Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(6):1212-1217

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Development and validation of analytical method for simultaneous estimation of amoxycillin trihydrate and probenecid in combined dosage form

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ABSTRACT

A simple, fast and precise reverse phase high performance liquid chromatographic method was developed for the simultaneous estimation of Amoxycillin trihydrate and Probenecid in combined dosage form. The chromatographic separation was achieved on Zorbax RX-C18 (150x4.6) mm; 5µm column with an isocratic mixture of 0.05M potassium dihydrogen phosphate buffer pH 5.6 adjusted with a mixture of trifluoro acetic acid and tetrahydrofuran (0.5%): acetonitrile in the ratio of 86:14 v/v. The mobile phase was kept at a flow rate of 0.8ml/min with injection volume of 20µl and wavelength of detection 227nm at room temperature. The retention times for AMOX and PRO was found to be 1.85±0.1min and 6.90±0.1min, respec tively. The linearity was obtained in the range of 50-250µg/ml for both Amoxycillin trihydrate and Probenecid with correlation coefficient 0.999 for both. On carrying out degradation studies it was found that products did not interfere with the detection of AMOX and PRO. The proposed method was found to be linear, accurate, precise, stable, robust and specific and was successfully applied for the determination of investigated drugs in combined dosage form.

Keywords: Amoxicillin, Probenecid, RP-HPLC, Stability, Validation.

INTRODUCTION

Amoxycillin (AMOX) is chemically (2S.5R.6R)-6-{[(2R)-2-amino-2-(4-hydroxyphenyl)-acetyl] amino}-3, 3-dimethyl-7-oxo-4-thia-l-azabicyclo [3.2.0] heptane-2-carboxylic acid [1]. Amoxycillin is amino Penicillin with spectrum similar to that of Ampicillin [2]. AMOX is a moderate-spectrum bacteriolytic β-lactum antibiotic used to treat bacterial infections caused by susceptible microorganisms. It is usually the drug of choice within the class because it is better absorbed following oral administration. AMOX acts by inhibiting the synthesis of bacterial cell wall. Probenecid (PRO) decreases the renal tubular secretion of amoxicillin. Concurrent use of amoxicillin and probenecid may result in increased and prolonged blood levels of Amoxycillin [3].

Rationale

Literature survey has revealed that a number of methods have been reported for estimation of AMOX, for example, spectrometric, HPLC and HPTLC for AMOX [4-6] or in combination with other drugs for example HPLC, spectrometric method and HPTLC for AMOX[7-15] and for probenecid [16-18]. Only one spectrophotometry method [19] has been reported for the quantitative estimation of AMOX and PRO in pharmaceutical dosage form. Therefore, an attempt has been made to develop new HPLC method for simultaneous estimation of AMOX and PRO in combined dosage form.

In the proposed method forced degradation studies for the drug substances and drug product will also be carried out under different stress conditions like acidic, basic, oxidative, thermal, and UV exposure and the stressed samples will be analyzed by the developed and validated method.

EXPERIMENTAL SECTION

The tablet MOXYLONG DT of AMOX and PRO, (label claim: AMOX 500mg and PRO 500mg), manufactured by American remedies ltd. and API were procured from Astron Research Limited, Ahmadabad. All the chemicals used which are analytical grade purchased from MERCK Chem. Ltd., Mumbai. HPLC instruments (Shimadzu) LC2010CHT with SPDM20A diode detector was used for estimation of AMOX and PRO in combined dosage form. LC solution software was applied for data collecting and processing. Other instruments used were FTIR Spectrophotometer (Brukeroptics), digital balance (Sartorius) (0.1 mg – 205 gm) and pH meter (ELICO).

METHOD DEVELOPMENT

The chromatographic separation was performed with isocratic elution on a Zorbax RX-C18 (150x 4.6) mm; 5µm as a stationary phase with mobile phase which is a mixture of 0.05M KH_2PO_4 buffer (pH 5.6) adjusted with TFA + THF 0.5%: acetonitrile (86:14 v/v) pumped at a flow rate of 1ml/min. The samples were analyzed by a PDA detector at 227 nm with the injection volume of 20µL.

Preparation of working standard solution for AMOX and PRO

Accurately weighed 100mg of standard AMOX and PRO and was transferred to a 100ml volumetric flask separately and dissolved in 100ml of solvent (methanol: acetonitrile in the ratio of 80:20 v/v). The flask was shaken and volume was made up to the mark with solvent to give a solution containing 1000µg/ml AMOX and PRO. From this solution 5ml solution was taken and diluted up to 10 ml with solvent in a volumetric flask to give solution of 500µg/ml of AMOX and PRO. Further, 2ml of this solution was taken out and diluted up to 10ml with solvent to give working standard solution containing 100µg/ml of AMOX and PRO.

