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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ACECLOFENAC AND CYCLOBENZAPRINE HYDROCHLORIDE IN PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

A simple, precise and accurate stability indicating RP-HPLC method has been developed and subsequently validated for the simultaneous estimation of Aceclofenac and Cyclobenzaprine Hydrochloride. The separation was carried out using Inertsil ODS C_{18} column (250mm x 4.6mm, 5µm), mixture of 0.05 M Potassium dihydrogen phosphate (PH 5.0): Methanol:Trietylamine 70:30:01 % v/v as a mobile phase with a flow rate of 1 ml/min and the effluent was monitored at 210 nm using UV detector. The retention time of Cyclobenzaprine Hydrochloride and Aceclofenac were 3.200 min and 5.093 min respectively. The method is linear over the range of 1.5-4.5µg/ml and 20-60µg/ml for Cyclobenzaprine Hydrochloride and Aceclofenac respectively. The method was found to be precise, accurate and specific during the study. The percentage recoveries were found to be 99.140%-100.971% and 100.495%-101.487% for Cyclobenzaprine Hydrochloride and Aceclofenac respectively. Cyclobenzaprine Hydrochloride and Aceclofenac were subjected to stress condition to check the degradation behaviour of them. The drugs undergo degradation under acidic, basic, oxidative, thermal and photolytic condition. The proposed method enables rapid quantification and simultaneous analysis of both drugs from commercial formulations without any interference of excipients. So, the method can be used for routine analysis of Cyclobenzaprine Hydrochloride and Aceclofenac in combined tablet formulation.

KEYWORDS: Cyclobenzaprine Hydrochloride and Aceclofenac, RP-HPLC, Stability indicating.

INTRODUCTION

Aceclofenac (ACE) is designated chemically as 2-[2-[2-(2,6-dichloroanilino)phenyl]acetyl]oxyacetic acid.[1] (Figure 1) Aceclofenac is a non-steroidal anti-inflammatory drug (NSAID) with marked anti-inflammatory and analgesic properties. It is orally administered for the relief of pain and inflammation in osteoarthritis, rheumatoid arthritis and ankylosing spondylitis. Aceclofenac belongs to BCS Class II as it possesses poor aqueous solubility. Aceclofenac is also reported to be effective in other painful conditions such as dental and gynaecological conditions.[2]

Cyclobenzaprine Hydrochloride (CBP) designated chemically as 3-(dibenzo[1,2-a:1',2'-e][7]annulen-11-ylidene)-N,N-dimethylpropan-1-amine;hydrochloride[3] (Figure 2) Cyclobenzaprine Hydrochloride is a centrally acting muscle relaxant, chemically similar to amitriptyline hydrochloride with antidepressant activity. The exact mechanism of action of cyclobenzaprine hydrochloride has not been fully determined. However, it primarily acts at the brain stem to reduce tonic somatic motor activity, influencing both gamma and alpha motor neurons. This leads to a reduction in muscle spasms. Cyclobenzaprine Hydrochloride exhibits anticholinergic activity, potentiation of norepinephrine and antagonism of reserpine.it does not directly act on the neuromuscular junction or the muscle but relives muscle spasms through a central action, possibly at the brain level.[4]

Various analytical methods have been reported for the estimation of Aceclofenac and Cyclobenzaprine Hydrochloride as alone in formulation, as well as in combination with other drugs.[5-7]

Literature review reveals methods such as RP- HPLC[8] for Aceclofenac and Cyclobenzaprine Hydrochloride in combination but it does not reveal stability indicating RP-HPLC method of Aceclofenac and Cyclobenzaprine Hydrochloride Therefore, attempt was made to develop and validate simple, precise and accurate, stability indicating RP-HPLC method for estimation of both the drugs in their combination. The parent guideline on drug stability testing Q1A (R2) issued by international conference on harmonization stipulates stress studies be carried out on a drug in order to establish the drug's inherent stability characteristics.[9]

