Journal of Pharma Research
Available online through
www.jprinfo.com

Research Article
ISSN: 2319-5622

High Performance Liquid Chromatographic Determination of Amoxicillin, Cloxacillin and Serratiopeptidase in their Combined Tablet Dosage form

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Received on: 26-07-2015; Revised and Accepted on: 05-08-2015

ABSTRACT

A new, simple, precise and accurate high performance liquid chromatography method is developed and validated for the estimation of amoxicillin, cloxacillin and serratiopeptidase as the bulk drug and in pharmaceutical dosage forms. Chromatographic separation of the drugs was performed on Zorbax C8 (250 x 4.6 mm; 5 µm particle size) analytical column as the stationary phase. The solvent system consisted of 0.1M NaH_2PO_4 and Acetonitrile in the ratio of 55:45 (v/v) as mobile phase. Evaluation of the separated drugs was performed using a PDA detector covering the range of 200-400 nm. All the three drugs were resolved with the retention time of 3.421 min, 6.174 min and 7.288 min for amoxicillin, cloxacillin and serratiopeptidase, respectively. The method was validated with respect to linearity, sensitivity, precision, accuracy and robustness in accordance with ICH guidelines. The validated method was successfully applied to the commercially available pharmaceutical dosage form, yielding good and reproducible result.

Keywords: *Amoxicillin; Cloxacillin; Serratiopeptidase; High-performance liquid chromatography Simultaneous Determination.*

INTRODUCTION

Amoxicillin [1, 2] is a broad-spectrum semi synthetic antibiotic belonging to o the class of organic compounds known as penicillins.Chemically, amoxicillin is described as (2*S*,5*R*,6*R*)-6-{[(2*R*)-2-amino-2-(4-hydroxyphenyl)-acetyl]amino}-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-24-carboxylic acid. Amoxicillin binds to penicillin-binding protein 1A (PBP-1A) located inside the bacterial cell well. Penicillins acylate the penicillin-sensitive transpeptidase C-terminal domain by opening the lactam ring. This inactivation of the enzyme prevents the formation of a cross-link of two linear peptidoglycan strands, inhibiting the third and last stage of bacterial cell wall synthesis. Cell lysis is then mediated by bacterial cell wall autolytic enzymes such as autolysins; it is possible that amoxiclin interferes with an autolysin inhibitor.

Cloxacillin [3, 4] is a semi-synthetic antibiotic that is a chlorinated derivative of oxacillin.. Chemically, cloxacillin is known as (2S,5R,6R)-6-[3-(2-chlorophenyl)-5-methyl-1,2-oxazole-4-amido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid. Cloxacillin binding to specific penicillin-binding proteins (PBPs) located inside the bacterial cell wall; cloxacillin inhibits the third and last stage of bacterial cell wall synthesis. Cell lysis is then mediated by bacterial cell wall autolytic enzymes such as autolysins; it is possible that cloxacillin interferes with an autolysin inhibitor.

Serratiopeptidase [5, 6] is a proteolytic enzyme produced by enterobacterium *Serratia* sp. E-15. It is isolated from the in the late 1960s from silkworm Bombyx moriL. (intestine), Serratiopeptidase is present in the silkworm. Serratiopeptidase having anti inflammatory, anti-oedemic and fibrinolytic activity and it also acts rapidly on localized inflammation. Serratiopeptidase has been widely used in the treatment of acute pain. Figures 1, 2 and 3 shows the chemical structure of amoxicillin,cloxacillin and serratiopeptidase, respectively.

