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VALIDATED RP-HPLC FOR SIMULTANEOUS ESTIMATION OF CEFOTAXIME AND SULBACTUM IN RECONSTITUTED SOLIDS

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ABSTRACT

Background: Cefotaxime (CTX) is a third generation cephalosporin antibiotic. Sulbactum (SBT) is semi-synthetic parenteral penicillin with a broad spectrum of antibacterial activity. The combination of Cefotaxime and Salbactum is more effective for the treatment of moderate to severe infections than single drug therapy alone. There are so many combinations of sulbactum and other cephalosporin on which RP-HPLC method was developed. Till date no HPLC method is available for simultaneous estimation of sulbactum and cefotaxime in combined dosage form. **Objectives:** The present work involves the development of new and validated RP-HPLC method for simultaneous estimation of CTX and SBT in reconstituted solids as combined dosage form. **Material and Methods:** The simultaneous estimation of Cefotaxime and Sulbactum has been done by RP-HPLC on Hypersil C-18 (Gold) column (250 mm x 4.6 mm) using 10mM Potassium dihydrogen phosphate buffer (pH- 5.1): Acetonitrile (90:10 v/v) as a mobile phase at the flow rate of 1.0 ml/min. and quantified at 228 nm using UV detector. **Results and Discussion:** Cefotaxime and Sulbactum were satisfactorily resolved with Retention time (R_t) of 8.56 min. and 4.00 min. respectively. The accuracy and reliability of the method was assessed by evaluation of linearity (Cefotaxime 20-140 µg/ml and Sulbactum 10-70 µg/ml respectively), precision intra-day and inter-day RSD values were always less than 2 for them, accuracy (99.66% ±3% for Cefotaxime and 99.51 ±2% for Sulbactum) and specificity, in accordance with ICH guidelines. **Conclusion:** The statistically validated results indicate that the proposed new method has good accuracy and precision. Thus new HPLC method has been successfully applied for the simultaneous estimation of Cefotaxime and Sulbactum in combined dosage form as reconstituted solids.

KEYWORDS

Cefotaxime, Sulbactum, RP-HPLC and Validation.

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INTRODUCTION

Infectious diseases have always been created the threat to human being and animals[1]. Therefore, the treatment is necessary using suitable antimicrobial agents. Although various antibiotics have been developed but cephalosporin group of antibiotics are widely used[2]. Cefotaxime is a third generation cephalosporin antibiotic indicated for the treatment

of patients infected with Septicaemia (Bacterial infection of blood), Bacterial Meningitis, Bacterial Endocarditis, Bacterial infections of lungs and respiratory tract, Bacterial infections of bones and joints. Cefotaxime Sodium (CTX) is chemically (5-Thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylic acid- 3-(acetyloxy) methyl-7-[(2-amino-4-thiazolyl) methoxyimino acetyl] amino] -8-oxo monosodium salt) and structurally represented in Figure No.1. It has highly augmented activity against gram-negative enterobacterias. Sulbactum sodium (SBT) is semi-synthetic parenteral penicillin which inhibits the action of enzyme β lactamase with a broad spectrum of antibacterial activity[3]. Sulbactum sodium (SBT) is chemically known as 4-Thia-1-azabicyclo [3.2.0] heptene-2-carboxylic acid-3, 3-dimethyl-7-oxo-4, 4-dioxide sodium salt[4] and structurally represented in Figure No.2. Sulbactum is official in British Pharmacopoeia while Cefotaxime is official in Indian Pharmacopoeia, as well as in British Pharmacopoeia[5-6]. The combination of CTX and SBT is more effective for the treatment of moderate to severe infections than single drug therapy alone. Although there are so many combination of sulbactum and other cephalosporin on which RP-HPLC method was developed[7-9]. Till date no HPLC method is available for simultaneous estimation of sulbactum and cefotaxime in combined dosage form. Therefore the present work involves the development of a new RP-HPLC method for simultaneous estimation of CTX and SBT in reconstituted solid as combined dosage forms.

MATERIAL AND METHODS

Chemicals and reagents

The reference standards Cefotaxime sodium and Sulbactum sodium were collected as gift samples from R and D Lab of Shreya Life Sciences Pvt. Ltd. Aurangabad. The samples of combined preparation (Taximax Injection of Alkem Laboratories) of Cefotaxime (1g) and Sulbactum (0.5g) were collected from local pharmacies of Aurangabad. Acetonirile (HPLC grade) and potassium dihydrogen phosphate (AR grade) were obtained from Merck, Mumbai, India. Other reagents were of AR grade.

Instruments

Shimadzu 2010 (Japan) Binary Gradient HPLC system consists of G1330B cooler with auto sampler and Quaternary gradient type pumps connected with UV-Visible detector. The data acquisition was performed by Lab Solutions software and chromatographic separation was performed on *a Hypersil C-18 (Gold)* stainless steel column with dimensions of 250×4.6 mm, 5 µm particle size.