MATERIALS AND METHODS

Instruments and Chromatographic Conditions

Young lin HPLC system was used for method development, degradation studies and validation. Data acquisition was performed on YL 9100 HPLC software. The separation were achieved on Inertsil ODS C_{18} (250 × 4.6 mm, 5µm) column. The column was maintained at room temperature and the eluent was monitored at 210 nm using UV detector. The mixture of 0.05 M Potassium dihydrogen phosphate (PH 5.0): Methanol: Trietylamine 70:30:01 % v/v as a mobile phase with a flow rate of 1 ml/min was used as a mobile phase. The injection volume was 20µl.

Reagents and Chemicals

Aceclofenac and Cyclobenzaprine Hydrochloride were obtained as gift samples from RMR Pharma, Ahmedabad. ACE and CBP combined dosage form tablets were purchased from local market. HPLC grade Water and Methanol, and Potassium Dihydrogen Phosphate of AR grade were obtained from Finar Chemicals Ltd.

Preparation of mobile phase

- **Preparation of 0.05 M phosphate buffer (pH 5):** Weigh accurately and transferred 6.80 gm of KH_2PO_4 into 1000 mL of Volumetric flask. Added 500 mL of HPLC grade water & shaken to dissolved. Diluted up to the mark with HPLC grade water and mixed. Adjusted pH 5 with diluted Adjusted pH 5.0 with 0.1N NaOH.

- **Preparation of mobile phase:**

Composition: 0.05 M PhosphateBuffer (pH 5.0): Methanol: Triethylamine (70:30:0.1).

Take 70ml of Buffer (pH 5.0) and 30ml of Methanol, Mix well and Sonicate in Sonicator for 15 minutes to degas it. Then Filter it with 0.45µ Membrane Filter Paper.

Preparation of stock solutions

- **ACF standard stock solution: (400µg/mL)**

40 mg of ACF was weighed and transferred to a 100 mL volumetric flask. Volume was made up to the mark with mobile phase.

- **CBP standard stock solution: (30µg/mL)**

30 mg of CBP was weighed and transferred to a 100 mL volumetric flask. Volume was made up to the mark with mobile phase, take 10ml from this solution and transfer to 100ml volumetric flask and volume was made up with mobile phase.

- **Preparation of standard Working solution of binary mixtures of ACF (40µg/mL) and CBP (3µg/mL)**

1 mL from the ACF stock solution and 1mL from CBP stock solution was taken and transferred to 10 mL volumetric flask and volume was made up to the mark by mobile phase.

- **Preparation of peak ID solution of Aceclofenac (40 µg/ml)**

40 mg of ACF was weighed and transferred to a 100 mL volumetric flask. Volume was made up to the mark with mobile phase. 1 ml of this solution was taken and dilute upto mark with mobile phase in 10 ml volumetric flask.

- **Preparation of peak ID solution of Cyclobenzaprine Hydrochloride (3 µg/ml)**

3 mg of CBP was weighed and transferred to a 100 mL volumetric flask. Volume was made up to the mark with mobile phase. 1 ml of this solution was taken and dilute upto mark with mobile phase in 10 ml volumetric flask.

- **Preparation of formulation solution**

Tablet Powder equivalent to 200 mg of ACE and 15 mg of CBP was transferred to a 100 ml volumetric flask, 60 ml of Mobile phase was added and Shaken for 15 minutes and made up volume up to the mark with mobile phase. The solution was filtered through Whatman filter paper no. 42 and first few drops of filtrate were discarded. 2 ml of solution was diluted to 10 ml. and again 1 ml of this solution was diluted to 10 ml with mobile phase to get 40 µg/ml ACE and 3 µg/ml CBP.

System Suitability testing

System suitability tests were performed to verify that the resolution and repeatability of the system were adequate for the analysis intended. The parameters monitored for system suitability includes retention time, theoretical plate number, peak area, tailing factor and resolution. The repeatability of these parameters was checked by injecting three times the test solution of 40µg/ml of ACE and 3µg/ml of CBP. The results shown in Table-1 were within acceptable limits.