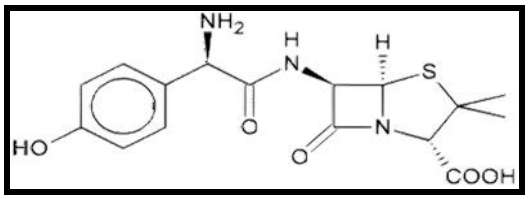

Fig. 1: Chemical structure of amoxicillin

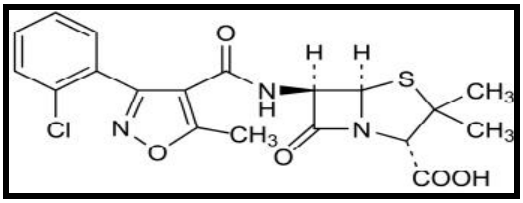

Fig. 2: Chemical structure of cloxacillin

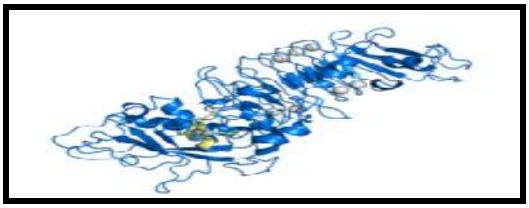

Fig. 3: Chemical structure of serratiopeptidase

The literature reports, many methods for simultaneous quantitative determination of amoxicillin, cloxacillin and serratiopeptidase in bulk, tablet dosage form, capsule dosage form and human plasma. These methods include simultaneous estimation of amoxicillin, cloxacillin and serratiopeptidase by UV spectrophotometry [7-11], HPLC [12-14], HPLC–ESI-MS/MS [15] and LC-MS [16].

The aim of the present investigation is to develop and validate a sensitive, precise and accurate RP-HPLC method for the simultaneous quantification of amoxicillin, cloxacillin and serratiopeptidase in bulk and in its combined pharmaceutical formulation.

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MATERIALS AND METHODS

Apparatus:

A Waters 2695 alliance with binary HPLC pump equipped with Waters 2998 PDA detector and Waters Empower2 software was used in the present investigation.

Mobile phase:

The solvents and chemicals used in the preparation of mobile phase were of HPLC grade and analytical grade, respectively. The mobile phase used was 0.1M NaH_2PO_4 and acetonitrile in the ratio of 55:45 *v/v*. Before use, the mobile phase was filtered through millipore membrane filter and degassed for 15 minutes by sonication.

Chromatographic conditions:

Zorbax C8 (250 x 4.6 mm; 5 µm particle size) analytical column was used for separation and simultaneous analysis of amoxicillin, cloxacillin and serratiopeptidase. The column temperature was maintained at 30±1^{0}C. The separation was carried out under isocratic elution. The flow rate was maintained as 1.0 ml/min. The injection volume was 10 µl. The eluents were detected at 236 nm.

Standard solutions:

The standard stock solution was prepared by dissolving 250 mg of amoxicillin, 250 mg of cloxacillin and 10mg of serratiopeptidase in 100 ml mobile phase. Working standard solutions equivalent to 62.5-187.5 µg/ml amoxicillin, 62.5-187.5 µg/ml cloxacillin and 2.5-7.5 µg/ml serratiopeptidase was prepared from stock solution by appropriately diluting the stock standard solution with the mobile phase.

Sample Solution:

Ten tablets were weighed and crushed to a fine powder. The powder equivalent of 250 mg of amoxicillin, 250 mg of cloxacillin and 10mg of serratiopeptidase was taken in a 100 ml volumetric flask containing 20 ml of mobile phase, sonicated for 20 minute and made up to mark with the same solvent. The resultant mixture was filtered through 0.45 µm filter paper. The filtrate was diluted appropriately with the mobile phase to get a final concentration of 250 mg of amoxicillin, 250 mg of cloxacillin and 10mg of serratiopeptidase.

RESULTS AND DISCUSSION

HPLC parameters optimization:

So as to study the simultaneous elution of more than one drug under isocratic conditions, different chromatographic conditions (type of the column, mobile phase composition, flow rate and pH) have been investigated. The objective of the simultaneous HPLC method development was to achieve a peak tailing factor <2, USP plate count ≥ 2000, retention time in between 4,7 and 8 minutes, along with good resolution. This objective was obtained using mobile phase consisting of 0.1M sodium dihydrogen phosphate – acetonitrile in the proportion of (55/45, *v/v*). The pH of the mobile phase was adjusted to 4.2with orthophosphoric acid. Under the above described conditions, the analyte peaks were well defined, resolved and free from tailing. The tailing factors were <2 for both the peaks. The elution orders were amoxicillin (retention time- 3.420 min), cloxacillin (retention time- 6.172 min) and serratiopeptidase (retention time- 7.287 min) at a flow rate of 1.0 ml/min (Figure 4). The optimum wavelength for detection was 236 nm at which much better detector responses for the selected drugs were obtained.

