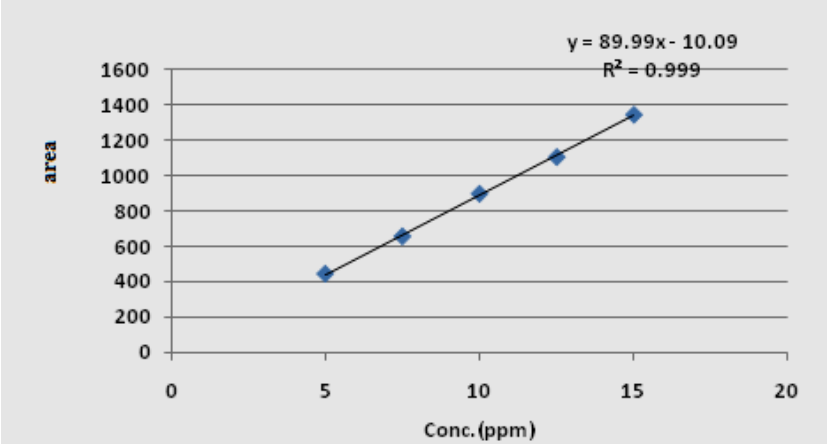


Fig-3: Calibration Curve for AMX

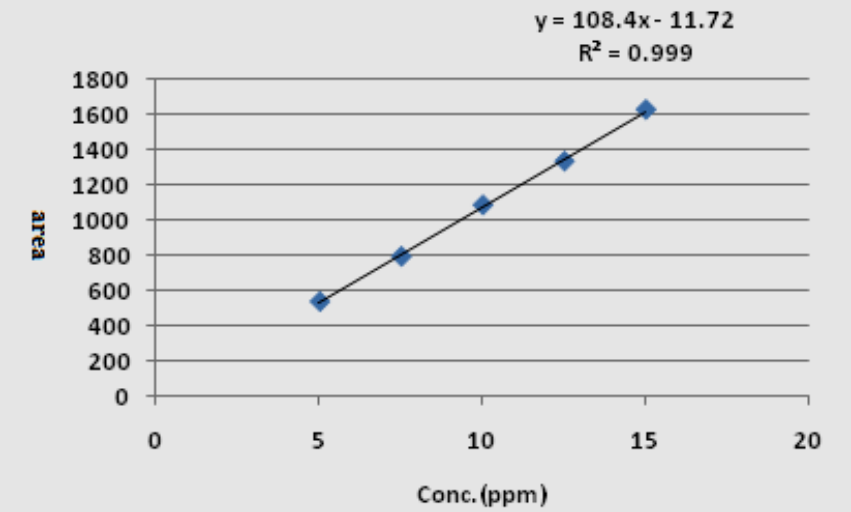


Fig- 4: Calibration Curve for PRB

Table - 3: Intraday and Interday variability of AMX and PRB (Concentration (µg/ml)

	AMX		PRB			
	5	**10**	**15**	**5**	**10**	**15**
Inter day	1.300	0.696	0.594	0.716	0.386	0.1006
Intra day	0.8020	0.6822	0.8124	1.154	0.587	0.498

Table no. 4: Recovery Study of AMX and PRB

Drug	**Level of recovery**			
		80%	100%	120%
AMX	Mean % Recovery	99.904	99.579	99.745
	% RSD (n=3)	0.966	0.416	0.490
PRB	Mean % Recovery	99.459	99.368	99.543
	% RSD (n=3)	0.734	0.309	0.266

Table no. 5: Robustness study of AMX and PRB

Drug	**% RSD**					
	Flow rate (1 ml/min)		**pH (3.5)**		**Ratio of mobile phase (65:35 v/v)**	
	+0.2	-0.2	+.02	-0.2	+2	-2
AMX	1.105	0.990	1.165	0.785	1.663	0.754
PRB	0.728	0.714	1.165	0.772	0.632	0.497

Table no. 6: Result of System Suitability Parameters

Parameters	**Data obtained**		**Specification**
	AMX	**PRB**	
Retention time (R_t)	3.637 min	5.417 min	-
Resolution (R_s)	8.978		More than 1.5
Theoretical plates (N)	6862	9618	More than 2000
Tailing factor (T_f)	1.391	1.355	Not more than 2

Table no. 7: Results of assay

Parameters	**Moxylong tablet**	
	AMX	**PRB**
Actual Concentration (µg/ml)	10	10
Concentration Obtained (µg/ml)	10	10
% Assay	102.31%	97.533%
%RSD	0.420	0.343
Limit	90-110 %	90-110 %

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 101.82 % - 102.61 % for AMX and that for PRB was 97.26 – 97.90 %. Results of tablet analysis are shown in [Table no.7].

CONCLUSION

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

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