Forced Degradation Studies

Forced Degradation Studies of the drugs, in combination, were performed under different stress conditions as mentioned in ICH guideline Q1A (R2). The standard solution containing 40µg/ml ACE and 3µg/ml CBP was subjected to acidic, alkaline, oxidative, thermal and photolytic stress condition. Acidic and alkaline degradation were performed using up to 0.1N strength of acid/base at different temperature. Oxidative stress studies were carried out for using 3-20% H_2O_2. The active pharmaceutical ingredients i.e. both ACE and CBP were subjected to thermal and photo degradation as explained in detail below.

A) Acid Degradation

Accurately measured 1 ml of sample stock solution was transferred into 50 ml volumetric flask and 1 ml 0.1 N HCl was added and solution was heated for different time period for acid hydrolysis. Then the solution was neutralized with 1 ml 0.1 N NaOH made volume up to mark with diluent to get 40µg/ml of Aceclofenac and 3µg/ml of Cyclobenzaprine Hydrochloride and filtered through 0.45µm membrane filter paper and injected in to HPLC system. Similarly solutions were prepared for respective conditions.

B) Alkali Degradation

1 ml of Aceclofenac standard stock solution and 1 ml of Cyclobenzaprine Hydrochloride standard stock solution were transferred in 10 ml volumetric flask. 1 ml of 0.1N NaOH was added and reflux for 8 hours. Cooled to room temperature and added 1 ml of 0.1N HCl and make up the volume upto the mark with diluent and mixed. Inject into HPLC system. Similarly solutions were prepared for respective conditions.

C) Peroxide Degradation

Oxidation decomposition studies were performed by Transferring 1ml of stock solution in to 10 ml of volumetric flask. 1 ml of 3% H_2O_2 solutions was added and mixed well and put for 6 hrs. After time period the volume was adjusted with diluents to get 40µg/ml for ACF and 3µg/ml for CBP.

D) Thermal Degradation

Thermal decomposition studies were performed by transferring 400mg ACE and 30mg CBP drug powder in Petri dish. Petri dish was stored in oven at 80°C for 3hrs thermal hydrolysis. Powder equivalent to 40 mg ACE and 3 mg CBP was weighed and transferred into 100 ml volumetric flask then volume was made up to the mark with diluent.(400µg/ml ACE and

30µg/ml CBP) One ml of stock solution was transferred in to 10 ml of volumetric flask. The volume was adjusted with diluent to get 40µg/ml for ACE and 3µg/ml for CBP and filtered through 0.45 µm membrane filter paper and injected in to HPLC system. Similarly solutions were prepared for respective conditions mentioned in table for thermal degradation optimization.

E) Photo Degradation

1 ml of ACE standard stock solution and 1 ml of CBP standard stock solution were transferred in 10 ml volumetric flask. Exposed under U V chamber for 6,12,24 hrs. diluted up to the mark with diluent and mixed Inject into HPLC system. Similarly solutions were prepared for respective conditions.

Method Validation

1) Specificity

Specificity of method can be termed as absence of any interference at retention times of samples. Specificity was performed by injecting blank and standard preparations. Chromatograms were recorded and retention times from sample and standard preparations were compared for identification of analytes. Specificity graph of ACE and CBP given in figure 4-6.

2) Linearity and Range

The linearity for ACF and CBP were assessed by analysis of combined standard solution in range of 20-60µg/ml and 1.5-4.5µg/ml respectively. An aliquot of 20µl of each solution was injected 3 times for each standard solutions and peak area was observed. Plot of average peak area versus the concentration is plotted and from this the correlation coefficient and regression equation were generated. The calibration data of ACE and CBP is given in Table 3 and 4.