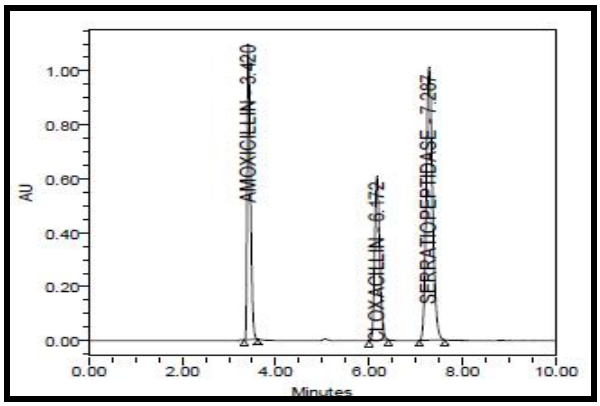

Fig. 4: Typical chromatogram of amoxicillin, cloxacillin and serratiopeptidase

Method validation:

The optimized RP-HPLC method for simultaneous assay of amoxicillin, cloxacillin and serratiopeptidase was validated according to ICH guidelines [18] with respect to system suitability, linearity, sensitivity, accuracy, precision and robustness.

System suitability:

In relation to U.S. Pharmacopeia, system suitability tests are an integral part of a liquid chromatographic method. System suitability tests are used to confirm that the column efficiency, resolution and reproducibility of the chromatographic system are sufficient for the analysis. System suitability test was assessed from five replicate injections of the standard solution containing 250 mg of amoxicillin, 250 mg of cloxacillin and 10mg of serratiopeptidase. The results of system suitability in comparison with the required limits are shown in Table 1. The results are found to be suitable and are within the accepted limits.

Table No. 1: System suitability test of the HPLC method

Parameters	Results			Recommended limits
	Amoxicllin	Cloxacillin	Seeratiopeptidase	
Retention time	3.420	6.172	7.287	-
Peak area	5184858 (%RSD – 0.6)	7196600 (%RSD – 0.5)	9202007 (%RSD – 0.5)	RSD ≤1
USP resolution	-	16.61	4.92	> 1.5
USP plate count	11424	16049	13904	> 2000
USP tailing factor	1.33	1.20	1.20	≤ 2

Linearity and range:

The linearity of the method was determined by analyzing five different concentrations of each drug. The calibration curve was plotted by area under the peak responses of the drugs against their corresponding concentrations. Calibration curves were linear over the concentration range of 62.5-187.5µg/ml amoxicillin, 62.5-187.5µg/ml cloxacillin and 2.5-7.5µg/ml serratiopeptidase. The parameters such as a regression equation and regression coefficient are given in Fig. 4 & 5. The results show a good correlation between the peak areas of the drugs and their corresponding concentrations.

