Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(8):695-705

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Experimental design based development of a liquid chromatographic method for the quantification and validation of related substances in bromfenac Sodium in Bulk drugs

Vishnu Vardhana Rao G.[1], V. Srinivasa Rao[2] and K. Ramakrishna[1]*

[1]*Department of Chemistry, GITAM Institute of Science, GITAM University, Visakhapatman, India*
[2]*Analytical Research and Development, Syngene International Ltd, Biocon Park, Bommasandra Industrial Estate, Bangalore, India*

ABSTRACT

A novel and stability indicating, experimental design assisted liquid chromatographic method developed for the estimation of related substances of bromfenac sodium sesquihydrate. Stability indication of method established by forced degradation study. The chromatographic separation was attained with Kromosil C18,125 x 4.0mm, 5µm using gradient elution using mobile phase-A consists of a mixture of pH 4.8 ammonium acetate buffer and the mobile phase-B consists a mixture of methanol: Acetonitrile (500:500 v/v), respectively. Column temperature maintained at 30◌ C with wavelength detection at 265nm.The develop method is validated as per International Conference on Harmonization (ICH) norms. Central composite experimental design (CCD) was employed to check the robustness of the method.

Keywords: Bromfenac sodium, Liquid Chromatography , Validation, International Conference on Harmonization (ICH), Central composite design (CCD).

INTRODUCTION

Bromfenac is chemically 2-[2-amio-3-(4-bromobenzoyl) phenyl] acetic acid (Figure-1) with the molecular formula is $C_{15}H_{12}BrNO_3$.The yellow powder with molecular mass is 334.16 g/Mol [1,2]. An anti-inflammatory drug for a non-steroidal category for ophthalmic use. It has the ability to block prostaglandin synthesis by inhibiting cyclooxygenase 1 and 2. Bromfenac antagonizes COX by binding to the upper portion of the active site, preventing its substrate, arachidonic acid, from entering the active site. Prostaglandins have been shown in many animal models to be mediators of certain kinds of intraocular inflammation. In studies performed in animal eyes, prostaglandins have been shown to produce disruption of the blood-aqueous humor barrier, vasodilation, increased vascular permeability, leukocytosis, and increased intraocular pressure. The analgesic and anti-inflammatory effects of Bromfenac occur as a result of decreased prostaglandin synthesis [3-5].

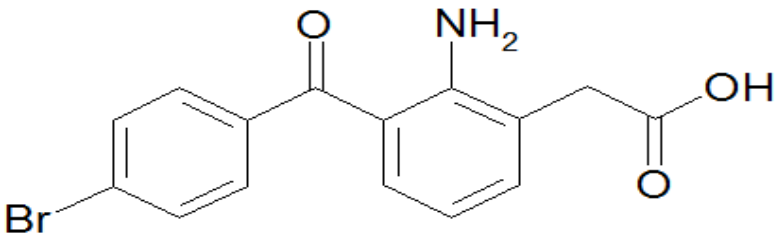


Figure- 1: Bromfenac structure

Various methods in the literatures reveal that several methods have been reported for determination of Bromfenac in bulk drug and dosage forms [6-12]. However, there is no method available for the stability-indicating chromatographic method with experimental design approach for bromfenac. The aim of the present work was, experimental design based development and validation of a selective, specific and stability indicating LC method for the estimation of bromfenac impurities.

EXPERIMENTAL SECTION

Materials and Chemicals

Analytical grade reagents are used in method development and validation activity. Bromfenac sodium drug substance and its impurities were obtained as gift samples. Ammonium acetate, Triethylamine, O- phosphoric acid , Glacial acetic acid , Hydrochloric acid, Sodium hydroxide and Hydrogen peroxide ware purchased from Merck. Acetonitrile and methanol were purchased from Rankem Chemicals.

Chemical names of Bromfenac and its Impurities

a) Bromfenac: 2-[2-amio-3-(4-bromobenzoyl) phenyl] acetic acid
(b) Impurity-A: Sodium salt of [2-amio-3-bromobenzoylphenyl] acetic acid
(c) 7-(4-bromobenzoyl)-3-(methylthio)-1,3-dihydro-2H-indole-2-one
(d) 7-(4-bromobenzoyl)-1,3-dihydro-2H-indole-2-one
(e) 7-benzoyl-1,3-dihydro-2H-indole-2-one

Chromatographic Conditions

Shimadzu HPLC- LC-20AT Prominence equipped with SPD-20A UV detector with LC-Solutions software and Waters Alliance with PDA detector used for analysis.

Buffer preparation for Diluent: Add 1 ml of Triethylamine in 1000 ml of water, adjust p H 9.0 with dilute Ortho-phosphoric acid.

Diluent: Buffer (pH 9.0): Acetonitrile: Methanol: Tetrahydrofuran (50:25:24:1) (v/v).

Mobile phase-A: Dissolve 0.77 g of Ammonium acetate in 1000 ml of water. Added 1.0 ml of Triethyl amine and adjust p H 4.8 with dilute glacial acetic acid.

Mobile phase-B: Prepare a homogenous mixture of Acetonitrile and Methanol (500:500) v/v

*Gradient programme: (*T/%B): 0/40,2/40,25/85,35/85,36/0,40/40
The flow rate of the mobile phase was 1.0 m/min. The column temperature maintained at 30◌ C and the wavelength was monitored at 265 nm. The injection volume was 20µLwith sample cooler temperature 10◌ C.