Shimadzu UV 1800 with UV Probe data processor was used for determination of an isopiestic point for CTX and SBT in mobile phase.

The pH meter (Labindia) was used for checking the pH of mobile phase.

Shimadzu electronic micro weighing balance was used for weighing.

Preparation of standard stock solution

Accurately weighed quantities of CTX (20mg) and SBT (10 mg) were transferred to a single 100 ml volumetric flask and dissolved in sufficient mobile phase. Then volume was made up to100 ml with mobile phase. 5 ml of above stock solution was pipette out and diluted up to 10 ml with mobile phase to give concentration of 10µg/ml and 5µg/ml of CTX and SBT respectively.

Preparation of sample solution

Sample solutions were prepared by dissolving accurately weighed quantity 100 mg and 50 mg CTX and SBT in 100 ml of volumetric flask and dilute them separately with mobile phase to get the concentration of 1000µg/ml and 500µg/ml respectively. 5 ml of sample stock solution was pipette out and diluted to 50 ml with the mobile phase to give the concentration of CTX is 100µg/ml and SBT is 50µg/ml respectively (Working solutions).

Chromatographic Method Optimization

The sensitivity of the HPLC Method depends upon the proper selection of the detection wavelength. An ideal wavelength is one where compound shows maximum absorbance. Detection wavelength was

selected using isobestic point method where CTX and SBT show optimum absorbance (Figure No.3).

Chromatographic Conditions

HPLC : Shimadzu (Japan) 2010 Binary Gradient system
Column : *Hypersil C-18* (Gold) Stainless steel, 250 x 4.6 mm, 5µm
Column Temperature : 40^0C
Wavelength : 228 nm
Mobile Phase : Acetonitrile: 10mM potassium dihydrogen Phosphate buffer (pH 5.1) (10:90 %v/v)
Flow rate : 1 ml/min
Drug Concentration : CTZ (100 µg/ml) and SBT (50 µg/ml)
Injection Volume : 20 µl
Run time : 15 minutes

A binary mobile phase consisting 10mM potassium dihydrogen phosphate buffer, pH 5.1 and Acetonitrile (90:10 % v/v) was delivered through a column at a flow rate of 1 ml/min. The phosphate buffer, pH 5.1 and Acetonitrile were filtered separately through a 0.45µm membrane filter paper. The mobile phase was degassed before use. HPLC analysis was performed at ambient temperature with detection at 228 nm.

Separately inject equal volumes (about 20 µl) of blank, the Standard preparations of SBT and CTX, and the sample preparation (Assay of Injection) into the chromatograph, record the chromatograms, and measure the peak responses for CTZ and SBT as shown in Figure No.4, 5, 6, 7 and 8.

METHOD VALIDATION

Validation of an analytical procedure is the process by which it is established by laboratory studies that the performance characteristics of the procedure meet the requirements for the intended analytical application. The developed chromatographic method was validated for system suitability, linearity and range, robustness, accuracy, precision, and specificity, as per ICH guidelines[10].

System suitability test

The system suitability test was performed by five replicate analyses of working standard solution. The relative standard deviation of retention times from five replicate injections of standard preparations of CTX and SBT should not be more than 1.0%. The tailing factor for CTX and SBT peak should not be more than 1.5 and the number of theoretical plates should be more than 1000.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to present in the formulation. These might include impurities, degradents, etc.

Specificity of the analytical procedure was demonstrated by performing injections of blank, placebo and sample solution. There was no interference from the blank at the retention time of analyte peak and peak purity data reveals that there are no co-eluting peaks and no interference of impurity at the retention time of CTX and SBT.

Solution Stability

The solution stability is a measure of the capacity of the analyte variation with respect to different time intervals and performed by preparing standard and sample solutions and injecting them separately into HPLC at initial and at different time intervals up to 8 hrs by storing these solutions at 2-8ºC and 25ºC. Cumulative mean was calculated from peak responses and % RSD was calculated from cumulative mean.

Linearity and range

A series of increasing concentrations of working solution were injected under the operating chromatographic conditions and peak areas for each drug were integrated at 228 nm. The calibration curve was plotted between areas against corresponding concentrations of each drug. The concentration range of solution has been decided according to correlation coefficient of regression equation. The regression equations were calculated from the calibration graphs with the standard deviations of the slope and intercept.

Precision (Intra-day and Inter-day Precision)

Precision of the assay was determined by repeatability (within-day) and intermediate precision (between-day). Intermediate precision was

assessed by comparing the assays on two different days.