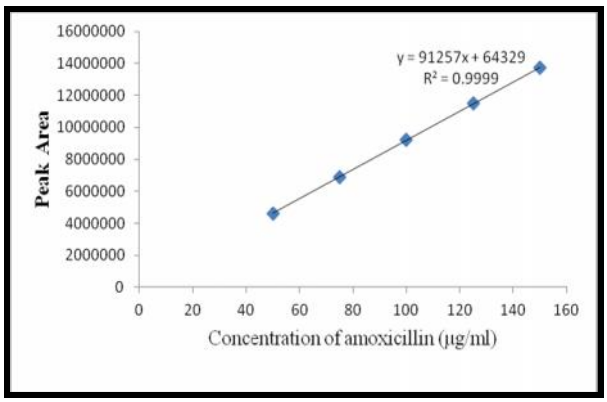

Fig. 5: Linearity curve of amoxicillin

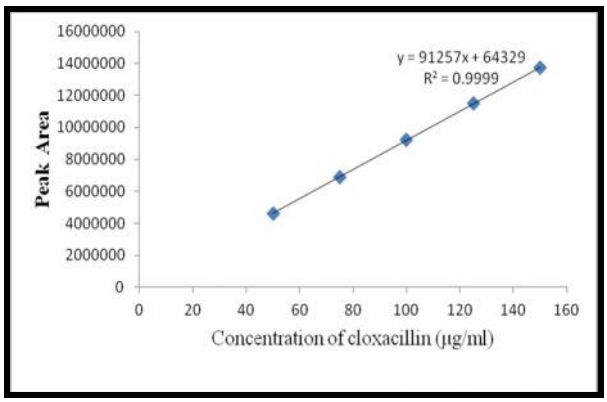

Fig. 6: Linearity curve of cloxacillin

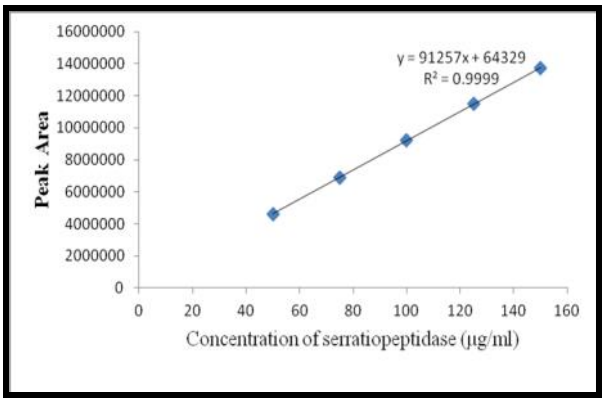

Fig. 7: Linearity curve of serratiopeptidase

Sensitivity:

The sensitivity of the method was assessed by calculating limit of detection (LOD) and limit of quantification (LOQ) according to ICH guidelines. The results are summarized in Table 2. The low values of LOD and LOQ demonstrate the sufficient sensitivity of the method. The chromatograms of amoxicillin, cloxacillin and serratiopeptidase at LOD and LOQ levels are presented in Fig. 8 & 9.

Table No. 2: Sensitivity of the HPLC method

Parameters	Results		
	Amoxicillin	Cloxacillin	Serratipeptidase
LOD	3.421	6.201	7.364
LOQ	3.439	6.226	7.391

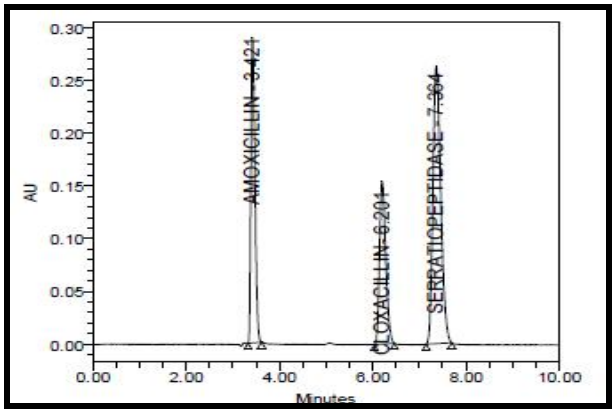

Fig. 8: Chromatogram of amoxicillin, cloxacillin and serratiopeptidase at LOD level

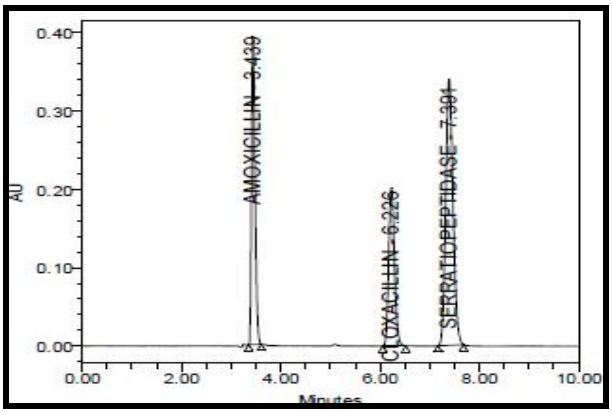

Fig. 9: Chromatogram of amoxicillin, cloxacillin and serratiopeptidase at LOQ level

Precision:

Precision was determined by injecting six standard solutions of amoxicillin (250µg/ml),cloxacillin(250µg/ml) and serratiopeptidase (10 µg/ml). The peak areas were determined. Relative standard deviation of peak areas of the two drugs was then calculated to represent precision. The results are summarized in Table 3. The low % RSD values indicated that the method was precise.

Table No. 3: Precision of the HPLC method

Amoxicillin		Cloxacillin		Serratiopeptidase	
Peak area	%RSD	Peak area	%RSD	Peak area	%RSD
5093555	0.05	4322301	0.07	9208536	0.04
5098076		4327817		9201274	
5096518		4323733		9205371	
5092035		4328736		9209814	
5096836		4323199		9207159	
5093571		4320535		9202418	

Accuracy:

Accuracy of the method was evaluated by recovery studies at three concentration (50%, 100%, and 150%) levels by standard addition method. The mean percentage recoveries obtained were shown in Table 5. The good % recovery values showed that the method was highly accurate.