Procedure

Preparation of Impurity stock solutions and Reference solutions

A stock solution of each impurity at 15µg/ml was prepared in diluent. Prepared reference solution (a) consists of bromfenac at 500 µg/ml and each impurity at 0.75 µg/ml in diluent and also prepared reference solution (b) which consists of bromfenac at 0.5 µg/ml in diluent.

Preparation of sample solutions

Prepared the sample solution containing bromfenac at 500µg/ml in diluent.

RESULTS AND DISCUSSION

Method Optimization

The method conditions were optimized after testing with different parameters such as column and buffer, mobile phase ratio, column temperature and flow rate to improve the resolution between Impurity-D and bromfenac and also maintain optimum resolution between impurity-C and Bromfenac. The initial trials were taken with different buffers with various pH values. At the combination of Mobile phase-A having pH 4.8 buffer with Mobile phase-B having 50:50v/v of Methanol and Acetonitrile gives an optimum resolution between peaks of interest with the maximum plate count. Diluent A mixture of Buffer (pH 9.0): Acetonitrile: Methanol: Tetrahydrofuran (50:25:24:1) (v/v) used as diluent. Detection was performed at 265 nm, where the expected degradation peaks and impurities were expected to absorb. Forced degradation samples and impurities blend solution provides optimum resolution with the Gradient programme of *(*T/%B): 0/40,2/40,25/85,35/85,36/0,40/40 at sample temperature 10◌ C.

***Method validation**:* The recommended method was validated as per ICH procedures [13]

System suitability and Specificity

A blank,system suitability solution and diluted standard solution,all individual impurities at the specification level,impurity spiked solution and sample solution of bromfenac sodium were prepared and injected. The system suitability parameters and retention times and relative retention times of known impurities are recorded in Table-3 and Table-7.

To establish the non-interference of blank, Standard and sample solutions prepared as per procedure and injected into the chromatograph system. A typical chromatogram of blank and reference solution (a) were shown in Figure-2 and Figure-3 respectively. Specificity is the ability of the method to measure the analyte response in its degradation studies. Significant degradation was observed only in acid (0.1N HCl) degradation. Optimum degradation observed in 0.5% peroxide. No significant degradation was not observed in Base (0.1 N NaoH), Photo, Humidity conditions. In thermal degradation 0.32% degradation was observed. Peak purity criteria meet the requirement for all impurities and bromfenac. All the degradation impurities and co-eluting peaks are well separated with optimum resolution. It was observed that the % of impurity-C is increased by acid and Thermal degradations. Results of degradation study and impurity data of degradations are given in Tabel-1, Table-2 respectively, and the typical chromatograms of degradation samples are shown in Figure-4

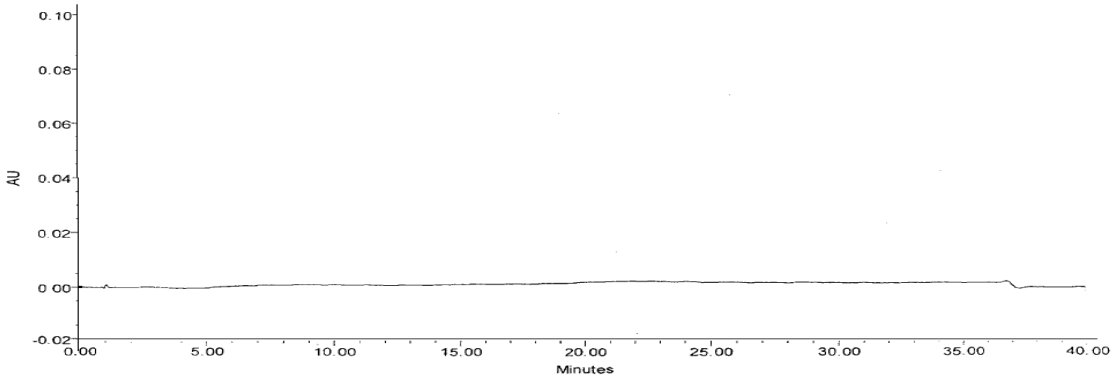


Figure-2: Chromatogram of blank

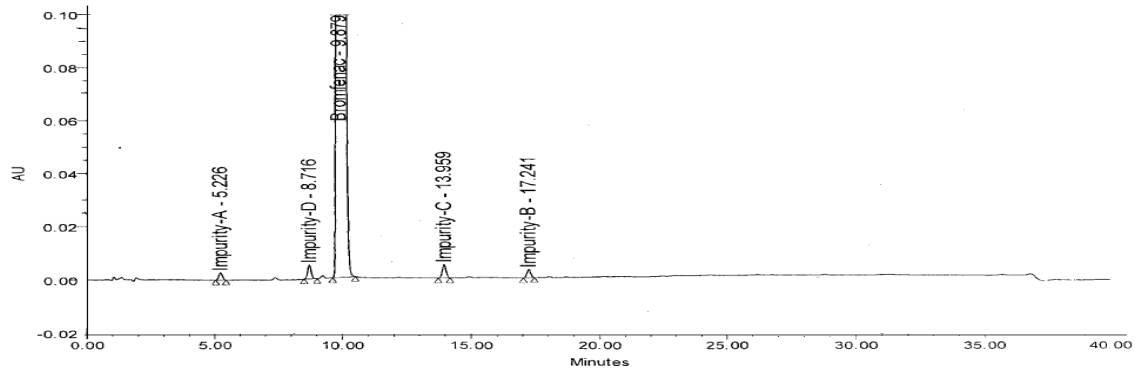
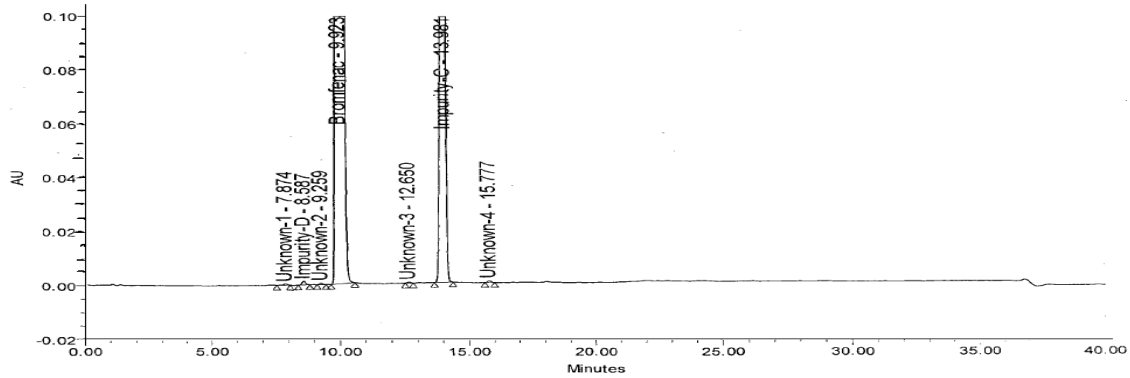