3) Precision

The method was validated in terms of intra-day inter-day precision. The solution containing 40µg/ml of ACE and 3µg/ml of CBP was injected six times for repeatability study. Inter-day and Intra-day study was performed by injecting 20, 40 and 60µg/ml of ACE and 1.5, 3 and 4.5µg/ml of CBP solutions three times for each aliquots. The % RSD for precision study was found less than 2% as shown in Table 5.

4) Accuracy

The accuracy of the method was determined at 50%, 100%, and 150% level by calculating recoveries of ACE and CBP by the standard addition method. Known amount of standard solutions of ACE (10, 20, 30µg/ml) and CBP (0.75, 1.5, 2.25µg/ml) were added to a pre-quantified sample solution of ACE (20 µg/ml) and CBP (1.5µg/ml). Each solution was injected in triplicate and the percentage recovery was calculated by measuring the peak areas and fitting these values into the regression equation of the respective calibration curves. The result given in Table no-6-7.

5) LOD and LOQ

LOD and LOQ for ACE and CBP were calculated as suggested by ICH guidelines using equations LOD = 3.3 σ/s and LOQ = 10 σ/s, respectively. Where, σ is the SD of the response and S is the slope of the calibration curve.

6) Rubustness

Following parameters were changed one by one and their effect was observed on system suitability for standard preparation. The Robustness data of ACE and CBP is given in Table-8.

1. Flow rate of mobile phase was changed (± 0.02 ml/min) 0.98 ml/min and 1.02 ml/min.
2. Ratio of Mobile phase was changed (±2) Buffer: Methanol (68:32) and Buffer: Methanol (72:28)
3. pH of buffer was changed (±0.2) pH 4.8 and pH 5.2

RESULT AND DISCUSSION

System suitability study

The Detection was carried out in the UV region at 210nm. The different composition of mobile phase was testing and the composition giving retention time of 3.200 min for CBP and 5.093 min for ACE with good resolution and theoretical plates was selected, that optimized mobile phase was 0.05 M KH_2PO_4 (pH 5.0):Methanol: Triethylamine (70:30:0.1% v/v). A chromatogram of the mixture in optimized conditions is shown Figure 3 and the system suitability parameters are shown in Table 1.

Forced degradation study

The mentioned percent degradation of both ACE and CBP is with respect to their decrease in the areas. Peak purity of the drug is not affected. There are few impurities peaks have been

observed for acidic, basic, oxidative, thermal and photolytic degradation. Thus, the conditions subjected to the drugs make them undergo forced degradation thereby being able to detect any difference in the response in terms of their areas and impurities. The final results for the stress conditions are shown in Table 2.

Method Validation

A) Specificity

The method was found to be specific as there was no interference observed in any of the parameters under observation.

B) Linearity and Range

The linearity for ACF and CBP were assessed by analysis of combined standard solution in range of 20-60µg/ml and 1.5-4.5µg/ml respectively.

C) Precision

The % RSD for repeatability study for ACE and CBP was found to be 1.83 and 1.76 respectively. The Inter-day and Intra-day study also show % RSD value for ACE and CBP within the acceptable limit. Results for precision study are shown in Table 5.

D) Accuracy

Accuracy of the method was confirmed by recovery study at three levels (50%, 100% and 150%) of standard addition. Percentage recovery for ACE was found to be 100.495%-101.487%, while for CBP it was found to be 99.140%-100.971%.as shown in Table 6-7.

E) LOD and LOQ

The LOD was found to be 2.187 µg/ml for ACE and 0.183 µg/ml for CBP, while the LOQ was found to be 6.626 µg/ml for ACE and 0.553 µg/ml for CBP.

F) Robustness

The typical variations studied under this parameter were mobile phase composition and detection wavelength. Overall % RSD was found to be less than 2% for all the variations which indicates that the proposed method is robust. Robustness data are shown in Table-8.

G) Analysis of marketed formulation by proposed method

Applicability of the proposed method was tested by analyzing the commercially available marketed formulation. The percentage of A C E and CBP was found to be 98.53% for ACE and 99.24% for CBP. Results as % Assay are shown in Table 9.