Preparation sample solution

Twenty tablets were weighed and triturated and weight-equivalent of powder containing 20 mg of AMOX and 20 mg of PRO was weighed accurately and transferred to a 20 ml volumetric flask and diluted up to 20 ml with methanol: acetonitrile (80:20 v/v). After this the solution was sonicated for 15min to dissolve compounds and further diluted similarly, same as working standard, to prepare a solution containing 100 µg/ml of AMOX and 100µg/mL of PRO, respectively.

Method optimization

The chromatographic separation was achieved with conditions shown in Table-1 and the chromatogram obtained is shown in Figure 1.

Calibration curve

Six different concentrations of AMOX and PRO i.e., 50µg/ml, 75µg/ml, 100µg/ml, 150µg/ml, 200µg/ml, 250µg/ml, were prepared from working standard solution of AMOX and PRO, respectively. Calibration curves constructed were linear over the prepared concentration of 50-250µg/ml for both AMOX and PRO. Calibration curves were prepared using ratio of analyte peak area to internal standard peak versus concentration of analyte. The calibration curves are shown in Figure 2 and 3.

METHOD VALIDATION [20]

The proposed method was validated in accordance with ICH guidelines. It was validated in terms of linearity, accuracy, precision, LOD, LOQ and % recovery.

Linearity

Linearity studies were carried out for AMOX and PRO at six different concentration levels. Calibration curves constructed were linear over the concentration range of 50-250µg/ml for both AMOX and PRO. Evaluation of two drugs was performed with UV detector at 227 nm and peak area was recorded for all the peaks. The correlation coefficient was found to be 0.999 for both AMOX and PRO.

Accuracy
The accuracy of the method was assessed by recovery studies of AMOX and PRO in combined dosage form at three concentration levels. A fixed amount of pre-analyzed sample was taken and standard drug was added at 80%, 100% and 120% levels. The samples were then analysed and each level was repeated for three times. The percentage recovery of AMOX and PRO was found to be 100.03% and 99.60%, respectively. The results are shown in Table 2.

Precision
The precision for the developed method was determined in terms of intraday and inter-day precision. For intraday precision evaluation, a standard solution of fixed concentration was injected at various time intervals on a particular day and %RSD for AMOX and PRO were found to be1.12% and 0.54%, respectively (limit %RSD <2.0%). In addition, the inter-day precision was studied by injecting the same concentration of standard solution on consecutive days and the %RSD for AMOX and PRO were 1.75% and 0.89%, respectively (limit %RSD < 2.0%).The results are shown in Table 3 and 4.

Limit of detection and limit of quantification
The LOD and LOQ were determined by injecting progressively low concentration of the standard solutions using the developed HPLC method. The LOD for AMOX and PRO were found to be 4.54µg/ml and 2.01µg/ml, respectively. The LOQ for AMOX and PRO were found to be 13.77µg/ml and 6.09µg/ml, respectively.

Assay
Sample solution (20 µl) was injected and analysed. The peak area of AMOX and PRO and the amount of each drug in samples was computed. The results of the assay show presence of 99.66% and 99.47% of AMOX and PRO, respectively**.** The results of the assay are shown in Table 5.

STABILITY INDICATING STUDY [21]
Force degradation studies
The stability indicating RP-HPLC assay method for simultaneous determination of AMOX and PRO were performed using above developed method. In order to establish stability-indicating nature of the method, drug product and solvent were subjected to various stress conditions to conduct force degradation studies. Stress studies were carried out under the conditions of acidic, basic, oxidative, thermal and UV exposure. Several trials with different severity of each stressed condition were conducted. Results are shown in Table 6.

RESULTS AND DISCUSSION

A new RP-HPLC method was developed for estimation of AMOX and PRO in combined dosage form. The HPLC method was optimized with a view to develop an accurate assay method for estimation of AMOX and PRO in combined dosage form. The samples were analyzed by a PDA detector at 227 nm with the injection volume of 20µL resulted in peak with good shape and resolution. The method was found to be linear in the range of 50-250µg/ml for both AMOX and PRO. The percentage recoveries of AMOX and PRO were 100.03% and 99.60%, respectively which shows that there is no interference from excipients and the lower values of RSD of assay indicate the method is accurate. The %RSD of AMOX and PRO for intraday precision studies were found to be 0.43%and 0.60%, respectively (limit %RSD< 2.0%) and % RSD of AMOX and PRO for inter-day precision studies were found to be 0.69% and 0.75%, respectively (limit %RSD < 2.0%).

The retention time of AMOX and PRO were found to be 1.851min and 6.906min, respectively with an asymmetry factor of 1.4 for AMOX and 1.49 for PRO which indicates efficient performance of the column. The LOD for AMOX and PRO was found to be 4.54µg/ml and 2.01µg/ml, respectively. The LOQ for AMOX and PRO was found to be 13.77µg/ml and 6.09µg/ml, respectively which indicates good sensitivity of the proposed method. Assay studies of the proposed method indicate 101.03% and 99.60% recovery for AMOX and PRO, respectively.