Figure-3: Chromatogram of Reference solution(a)

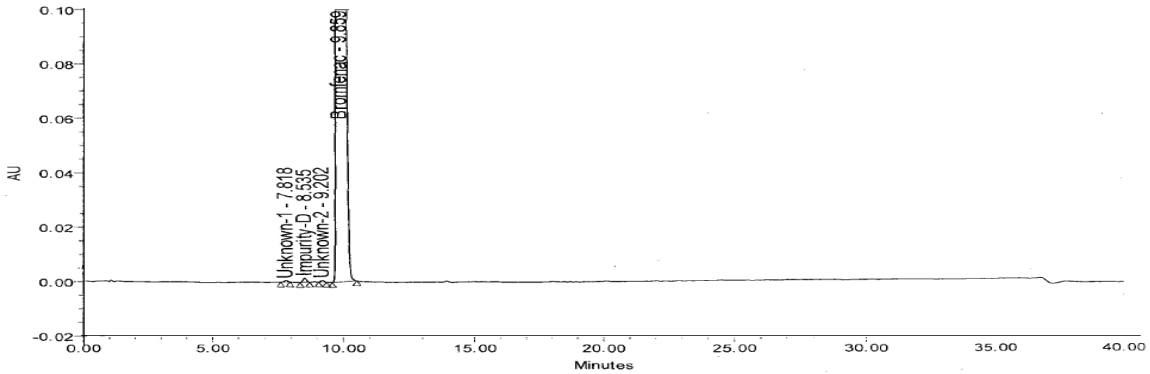
Table-1: Data of degradation study results of bromfenac

Stress condition	% Degradation	*Peak purity index of Bromfenac
Acid stressed(0.1 N HCl, 60^0 C, 30min)	19.26	Pass
Base Stressed (0.1 N NaoH, 60^0 C, 2Hrs)	0.11	Pass
Thermal stressed (150^0 C, 24Hrs	0.32	Pass
Oxidation (0.5% H_20_2, 30 min, Room Temperature)	11.98	Pass
Photolytic (200w.hr /m^2 in UV light and 1.2 M Lux fluorescent light)	0.12	Pass
Humidity	0.11	Pass

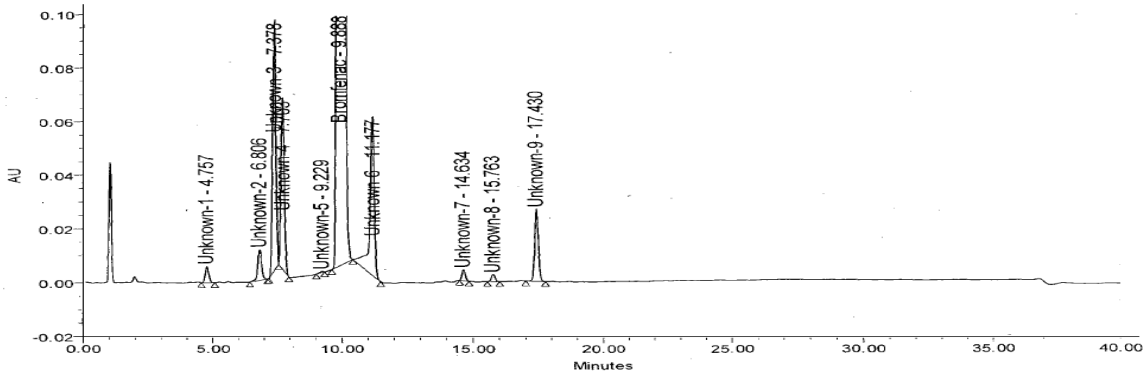
**Peak purity is considered as passing, when purity angle should be less than purity threshold.*