Table 1: Results for System suitability parameters.

Parameters	Aceclofenac (mean ± SD)(n=3)	Cyclobenzaprine Hydrochloride (mean ± SD)(n=3)
Retention time (min)	3.209 ± 0.04	5.097 ± 0.05
Theoretical plate	4532 ± 7.45	4944 ± 7.81
Asymmetry	1.302 ± 0.03	1.212 ± 0.029
Resolution	9.71 ± 0.18	

Table 2: Forced degradation results for ACE and CBP.

Degradation Method	Condition	% Degradation	
		CBP	ACE
Acid	1ml 2 N HCl For Reflux, 24 h	5.08%	13.40%
Base	1ml 1 N NaOH For Reflux, 12 h	4.18%	18.30%
Oxidation	1 ml 10 % H_2O_2, 24 h	18.02%	20.15%
Thermal	4 hours at 80ºC	1.16%	3.57%
Photo	UV light (254 nm) for 12 hours	0.56%	4.46%

Table 3: Linearity study data for ACE.

Sr. No	Concentration (µg/ml)	Area ± SD (n=3)	% RSD
1	20	2613.479±10.77	0.41
2	30	4015.012±39.91	0.99
3	40	5251.281±92.24	1.76
4	50	6377.316±10.01	0.16
5	60	7641.672±28.30	0.37

Table 4: Linearity study data for CBP.

Sr. No	Concentration(µg/ml)	Area ± SD (n=3)	% RSD
1	1.5	76.612±0.06	0.08
2	2.25	117.132±2.27	1.94
3	3	154.347±2.65	1.72
4	3.75	185.968±2.74	1.48
5	4.5	223.035±2.77	1.24

Table 5: Precision Study For ACE and CBP.

Parameters	Concentration (µg/ml)		%RSD	
	ACE	CBP	ACE	CBP
Repeatability	40	3	1.834	1.762
Intraday	20	1.5	0.238	1.066
	40	3	1.461	1.548
	60	4.5	0.774	1.534
Interday	20	1.5	0.372	0.720
	40	3	0.545	1.046
	60	4.5	0.526	1.263

Table 6: Recovery data for ACE.

SR. NO.	Conc. Level (%)	Sample amount (µg/ml)	Amount Added (µg/ml)	Amount recovered (µg/ml)	% Recovery	% Mean Recovery ± S.D
1	50 %	20	10	10.137	101.370	101.487 ± 0.155
2		20	10	10.166	101.663	
3		20	10	10.143	101.429	
4	100 %	20	20	19.832	99.160	100.495 ± 1.260
5		20	20	20.132	100.661	
6		20	20	20.333	101.663	
7	150 %	20	30	30.074	100.246	101.025± 0.699
8		20	30	30.369	101.231	
9		20	30	30.479	101.597	

Table 7: Recovery data for CBP.

SR. NO.	Conc. Level (%)	Sample Amount	Amount Added	Amount recovered (µg/ml)	% Recovery	% Mean Recovery ± S.D
1	50 %	1.5	0.75	0.759	101.204	100.890 ± 0.846
2		1.5	0.75	0.762	101.535	
3		1.5	0.75	0.749	99.931	
4	100 %	1.5	1.5	1.483	98.856	99.140 ± 1.097
5		1.5	1.5	1.505	100.352	
6		1.5	1.5	1.473	98.213	
7	150 %	1.5	2.25	2.250	99.213	100.971± 0.984
8		1.5	2.25	2.272	100.973	
9		1.5	2.25	2.294	101.954	

Table 8: Robustness study for CBP and ACE.