The Intra and Inter-day precision were determined by assay of sample solutions on the same day at different time intervals and on different days, respectively. Inter-day and Intraday precision were carried out in following concentration range 90, 100, 110 µg/ml of CTX and 40, 50, 60 µg/ml of SBT, respectively.

Accuracy (% recovery)

The accuracy of the method was determined by calculating percent recovery of each drug by standard addition method.

The known amount of sample was spiked in triplicate with Placebo at 80%, 100%, and 120% of target concentration. The concentration of CTX and SBT at each level spiked were quantified as per the proposed method. The percentage recovery was calculated from the amount found and actual amount added.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Therefore a study of robustness will involve an investigation into the effect of these small, but deliberate variations. To evaluate the robustness, the following small deliberate variations were made in the method and the samples were analyzed. The results were compared with method precision results. The mean, SD and % RSD were calculated. Again overall % RSD was calculated for the above results taking into consideration method precision results.

The method was studied by changing flow rate (±0.2 ml/min), change in pH (±0.2) change in mobile phase (±5%v/v) and column compartment temperature (±10˚C) during analysis. Sample solution of 100% concentration was prepared and injected for every condition and retention time was calculated for each condition for CTX and SBT Small deliberate variations made as follows:

Flow rate (± 10%)

Column oven temperature (± 5°C)

Wavelength (± 5 nm)

Change in mobile phase pH (± 0.2)

Change in mobile phase organic content (± 1%)

Assay of tablet dosage form was carried by injecting sample corresponding to equivalent weight of ALH into HPLC system.

Analysis of marketed formulation

Analysis of reconstituted solids of CTX and SBT of combined dosage form was carried out by injecting samples corresponding to equivalent weights of 100 mg and 50 mg CTX and SBT in HPLC system.

RESULTS AND DISCUSSION

System Suitability

All system suitability parameters meet the pre-established acceptance criteria as shown in Table No.1.

Specificity

No peak shall be eluted at the retention time of in blank and placebo or placebo-spiked samples. Hence, the method is specific for determination of CTX and SBT content in marketed sample of tablets as shown in Table No.2. Figure No.4 to Figure No.8 shows the chromatogram of mobile phase (blank), SBT, CTX, standard mixture and test sample respectively. As there is no interference found at retention time of Cefotaxime and Sulbactum peak, which is indicating the specificity of the method.

Stability in analytical solutions

Analytical solutions like standard and sample preparations need to be stored at recommended storage conditions during validation experiments. Therefore, with the objective of assessing storage stability at 5ºC±3ºC and 25ºC±2ºC, samples were prepared and stored in HPLC system and injected at different time intervals up to 8 hrs. The cumulative %RSD for the peak areas should be not more than 2.0. The cumulative % RSD for the peak areas meets the pre-established acceptance criteria (Table No.3).

Linearity

Calibration curve for CTX and SBT were constructed by plotting the concentrations of the sample solutions *versus* peak areas. Figure No.9 and Figure No.10 represent the calibration curves for

CTX and SBT respectively. The regression equation was calculated from the calibration graph, along with the standard deviations of the slope and intercept on the ordinate. The correlation coefficient (CC) value should not be less than 0.9999. The correlation coefficient (CC) value meets the pre-established acceptance criteria.

Precision

The percent Relative Standard Deviation (RSD) of the six replicate samples should not be more than 2.0 and overall % relative standard deviations should be not more than 2.0. The analytical method meets the pre-established acceptance criteria for precision (Table No.4 and Table No.5). Hence the method is highly precise.

Accuracy

The accuracy experiment was performed on five different concentrations (each injected three times) covering the linear range. Table No.6 shows the accuracy measurements represented % recovery at each spiking levels of measurement.

The % recovery of CTX and SBT content at each spiking level should be between 95 and 105%. The analytical method meets the pre-established acceptance criteria for spike recovery study. Hence the method is accurate and precise.

Robustness

Ideally robustness should be investigated as part of method development because a method is not complete without an evaluation of its reliability in routine use, but unfortunately it is often deferred, or completely overlooked, because of the time-consuming nature of the study. Result of variables such as Change in Flow Rate, Column Temperature, pH and organic content of Mobile Phase on drug content was summarized in Table No.7.

Analysis of marketed formulation

Results of assay were in good agreement with the label claim. The drug content in the unit was found to be 101.26% and 98.65% for Cefotaxime and Sulbactum, respectively, are shown in Table No.8.