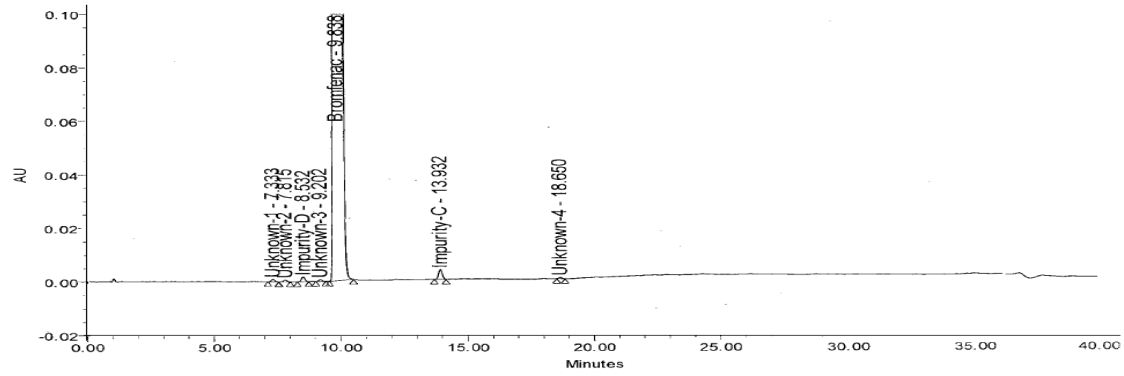
(a) Acid degradation



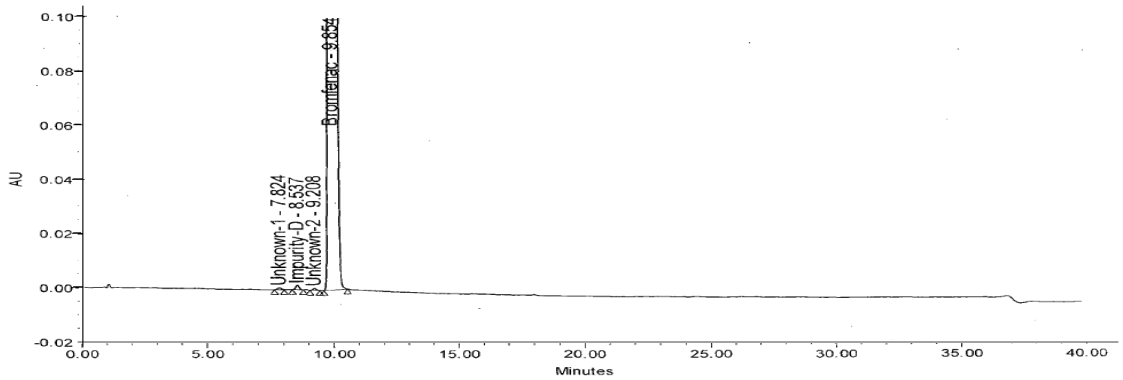
(b) Base degradation



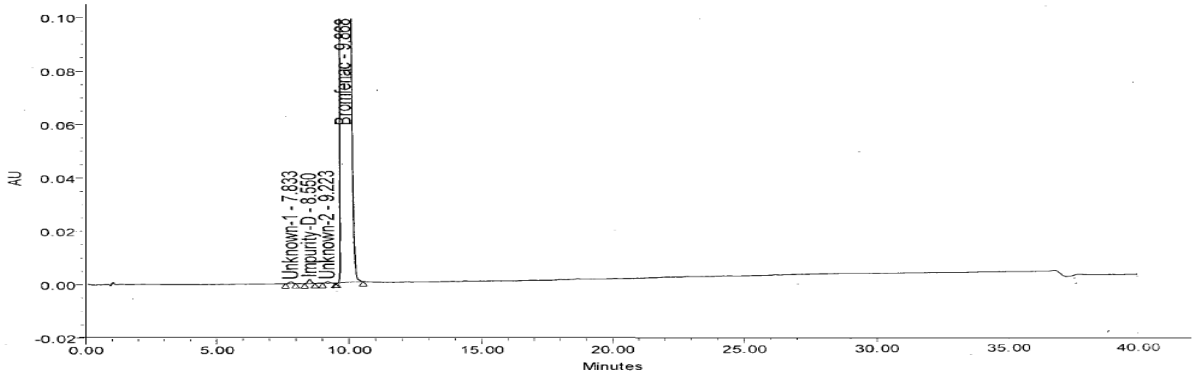
(c) Peroxide degradation



(d) Thermal degradation sample



(e) Photo degradation sample



(f) Humidity degradation sample

Figure-4: Typical chromatograms of Bromfenac degradation sample

Table-2: The impurity profile of bromfenac under degradation conditions

Stress condition	Impurities RRT's												
	0.48	0.69	0.75	0.78	0.87	0.93	1.13	1.27	1.41	1.48	1.59	1.76	Total Impurities
					Imp-D				Imp-C				
Acid	-	-	-	0.02	0.05	0.02	-	0.01	19.13	-	0.02	-	19.26
Base	-	-	-	0.02	0.06	0.02	-	-	-	--	-	-	0.11
Thermal	-	-	0.03	0.03	0.07	0.03	-	-	0.15	-	-	-	0.32
Oxidation	0.28	0.53	3.82	2.58	-	0.02	3.21	-	-	0.17	0.12	1.22	11.98
Photo	-	-	-	0.03	0.07	0.03	-	-	-	-	-	-	0.12
Humidity	-	-	-	0.03	0.06	0.03	-	-	-	-	-	-	0.11

Relative response factors (RRF) for known impurities

Relative response factor was established for known impurities were established by the linear co-relation coefficient of each impurity and bromfenac impurity. Slope value obtained with linearity calibration plot was used. Established RRF values for each impurity were tabulated in Table-3.