Figure 1: Typical chromatogram of standard for AMOX and PRO

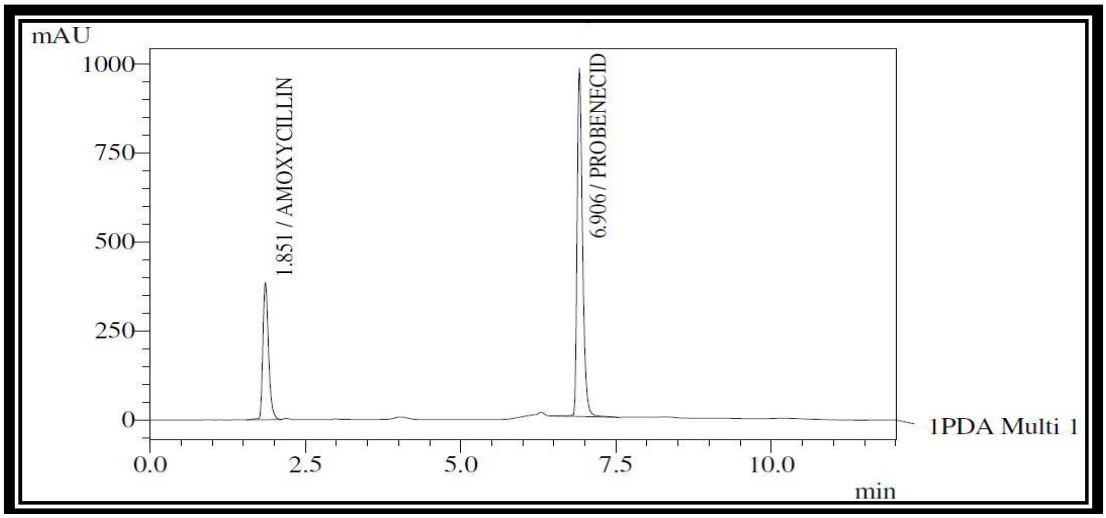

Figure 2: Calibration curve of AMOX at 227 nm

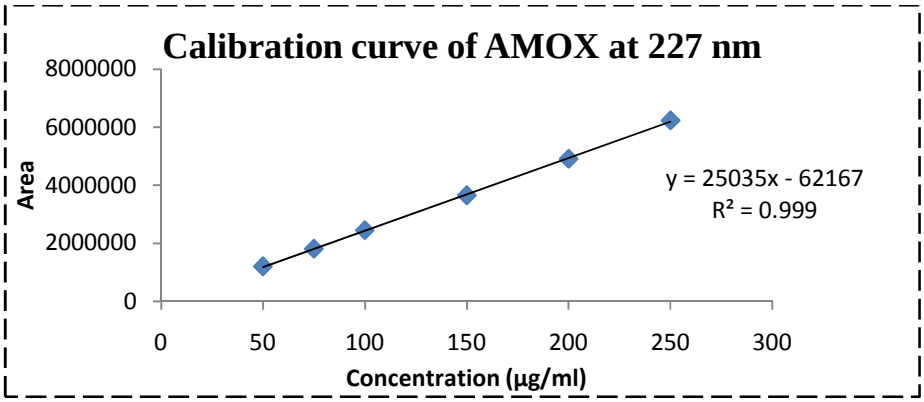

Figure 3: Calibration curve of PRO at 227 nm

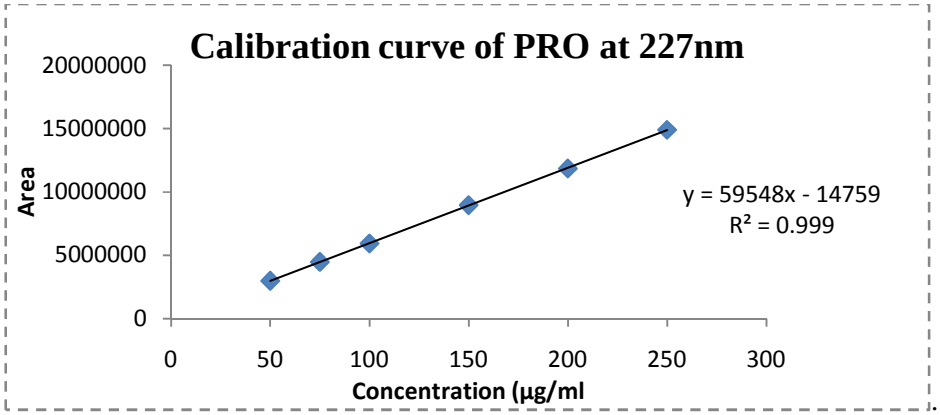

Table-1: Optimized chromatographic conditions

Sr. No.	Parameters	Condition
1	**Instrument**	Shimadzu LC 2010 CHT
2	**Stationary phase**	Zorbax RX C18 (250mm X 4.6 mm, 5 µm)
3	**Mobile phase**	0.05 M KH_2PO_4 Buffer pH 5.6 +THF 0.5%: Acetonitrile (86:14v/v) pH 5.6
4	**Pump mode**	Isocratic
5	**Flow rate (mL/min)**	0.8
6	**Run time (min)**	12
7	**Volume of injection (µl)**	20
8	**Detector**	UV
9	**Detection wavelength (nm)**	227
10	**Column temperature**	30˚ C
11	**Retention time (min)**	AMOX: 1.851 ± 0.1 min PRO: 6.906 ± 0.1 min

Table-2: Accuracy studies for the proposed method

Spiked level	Amount added (µg/ml)		Amount Found (µg/ml)		%Recovery	
	AMOX	**PRO**	**AMOX**	**PRO**	**AMOX**	**PRO**
80%	80	80	80.16	79.56	100.21%	99.45%
100%	100	100	99.66	99.47	99.66%	99.47%
120%	120	120	120.16	119.87	100.14%	99.89%

Table-3: Intraday precision for the analysis of AMOX and PRO

Sr. No.	Drug(µg/ml)	Peak Area			Mean	SD	%RSD
	AMOX	**1.**	**2.**	**3.**			
1.	75	1819521	1819543	1821349	1820138	1049.103	0.0576
2.	100	2551490	2541219	2497824	2530178	28485.84	1.1258
3.	125	3100820	3095895	3092593	3096436	4140.096	0.1337
Average %RSD							0.4390
Sr no.	PRO	1.	2.	3.	Mean	SD	%RSD
1.	75	4454120	4421051	4398259	4424477	28087.62	0.6348
2.	100	5924075	5928201	5982511	5944929	32612.28	0.5485
3.	125	7487490	7438215	7394398	7440034	46572.66	0.6259
Average %RSD							0.6031

Table-4: Inter-day precision for the analysis of AMOX and PRO

Sr. No.	Drug(µg/ml)	Peak Area			Mean	SD	%RSD
	AMOX	**1.**	**2.**	**3.**			
1.	75	1803289	1802351	1801892	1802511	712.055	0.0395
2.	100	2495831	2432925	2413598	2447451	42997.99	1.7568