Initially various mobile phases were tried in attempt to obtain the better separation and good resolution between CTX and SBT combined dosage form. Finally potassium dihydrogen phosphate (10*m*M KH_2PO_4) buffer (pH 5.1): Acetonitrile (90:10 % v/v) was found to be an appropriate mobile phase allowing good separation of both the compounds using, Hypersil C18 (Gold) column at 40°C±5 at 1 ml/min flow rate. As the CTX and SBT exhibit significant absorbance at wavelength 228 nm, therefore it was selected as detection wavelength for simultaneous estimation of CTX and SBT. These optimized conditions had acceptable system suitability parameters indicate good resolution for both the peaks (Table No.1). The value of % Relative Standard Deviation (% RSD) was 0.17 and 0.12 for CTX and SBT, respectively, indicates reproducibility of the method. The Number of theoretical plates for CTX and SBT were 4624 and 4927, respectively, in acceptance limit. Tailing Factors for CTX and SBT were 1.47 and 1.89, respectively, not more than 2 (Table No.2). These values were acceptable and therefore the optimized conditions were used for further analysis. The retention time for SBT and CTX were 4.00 and 8.56 minutes, respectively. The values of correlation coefficient for CTX and SBT (Table No.3) demonstrated the good relationship between peak area and concentration. Therefore the developed method was linear in concentration range of 20-140 µg/ml for CTX and 10-70 µg/ml for SBT (Figure No.9 and Figure No.10). The low value of % RSD in intra-day and inter-day precision (Table No.4 and Table No.5) indicated reproducibility of this method. Percent recovery was 99.71±0.30% for CTX and 99.51±0.51% for SBT demonstrated accuracy (Table No.6). The percentage assay of CTX and SBT in sterile dried powder samples were 101.26% and 98.65%, respectively (Table No.7). The high recovery obtained indicates that the proposed method is highly accurate. Finally, deliberate variations were made to check the significant variations in experimental conditions (Table No.8) suggested robustness of developed method.

Table No.1: Results of system suitability test

S.No	Parameter	Cefotaxime	Sulbactum
1	Retention Time R_t (min) ± SD*	8.54±0.17	3.95±0.12
2	Resolution	More than 2 %	
3	Theoretical Plate	8087	8983
4	Tailing Factor	1.40	1.36
5	Capacity Factor	Less than 2 %	

*****n=5

Table No.2: Results of specificity

S.No	Sample ID	Retention Times (minutes) in chromatograms	
		CTX	SBT
1	Blank	None	None
2	Placebo	None	None
3	Test Sample	None	4.00
4	Test Sample	8.62	None

Table No.3: Results of Solution Stability at 5^0C and 25^0C

S.No	Time of Injections	Reference Standard		Sample Preparation	
		AUC of Reference		AUC of Analyte	
		5^0C	25^0C	5^0C	25^0C
1	0 hr	1705306	1705388	176345	176358
2	2 hrs	1705958	1705775	176357	176469
3	4 hrs	1706705	1707425	176368	176575
4	6 hrs	1707861	1707861	176385	176892
5	8 hrs	1709095	1709025	177353	176905
6	**Mean**	1706985	1707095	176561.1	176639.8
7	**% RSD**	0.1158	0.1029	0.2234	0.1404

Table No.4: Intraday and Interday Precision of CTX

S.No	Conc (µg/ml)	Intraday Precision of CTX (Percentage)					Interday Precision of CTX (Percentage)				
		Trial I	Trial II	Trial III	Mean % Recovery	% RSD	Trial I	Trial II	Trial III	Mean % Recovery	% RSD
1	90	99.63	99.69	99.05	99.45	0.465	99.15	99.26	99.37	99.26	0.837
2	100	99.58	99.56	99.61	99.59	0.235	99.74	99.35	99.44	99.51	0.892
3	110	99.37	99.83	99.55	99.58	0.256	99.38	99.44	99.68	99.5	0.856

Table No.5: Intraday and Interday Precision of SBT

S.No	Conc (µg/ml)	Intraday Precision of SBT (Percentage)					Interday Precision of SBT (Percentage)				
		Trial I	Trial II	Trial III	Mean % Recovery	% RSD	Trial I	Trial II	Trial III	Mean % Recovery	% RSD
1	40	99.89	99.78	99.93	99.86	0.557	99.99	99.48	99.53	99.66	0.745
2	50	99.78	99.82	99.86	99.82	0.658	99.48	99.80	99.54	99.60	0.856
3	100	99.65	99.79	99.74	99.72	0.687	99.52	99.59	99.54	99.55	0.789

Table No.6: Statistical Validation of Recovery Data for HPLC Method

S.No	Level of % Recovery	Mean% Recovery		SD *		%RSD *	
		CTX	SBT	CTX	SBT	CTX	SBT
1.	80%	99.63	99.47	0.248	0.301	0.250	0.303
2.	100%	99.71	99.51	0.298	0.049	0.298	0.049
3.	120%	99.65	99.56	0.156	0.208	0.157	0.208