Table-3: RRF values of bromfenac known impurities

S.No	Description	Slope	RRF	Retention time of Individual impurities	RRT (Relative retention time)
1	Bromfenac sodium	50668.8	1.00	9.789	1.00
2	Impurity-A	41502.9	1.22	5.226	0.52
3	Impurity-B	49343.8	1.03	17.241	1.59
4	Impurity-C	70211.0	0.72	13.959	1.32
5	Impurity-D	71047.5	0.71	8.716	0.85

Method Precision

Method precision has been established by analyzing six sample preparations spiked with known impurities. To evaluate the method precision, study was established by repeatability and intermediate precision experiments. Intermediate precision was established by performing the precision study on a different day with a different analyst under same analytical conditions. Calculate each known impurity and single maximum unknown impurities. Mean % impurity and % RSD were calculated. The % RSD of known and total impurities are less than 15% indicates the method was precise. Results are tabulated in Table-4

Limit of detection (LOD) and Limit of Quantification (LOQ)

LOD, LOQ experiment was carried out from the lowest concentration of each impurity to bromfenac, to find out the quantification and detection limit for each impurity based on the standard deviation of response and slope method [14]. Precision at LOQ and LOD were performed by injecting six injections of LOQ concentrations to find the %RSD. The LOD, LOQ values and precision %RSD values are reported in Table-4. Less than 15% RSD was the acceptance criteria of each impurity at LOQ precision.

Table-4: Method precision, intermediate precision, LOD, LOQ and Linearity data of Bromfenac

Parameter	Impurity A	Impurity B	Impurity C	Impurity D	Single unknown	Total impurities	Bromfenac
Precision (n=6) %Mean (%RSD)	0.125 (0.42)	0.143 (1.17)	0.138 (0.90)	0.155 (0.64)	0.025 (4.22)	0.605 (0.28)	-
Intermediate precision (n=6) %Mean, (%RSD)	0.118 (1.12)	0.155 (3.14)	0.148 (1.12)	0.152 (0.57)	0.023 (0.99)	0.615 (0.45)	-
Overall precision (n=12) (%RSD)	3.10	4.62	3.79	1.04	7.06	0.89	-
Linearity range (μgmL^{-1})	0.0224-1.0087	0.0596-1.3415	0.0155-1.1598	0.0160-1.2011	-	-	0.0246-1.1066
Correlation coefficient	0.9994	0.9994	1.0000	1.0000	-	-	1.0000
[@]LOQ (%)	0.005%	0.010%	0.003%	0.003%	-	-	0.005%
[@]LOD (%)	0.0015%	0.004%	0.001%	0.001%	-	-	0.0015%
LOQ precision (%RSD)	2.69	3.49	1.99	2.95	-	-	1.99
LOD precision (%RSD)	11.8	9.9	9.1	10.9	-	-	13.0

@ Impurities % reported with respect to bromfenac concentration

Linearity

Linearity was established by preparing seven levels of concentrations from LOQ to 150% of specification limit for each impurity and bromfenac. A standard stock solution was prepared and further diluted to attain concentrations of seven levels. The obtained correlation coefficient was greater than 0.999 (Table-4).The linearity established with bromfenac is applicable to unspecified impurities.

Accuracy

Accuracy was performed by spiking all known impurities in the test preparation at LOQ to 150% of specification limit. Samples were prepared in triplicate at each level and analyzed. The % individual recovery and % mean recovery for each level was calculated and reported in Table-5.This indicates the method was more accurate for intended use. The recovery results at each level are well within the acceptable criteria of 85% to 115% and the %RSD for each level is found less than 15.

Table-5: Accurate data for bromfenac impurities

Level	LOQ		50%		100%		150%	
Name	% Mean recovery	% RSD	%Mean Recovery	% RSD	% Mean Recovery	% RSD	% Mean recovery	% RSD
Impurity-A	105.1	1.70	93.3	0.21	92.5	0.26	93.9	0.38
Impurity-B	91.3	3.54	96.3	3.85	100.1	0.47	102.3	1.24
Impurity-C	108.7	2.28	100.4	6.80	95.1	0.44	95.9	0.60
Impurity-D	99.4	6.19	104.7	0.59	103.6	0.40	105.9	0.51

Solution stability at 10^0C

To establish the solution stability of the sample solution, sample was weighed as per methodology and initial % impurity was determined. Separately weighed and preparation stored at 10◌̊ C for different time intervals like 1hr,2hrs,6hrs,12hrs,24hrs,36hrs and 48hrs.The impurity data at each time interval was reported in Table-6.From the results it was concluded that the sample solution was stable up to 48hrs.

Table-6: Impurity profile of bromfenac sample solution at 10◌ C in different time intervals

Time interval	Impurity A	Impurity B	Impurity C	Impurity D	Single unknown	Total impurities
Initial	ND	ND	ND	0.05	0.025	0.093
1hr	ND	ND	ND	0.05	0.024	0.095
% difference	-	-	-	0.00	0.001	0.00
2hr	ND	ND	ND	0.048	0.025	0.095
% difference	-	-	-	0.002	0.00	0.002
6hr	ND	ND	ND	0.049	0.025	0.097
% difference	-	-	-	0.001	0.00	0.004
12hr	ND	ND	ND	0.05	0.024	0.097
% difference	-	-	-	0.00	0.001	0.004
18hr	ND	ND	ND	0.049	0.025	0.097
% difference	-	-	-	0.001	0.00	0.004
24hr	ND	ND	ND	0.050	0.024	0.097
% difference	-	-	-	0.00	0.001	0.004
36hr	ND	ND	ND	0.050	0.025	0.098
% difference	-	-	-	0.00	0.00	0.005
48hr	ND	ND	ND	0.052	0.025	0.099
% difference	-	-	-	0.002	0.00	0.006

Robustness

As defined by ICH, robustness study was performed to establish the ability of method to remain unaffected for slight changes in the method conditions [15] like flow (1.0±0.1ml min^{-1}), Column temperature ($30+2^0C$), pH of mobile phase-A (pH 4.8±0.2). No substantial effect was observed on system suitability parameters like resolution and theoretical plates. The results were shown in Table-7.In all the above variable conditions the reproducibility results are found within the limit.