Table No. 4: Accuracy of the HPLC method

Drug	Spiked Level	µg/ml added	µg/ml found	% Recovery	% Mean
Amoxicillin	50%	61.875	62.01	100	100
	50%	61.875	61.94	100	
	50%	61.875	62.14	100	
	100%	123.750	124.15	100	100
	100%	123.750	124.10	100	
	100%	123.750	124.16	100	
	150%	185.625	186.22	100	100
	150%	185.625	186.22	100	
	150%	185.625	186.07	100	
Cloxacillin	50%	62.500	62.60	100	100
	50%	62.500	62.49	100	
	50%	62.500	62.50	100	
	100%	125.000	125.01	100	100
	100%	125.000	124.75	100	
	100%	125.000	124.98	100	
	150%	187.500	187.12	100	100
	150%	187.500	187.23	100	
	150%	187.500	187.37	100	
Serratiopeptidase	50%	2.475	2.48	100	100
	50%	2.475	2.48	100	
	50%	2.475	2.48	100	
	100%	4.950	4.96	100	100
	100%	4.950	4.96	100	
	100%	4.950	4.96	100	
	150%	7.425	7.45	100	100
	150%	7.425	7.45	100	
	150%	7.425	7.45	100	

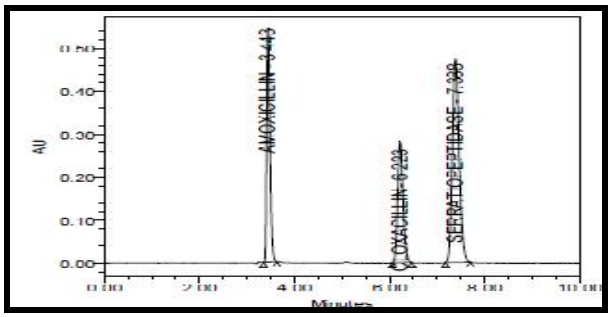

Fig. 10: Chromatogram of amoxicillin, cloxacillin and serratiopeptidase at 50% level

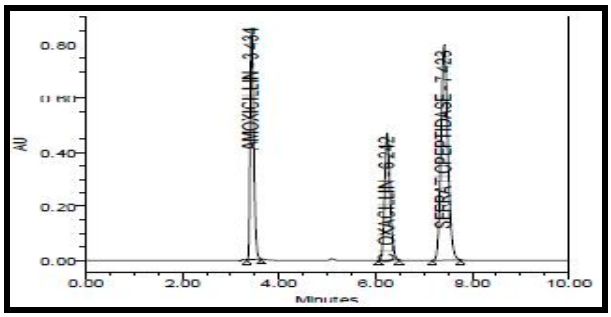

Fig. 11: Chromatogram of amoxicillin, cloxacillin and serratiopeptidase at 100% level

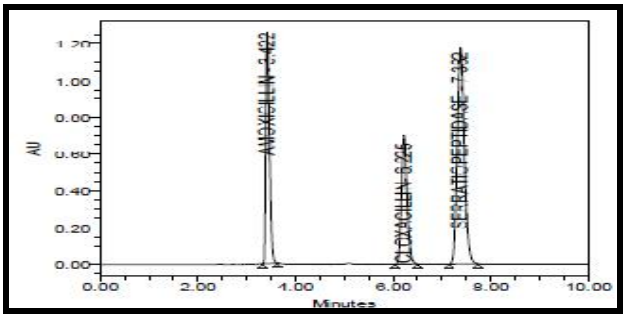

Fig. 12: Chromatogram of amoxicillin, cloxacillin and serratiopeptidase at 150% level

Robustness:

In order to show the robustness of the method, system suitability parameters were evaluated at different flow rate and different column temperature. The parameters used to define robustness are retention time, USP tailing factor and USP plate count. The results showed (Table 5) that slight variations in method parameters had a negligible effect on the analysis.