*****n=3

Table No.7: Robustness studies of CTX and SBT

S.No	-	Change in mobile phase Ph			Flow rate (ml/min)			Change in mobile phase composition(v/v)			Column Temperature	
1	-	4.9	5.1	5.3	0.8	1.0	1.2	15:85	10:90	05:95	30^0C	40^0C
2	**CTX**	10.88	8.66	10.5	13.16	8.54	7.80	5.73	8.55	5.30	11.88	8.55
3	**SBT**	4.17	3.99	4.09	5.17	4.12	3.43	3.75	4.12	3.93	4.29	4.09

Table No.8: Results of Assay of Marketed formulation

S.No	Parameter	Cefotaxime	Sulbactum
1	Label Claim (mg)	1000	500
2	Actual content found (mg)	1012.6	493.25
3	% Avg. Assay	101.26%	98.65%

*****n=3

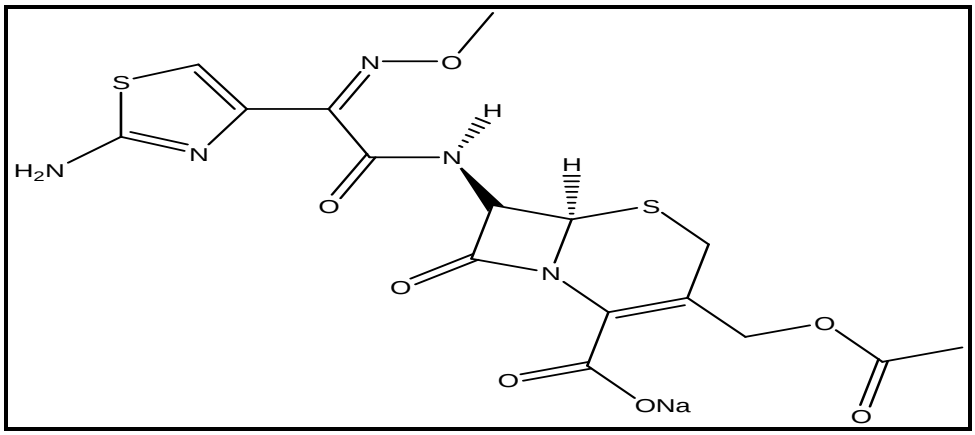

Figure No.1: Chemical structure of Cefotaxime Sodium (CTX)

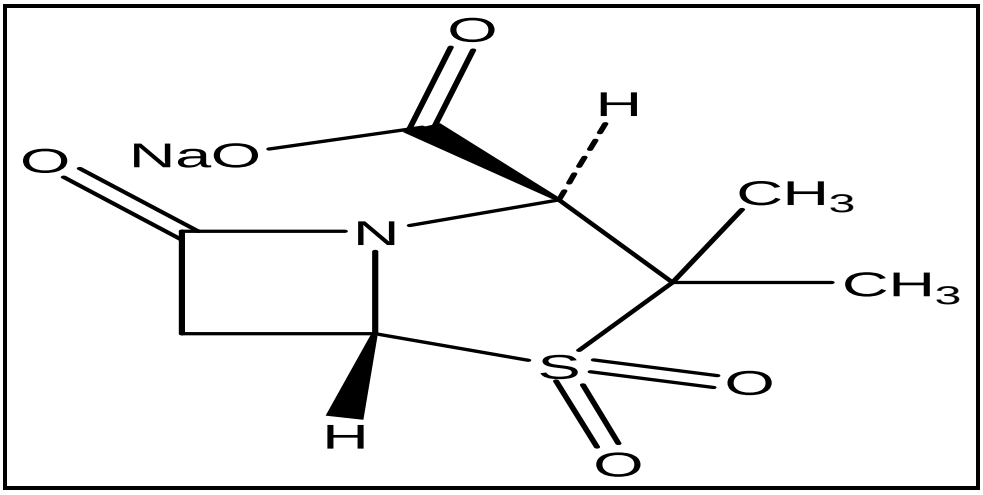

Figure No.2: Chemical structure of Sulbactum Sodium (SBT)