Table-7: System suitability parameters of Precision, intermediate precision and robustness data

Parameter	Resolution between Imp-D and Bromfenac (>3)	Resolution between Imp-C and Bromfenac (>6)	Theoretical plates of bromfenac from reference solution (a) (>8000)	% RSD n=6 (b) (<5%)
System suitability	3.87	13.30	11254	0.9
Precision	4.73	14.13	15847	0.2
Intermediate precision	3.96	11.47	11682	0.2
Flow-1.1 ml min^{-1}	4.60	13.24	11529	1.7
Flow-0.9 ml min^{-1}	5.66	12.19	15236	0.9
Column temperature 32^0C	5.74	12.31	14056	0.9
Column temperature 28^0C	5.98	12.11	15147	0.8
pH of mobile phase-A (4.6)	4.79	13.41	16392	2.3
pH of mobile phase-A (5.0)	4.73	13.44	16463	1.6

Experimental Design Approach

Design expert software (Stat-Ease Inc, Statistics made easy, Minneapolis, MN,USA, Version 9.0) was used for experimental design. In order to establish the simultaneous changes of factors on the considered responses, an approach using experimental design suggested for robustness study. A Response surface method was used to obtain maximum information and to observe the performance of response around the nominal values of the factors. Response surface methodology (RSM) has more advantages [16-17]. Generally a huge number of experiments required by standard design employed in RSM disenchant their use in the validation. If the method is fast and required a few factors, a good choice of robustness testing may be central composite design (CCD) [18], widely used due to its high competence with respect to the runs. In order to observe the variables at no more than 3 levels (-1,0,+1), the design used for the robustness of bromfenac was a CCD with D=±1.

Table-8: Factors and levels studied for robustness

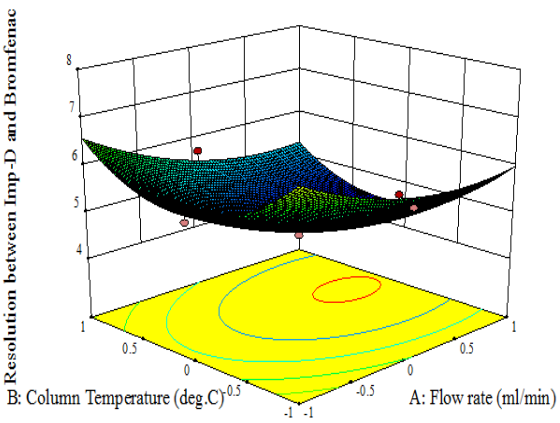
Factor	Levels		
	-1	0	+1
Flow rate mLmin^{-1} (A)	0.9	1.0	1.1
Column temperature ◌ C (B)	28	30	32
pH of mobile phase-A (C)	4.6	4.8	5.0

Three factors were considered :flow rate mLmin^{-1}(A): Column temperature ^{0} C (B) and pH of mobile phase-A(C). The factors and levels considered for study are shown in Table-8. Precision sample prepared by spiking all impurities at their bromfenac impurity concentration. The critical resolution between Impurity-D & bromfenac, Resolution between Impurity-C& bromfenac and Theoretical plates were studied as responses.

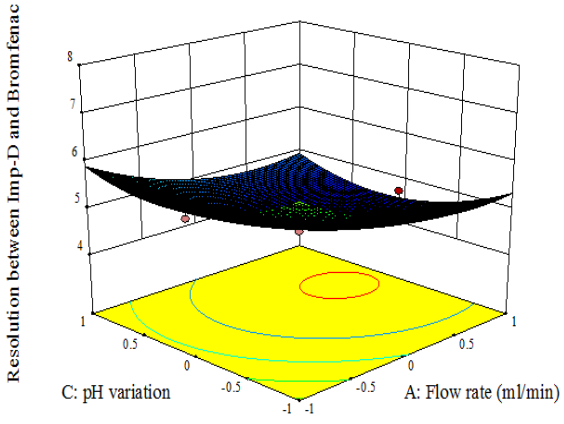
Table 9: The responses obtained from the standard run of robustness study

		Factor 1	**Factor 2**	**Factor 3**	**Response 1**	**Response 2**	**Response 3**
Std	Run	A: Flow rate	B: Column Temperature	C: pH variation	Resolution between Imp-D and Bromfenac	Resolution between Imp-C and Bromfenac	Theoretical plates
		ml/min	deg.C				
1	4	-1	-1	-1	7.9	11.6	15890
2	10	1	-1	-1	6.8	12.7	11745
3	3	-1	1	-1	7.5	12.9	14328
4	9	1	1	-1	6	13	11037
5	6	-1	-1	1	7.3	11.1	15673
6	16	1	-1	1	6.4	12.6	13256
7	11	-1	1	1	6.8	11.8	15781
8	12	1	1	1	5.6	12.9	13457
9	1	-1	0	0	5.5	12.1	15201
10	13	1	0	0	4.7	13.4	11453
11	15	0	-1	0	5.8	12.1	15087
12	5	0	1	0	5.7	12.3	14359
13	7	0	0	0	4.7	14.3	15768
14	14	0	0	0	4.6	14.7	15759
15	2	0	0	0	4.8	14.5	15972
16	8	0	0	0	4.5	14.4	15734