Parameters		Area (n=3)	
		ACE	CBP
pH (±0.2)	4.8	5236.766	152.887
	5.0	5125.604	153.604
	5.2	5142.927	151.215
	Mean ± SD	5168.432 ± 59.809	152.568 ±1.22
	% RSD	1.15	0.80
Flow Rate (±0.02 ml/min)	0.98 ml/min	5205.066	156.611
	1.0 ml/min	5125.604	153.604
	1.02 ml/min	5112.12	151.368
	Mean ± SD	5147.597± 50.224	153.861 ± 2.630
	% RSD	0.97	1.70
Mobile Phase Composition Buffer:Methanol (± 2 mL)	68:32	5179.178	154.313
	70:30	5125.604	153.604
	72:28	4989.964 79.964	150.931
	Mean ± SD	5091.582 ± 89.590	152.949 ± 1.783
	% RSD	1.75	1.16

Table 9: Analysis of Formulation of ACE and CBP by Proposed Method.

ACE			CBP		
Labelled amount (mg)	**Amount found (mg)**	**% Assay**	**Labelled amount (mg)**	**Amount found (mg)**	**% Assay**
200mg	193.53	97.24	**15 mg**	14.59	96.76
	196.53	98.74		14.81	98.26
	199.40	99.60		14.94	99.70
Mean ± SD	196.486 ±1.19	98.53±1.19	**Mean ± SD**	14.78 ±1.47	98.24 ±1.47
%RSD	1.21	1.21	**%RSD**	1.49	1.49

FIGURES

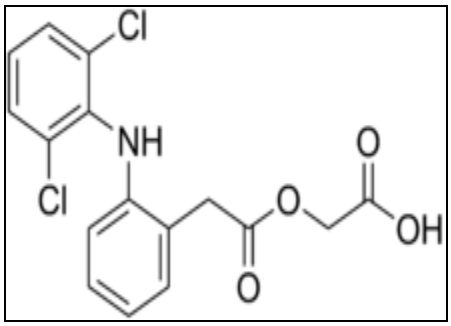

Fig.1 Chemical structure of Aceclofenac.

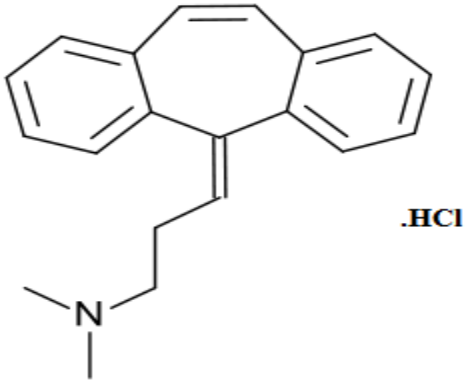

Fig.2 Chemical structure of Cyclobenzaprine Hydrochloride.

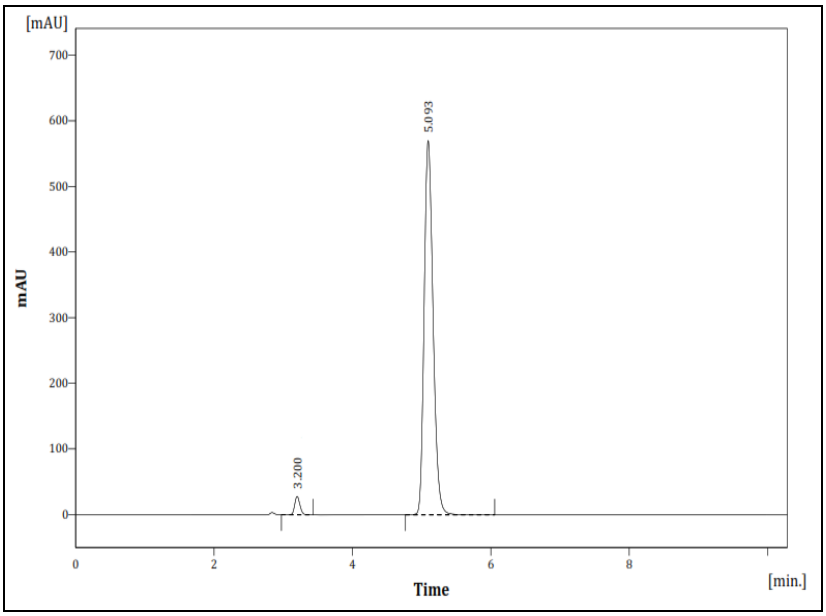

Fig.3 Chromatogram of ACF 40 µg/ml and CBP 3 µg/ml in 0.05M KH_2PO_4, pH 5.0: Methanol: Triethylamine (70:30:0.1).