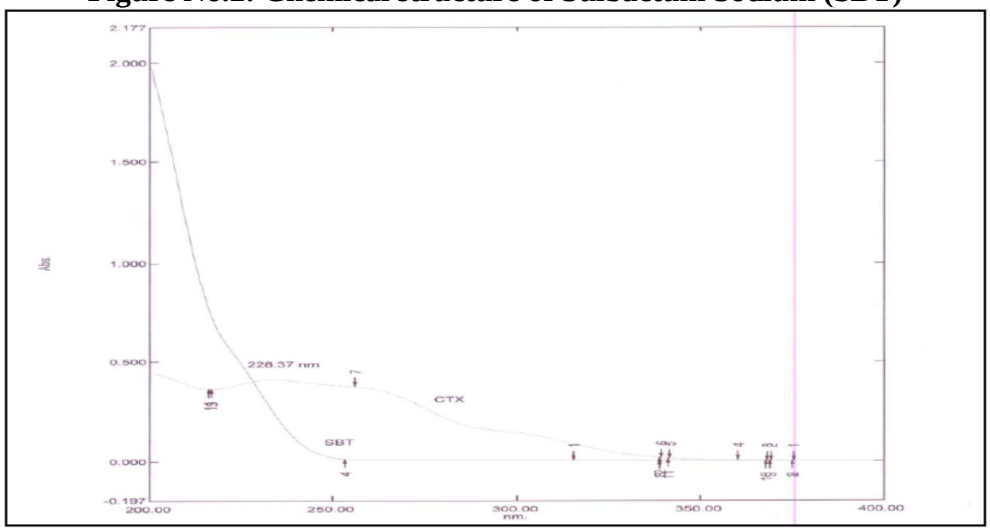

Figure No.3: Overlay UV Spectrum of Cefotaxime (CTZ) and Sulbactum (SBT) Gives an isobestic point at 228.37 nm in mobile phase