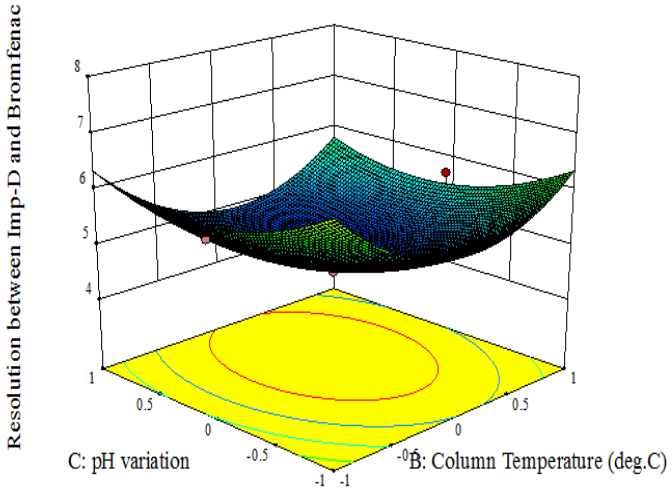
The ranges identified where small deviations from the method settings and the subsequent responses in the resolutions and the Theoretical plates considered (Y) were observed. A three factor CCD requires 16 experiments, including two center points. Standard run order created by design expert is reported in Table-9.By using the full quadratic model, a response surface regression data for every response factor was conducted using coded units.Table-10 shows the values calculated for the coefficient and p-values.The coefficient differs from zero significantly and the p-value<0.05 then the factor is considered to effect the response.



(a)

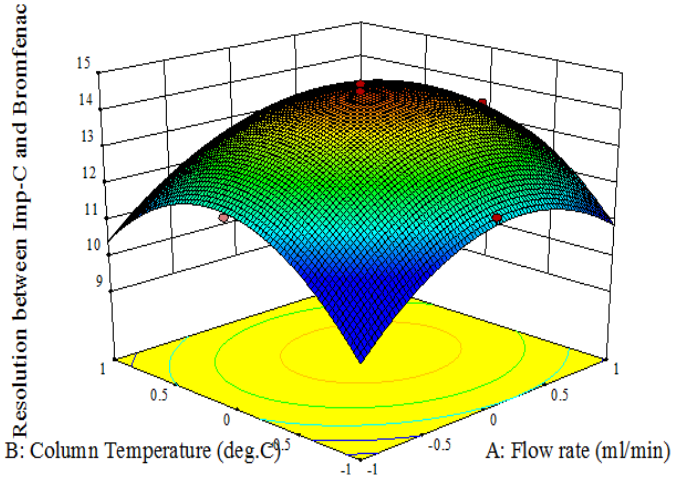


(b)

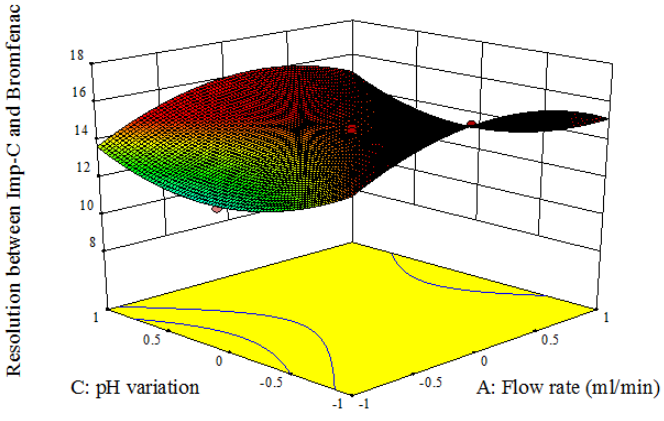


(c)

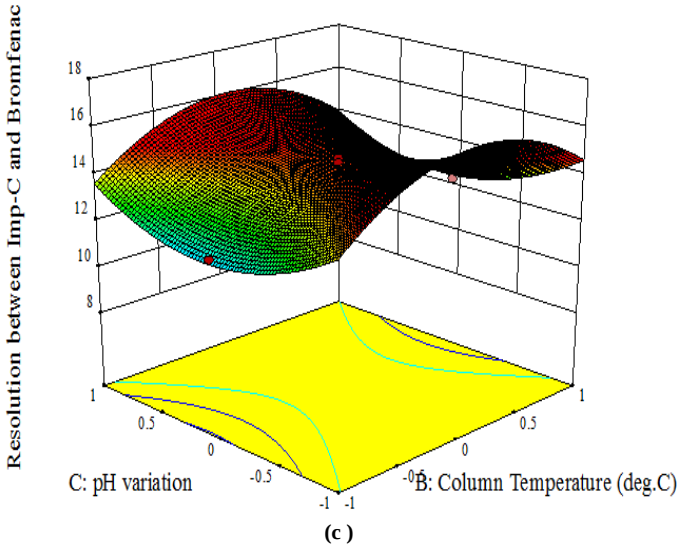
Figure 5:Three dimensional plot of the response surface for resolution between Impurity-D and Bromfenac
(a) Variation response as a function of A and B;fixed C
(b) Variation response as a function of A and C;fixed B
(c) Variation response as a function of B and C;fixed A



(a)



(b)



(c)