3.	125	3045902	3038512	3037970	3037970	8216.934	0.2704
Average %RSD							0.6889
Sr. No.	**PRO**	**1.**	**2.**	**3.**	**Mean**	**SD**	**%RSD**
1.	75	4454120	4392581	4428647	4425116	30921.08	0.6987
2.	100	5924075	5822195	5853148	5866473	52230.68	0.8903
3.	125	7487490	7397299	7407450	7430746	49402.87	0.6648
Average %RSD							0.7513

Table-5: Assay results of proposed methods

Sr. No.	Label claim		Amount taken		Amount Found		% Assay	
	AMOX	**PRO**	**AMOX**	**PRO**	**AMOX**	**PRO**	**AMOX**	**PRO**
1	500mg	500mg	500mg	500mg	498.32	497.37	99.66%	99.47%
2					495.51	498.32	99.10%	99.66%
3					497.94	497.2	99.58%	99.44%
4					497.9	499.71	99.58%	99.94%
5					498.59	498.37	99.71%	99.85%
6					498.43	499.29	99.68%	99.67%
SD							0.2291	0.2002
%RSD							0.2302	0.2009

Table-6: Force degradation study of AMOX and PRO

Degradation Condition	% ASSAY		% DEGRADATION	
	AMOX	PRO	AMOX	PRO
Acid Degradation	87.96%	89.97%	12.04%	10.30%
Base Degradation	82.43%	85.08%	17.57%	14.92%
Oxidative Degradation	70.75%	71.38%	29.25%	28.62%
Photo Degradation	96.91%	99.92%	3.09%	0.08%
Thermal Degradation	88.29%	90.05%	11.71%	9.95%

CONCLUSION

The developed RP-HPLC method is simple, specific, accurate and precise for the simultaneous estimation of AMOX and PRO in combined dosage form. The developed method provides good resolution between AMOX and PRO. It was successfully validated in terms of linearity, accuracy, precision, LOD, LOQ and recovery in accordance with ICH guidelines. Thus the described method is suitable for routine analysis and quality control of pharmaceutical preparations containing these drugs in combination.

Acknowledgement
The author is grateful to Dr. Harish Kakrani, Principal, Shivam Pharmaceutical Studies and Research Centre, Valasan, Assistant Professor Mr. Mayank Bapna and Miss Jigisha Patel for continuous support and guidance.

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