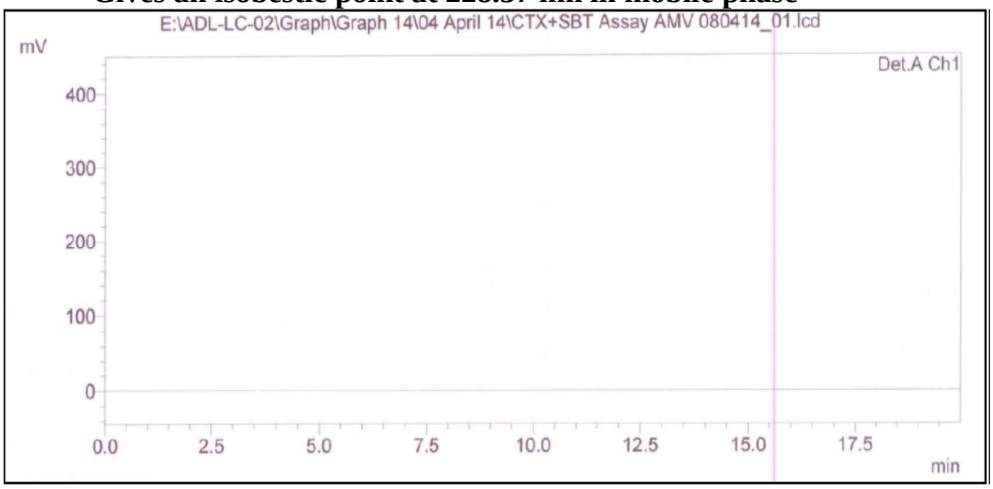

Figure No.4: Chromatogram of Mobile phase (blank) at 228 nm

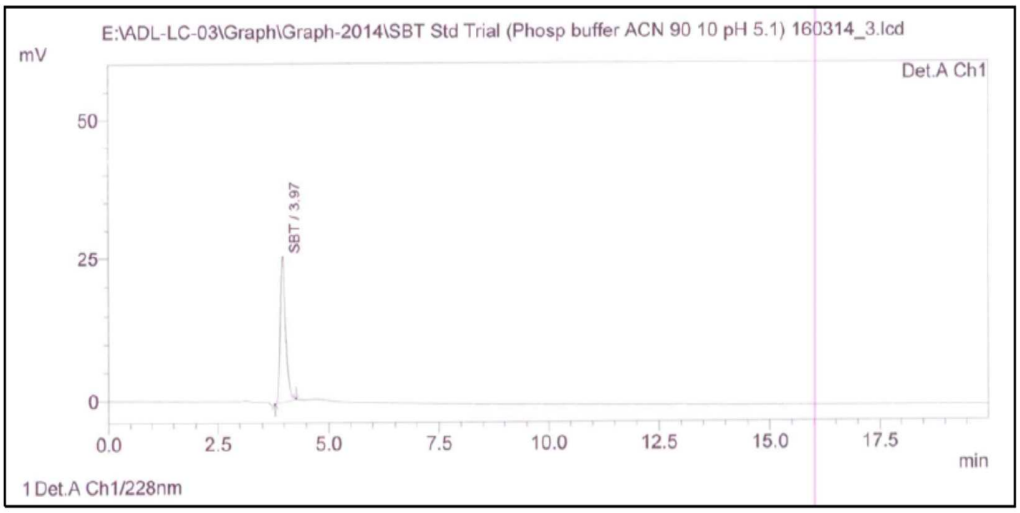

Figure No.5: Chromatogram of Salbactum (SBT) shows R_t 3.97 min

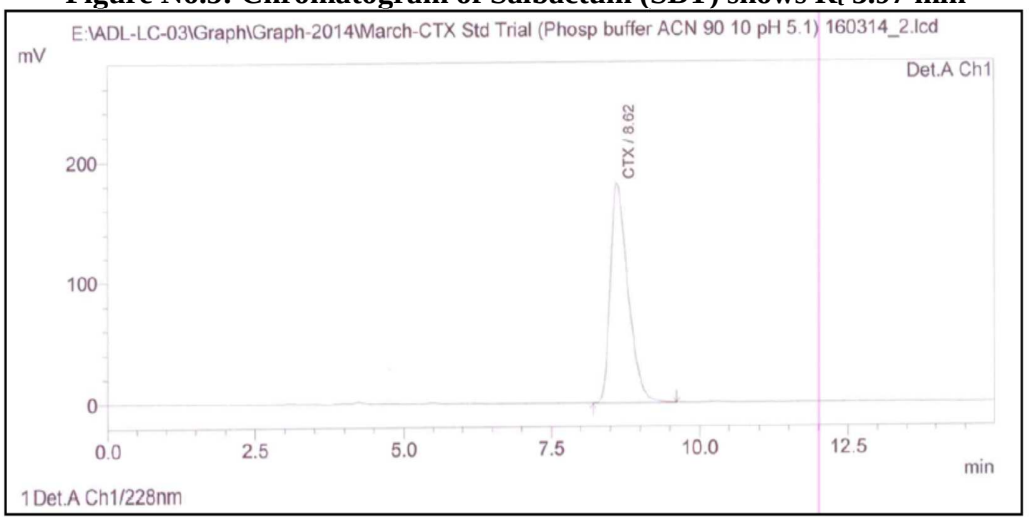

Figure No.6: Chromatogram of Cefotaxim (CTX) shows R_t 8.62 min

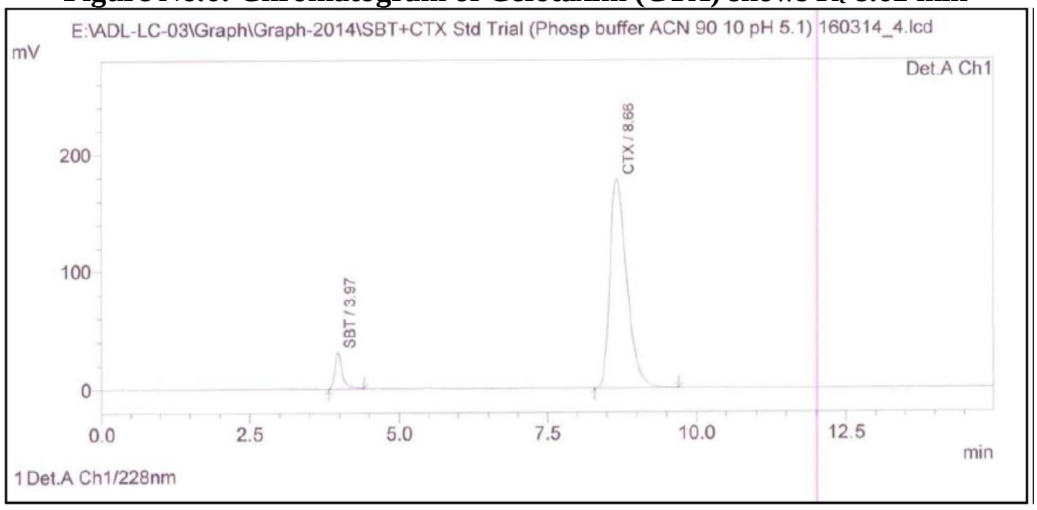

Figure No.7: Chromatogram of Standard mixture of CTX and SBT shows good resolution