Figure 6:Three dimensional plot of the response surface for resolution between Impurity-C and Bromfenac
(a) Variation response as a function of A and B;fixed C
(b) Variation response as a function of A and C;fixed B
(c) Variation response as a function of B and C;fixed A

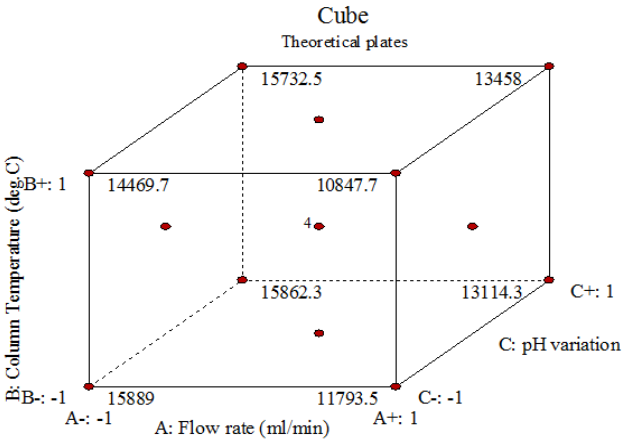


Figure 7: Three dimensional cubical representation of response for plate count

Table 10:Regression coefficient and probability values of responses

	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
Constant	4.65	0	14.47	0	15808.25	0
A-Flow rate	-0.55	0.0001	0.51	0.0002	-1592.50	0.0001
B-Column Temperature	-0.26	0.0050	0.28	0.0055	-268.90	0.0110
C-pH variation	-0.26	0.0080	-0.22	0.0227	645.88	0.0002
AB	-0.087	0.2416	-0.18	0.0558	118.38	0.2030
AC	0.063	0.3892	0.17	0.0558	336.88	0.0066
BC	-0.012	0.8589	-0.075	0.3496	322.38	0.0081
A^2	0.45	0.0343	-1.73	0.0001	-2481.25	0.0001
B^2	1.10	0.0006	-2.27	0.0001	-1085.25	0.0017
C^2	0.59	0.0391	1.85	0.0003	1654.13	0.0009

The model was validated by ANOVA. The statistical analysis shown in Table-10.The analysis produces three dimensional representations by plotting the response against two of the factors, and the third one kept constant at a desired level as shown in figure-5 and 6, and the cubical representation of plate count as a response in figure-7.From the Table-10,the p-values for any of the studied factors are noted. It shows that the method is highly robust for describing variations.

CONCLUSION

A novel and accurate stability indicating HPLC method for the estimation and quantification of bromfenac related substances in the presence of degradation products was established. The behavior of bromfenac under different degradation conditions was studied. The method validation data shows satisfactory results for all conditions. The key component's relation was studied through experimental design assessment. A good understanding of the factors effected chromatography method and great confidence in the ability of the method, and also this approach ensures great design of the product. The developed method is stability indicating and can be used for regular analysis analysis.

Acknowledgments
The authors would like to thank M/s Gitam University for supporting this work.

REFERENCES

[1] www. drugbank.ca/drugs/ DB00963
[2] www.chemicalbook.com
[3] https://www.medicineindia.org/pharmacology-for-generic/2922/bromfenac-sodium
[4] http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm116123.pdf
[5] http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_Public_assessment_report/human/001198/WC500107577.pdf
[6] Jia-yi K, Biao WU, Hong-xiao CUI. *Chinese Journal of Pharmaceutical Analysis* (**2009**), 04-05.
[7] Schmitz G, Lepper H, Estler CJ. *Journal of Chromatography B: Biomedical Sciences and Applications* **(1993),** 620(1),158-63.
[8]Gumbhir-Shah K, Cevallos WH, DeCleene SA, Halstenson CE, Korth-Bradley JM. *Ann Pharmacother* **(1997),** 31(4), 395-359.
[9] Boni JP, Cevallos WH, DeCleene S, Korth-Bradley JM. *Pharmacotherapy* **(1997),** 17(4),783-790.
[10] Osman M, Chandrasekaran A, Chan K, Scatina J, Ermer J, Cevallos W, et al, *J.Clin.Pharmacol*,**(1998),** 38,744–752.
[11] Bhavani Boddeda ,Lanka.Rama Prasad, J Vijaya Ratna, Asim Biswal, *International Journal of Pharmacy and Pharmaceutical Sciences*, **(2013)**,5(Suppl 2).
[12] Alpa N. Parmar, Rajesh R Parmar, Dr. Vishnu M. Patel , Dr. Dushyant A. Shah, , *JPSBR*, **(2012),**2(1), (36-39).
[13] ICH Q2 (R1),Validation of Analytical Procedures: Text and Methodology,(**2005)**
[14] M. Ribani, C. B. Grespan Bottoli, C. H. Collins, I. C. S. Fontes Jardim, and L. F. Costa Melo, *Quimica Nova*, **(2004),** 27(5), 771–780.
[15] Y. Vander Heyden, A. Nijhuis, J. Smeyers-Verbeke, B. G. M. Vandeginste, and D. L. Massart, *Journal of Pharmaceutical and Biomedical Analysis*, **(2001)**, 24(5-6),723–753.
[16] D. K. Lin, *Journal of Quality Technology*, **(1999),** 31,61–66.
[17] H. Fabre, *Journal of Pharmaceutical and Biomedical Analysis*, **(1996)**,14,1125–1132.
[18] Singh, R. Kumar. And N. Ahuja., *Critical Reviews in Therapeutic Drug Carrier Systems*,**(2004)**, 22, 27–105.