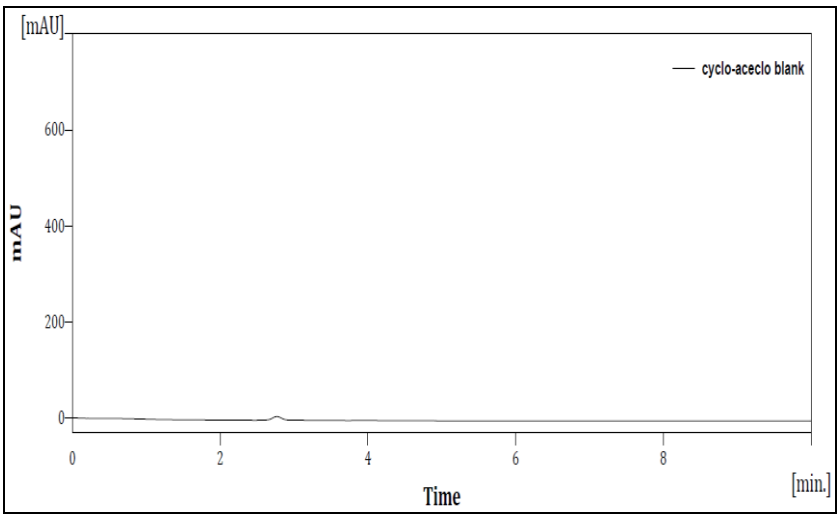

Fig. 4: Specificity chromatogram of diluents.

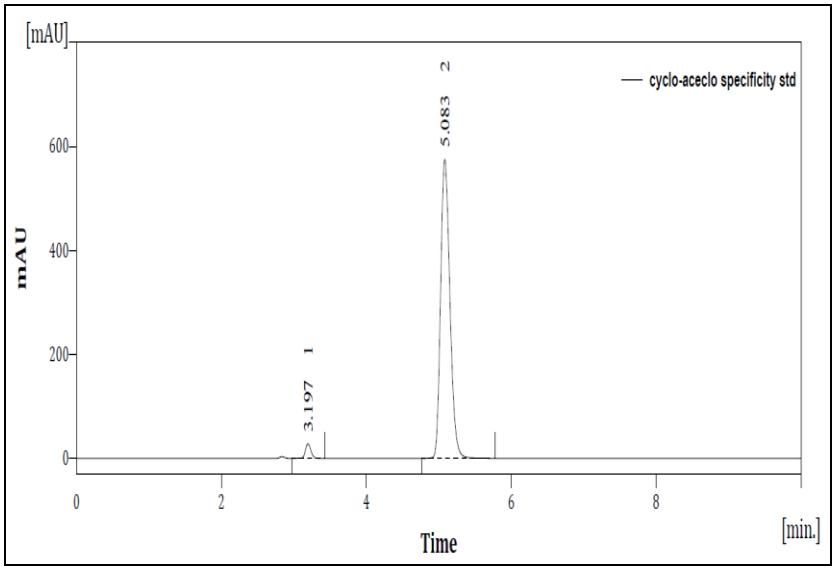

Fig.5: Specificity chromatogram of standard ACE and CBP.