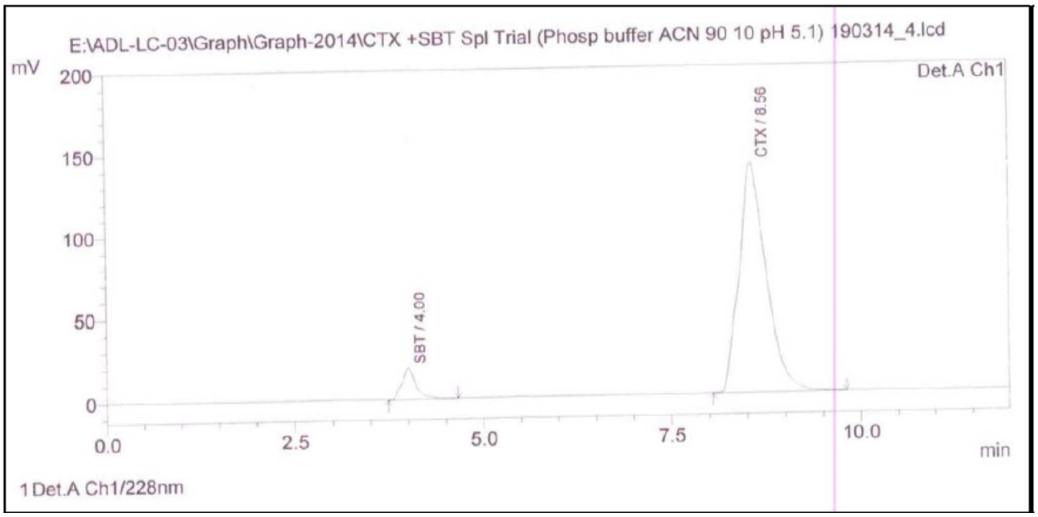

Figure No.8: Chromatogram of Sample mixture (Injection) of CTX and SBT shows good resolution

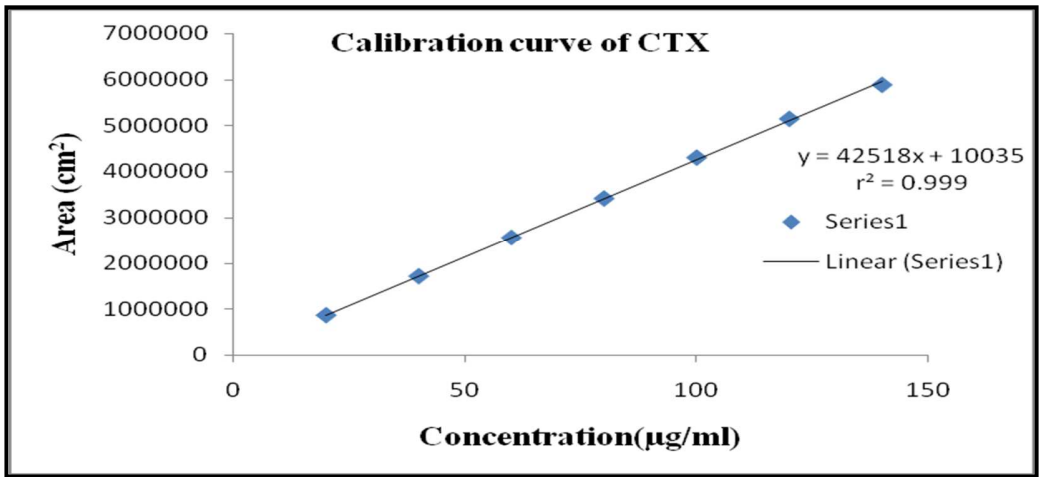

Figure No.9: Calibration curve during linearity experiment of CTX

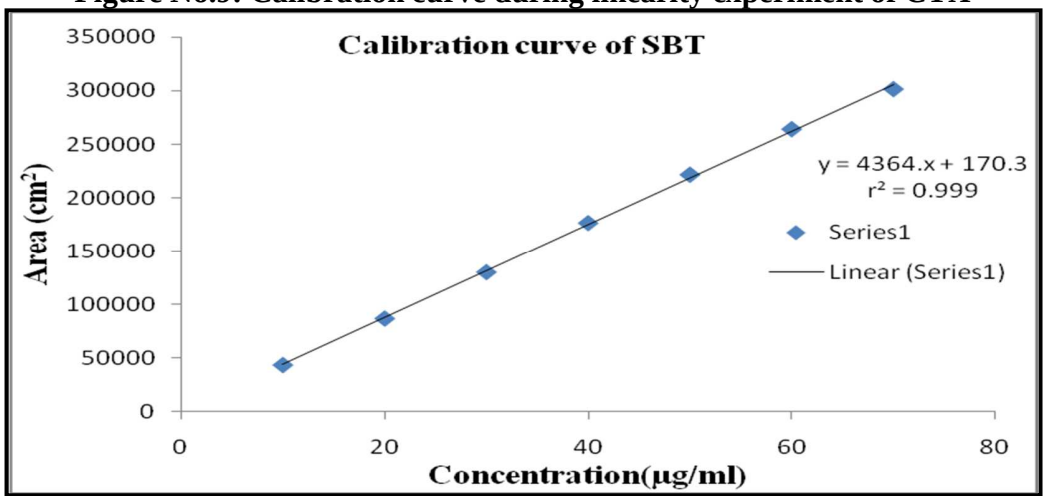

Figure No.10: Calibration curve during linearity experiment of SBT

CONCLUSION

The present work represents the first report that deals with simultaneous analysis of cefotaxime and sulbactum in bulk and sterile dried injection dosage forms using RP-HPLC. It can be concluded from the results that the proposed method is new, accurate, robust and precise. This method was validated as per ICH guidelines. Thus, it can be used for routine quality control studies for assay of Cefotaxime and Sulbactum.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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