Table No. 5: Robustness of the method

Drug	Parameter	Retention time	Peak area	USP Plate Count	USP Tailing
Amoxicillin	Flow 1	4.411	6151691	11843	1.43
	Flow 2	2.803	3792195	10207	1.37
	Temperature 1	3.426	4713356	13097	1.42
	Temperature 2	3.385	4669157	13779	1.35
Cloxacillin	Flow 1	8.023	5213247	16080	1.15
	Flow 2	5.096	3217671	14255	1.13
	Temperature 1	6.226	4031804	15736	1.12
	Temperature 2	6.107	3995385	16239	1.09
Serratiopeptidase	Flow 1	9.480	11171420	14509	1.15
	Flow 2	6.067	6893086	12481	1.10
	Temperature 1	7.390	8619106	13719	1.11
	Temperature 2	7.158	8500246	14333	1.08

CONCLUSION

A RP-HPLC method has been reported for simultaneous estimation amoxicillin, cloxacillin and serratiopeptidase. The proposed method gives good resolution of the above said drugs. The validation of developed method was done as per ICH guidelines and proved that method to be simple, sensitive, precise, accurate and robust. The validated method was successfully applied to the determination of commercially available pharmaceutical dosage form. Hence, the method can be used for the routine quality control analysis of pharmaceutical dosage forms containing amoxicillin, cloxacillin and serratiopeptidase.

ACKNOWLEDGEMENT

The authors are thankful to Department of Chemistry, Andhra Loyola College, and Vijayawada for providing instruments and analytical support and also for V. Ramakrishna, K. Srinivasa rao for supporting to this work.

REFERENCES:

1. Amoxicillin Susceptibility and Resistance Data. Retrieved, **2013**-06-20.
2. www.drugbank.ca/drugs/DB01060
3. David Greenwood. Antimicrobial drugs: chronicle of a twentieth century medical triumph. Oxford University Press US. **2008**; pp. 124
4. www.drugbank.ca/drugs/DB01147
5. Dr. David Jockers. Naturalnews for SerratioPeptidase is a potent anti-inflammatory enzyme, **2011**-09-04.
6. Shivani Bhagat, Monika Agarwa, Vandana Roy. *International Journal of Surgery*, **2013**; 11(3): pp 209-217.
7. Do T Giang and Vu D Hoang. *J. Young Pharm*., **2010**; 2(2): pp 190-195.
8. Saidul Zafar Qureshi,Talat Qayoom, Marud I. Helalet. *Journal of Pharmaceutical and Biomedical Analysis,* **1999**; 21(3): pp 473-782.
9. Dhoka Madhura Vishal, Gawande Vandana Tukaram, Joshi Pranav Pramod. *Int. Res. J. Pharma.,* **2011**; 2(3): 197-201.
10. Elmolla ES, Chaudhuri M. *J Hazard Mater.,* **2010**; 173(1-3): 445-9.
11. N. M. Nirale and Mala D. Menon. Ind. J. Pharm. Sci., **2010**; 72(1): pp 65-71.
12. A. Ashnagar, N. Gharib Naseri. *E-J. Chem.,* **2007**; 4(4): pp 539-545.
13. Angshuman Biswas, A Rajendiran, Inder Singh Rawat, Asit Kumar De, AyanKumar Dey. *Res. J. Pharma. Biol. Chem. Sci.,* **2010**; 1(4): pp 344-349.
14. Madhuradhoka,Vandanagawande,Pranavjosh. *Int. J. Pharma. Pharma. Sci*., **2010**; 2(1): pp 129-133.
15. Mónica Díaz-Bao, Rocío Barreiro, José Manuel Miranda, Alberto Cepeda, and Patricia Regal. *Journal of Anal. Meth. Chem.,***2015**; 1-8.
16. Rolf F. Straub, Robert D. Voyksner. J. Chromatography A, **1993**; 647(10): 167-181.
17. FDA Issues Safety Labeling Changes for Cobacta S, **2011**-01-19.
18. International Conference on Harmonization, ICH Guidelines, Validation of Analytical Procedures Technical Requirements for Registration of Pharmaceuticals for Human Use: Text and Methodology Q 2 (R1), International Conference on Harmonization, Geneva, Switzerland, November **2005**.
19. Hsieh SH, Huang HY, Lee S. *J. Chromatogr. A*., **2009**; 7186-94.
20. Samanidou VF, Evaggelopoulou EN. Papadoyannis IN, *J. Sep. Sci*., **2006**; 29: 1550-60.

How to cite this article:

K.L.N.N.S.V.K. Pavankumar et al.,: High Performance Liquid Chromatographic Determination of Amoxicillin, Cloxacillin and Serratiopeptidase in their Combined Tablet Dosage form, J. Pharm. Res., 2015; 4(7): 273-277.

Conflict of interest: The authors have declared that no conflict of interest exists.

Source of support: Nil