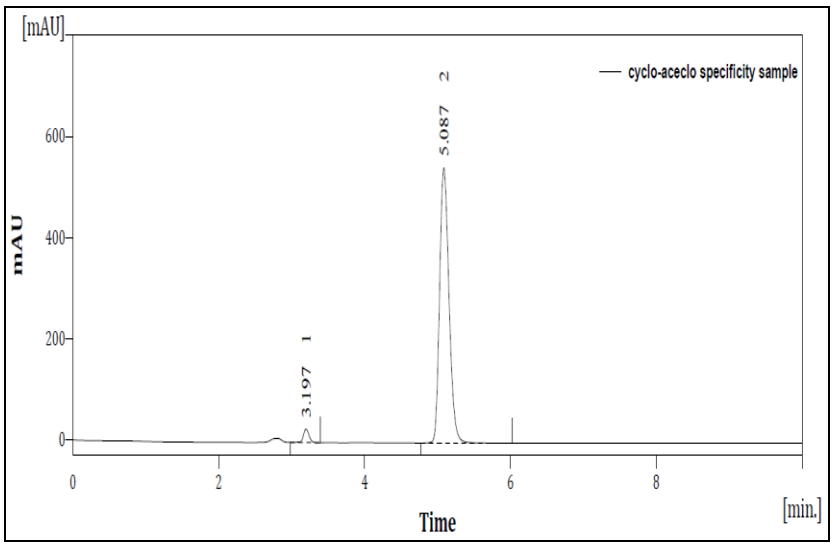

Fig.6: Specificity chromatogram of sample ACE and CBP.

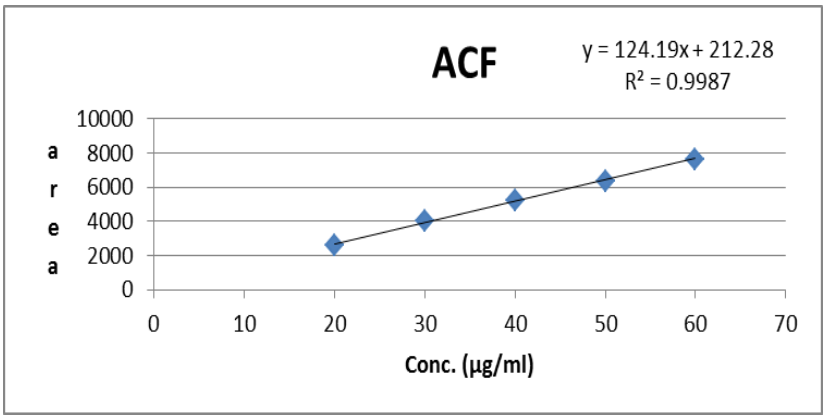

Fig. 4: Calibration Curve of ACF (20-60µg/ml).

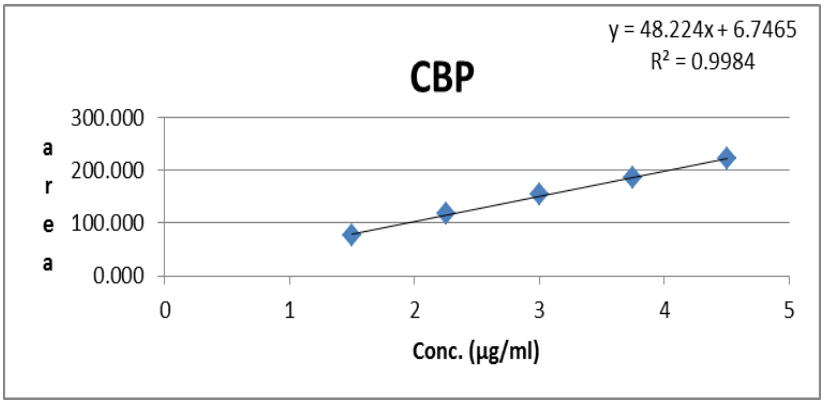

Fig.5: Calibration Curve of CBP (1.5-4.5 µg/ml).

CONCLUSION

From the above discussion it can be concluded that the proposed method is precise, accurate and stability indicating. Results are in good agreement with label claim which indicates there is no interference of exicipients. Therefore the proposed method can be used for routine analysis of Aceclofenac and Cyclobenzaprine Hydrochloride in combined tablet formulation.

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