ISSN: 2309-5288 (Print)
ISSN: 2309-6152 (Online)
CODEN: JCLMC4

Journal of Coastal Life Medicine

Development and Validation of Stability Indicating Assay Method By HPLC for Estimation Of Benzonatate

Received: 19 October 2022, **Revised:** 16 November 2022, **Accepted:** 26 December 2022

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Keywords:
Benzonatate, HPLC, stability indicating methods, Forced degradation

Abstract
Quality, purity, safety and efficacy of pharmaceuticals are important issues in drug therapy.A greater emphasis is being paid today on assurance of the quality and safety of a drug by monitoring and controlling the impurities and degradation products. The control of impurities and degradation products in dosage form became mandatory critical issue since their presence extensively diminishes both quality and safety of Active Pharmaceutical ingredient (API) and its dosage form. By the identification or characterization and quantification of the impurities and related substances, the risk of their impact on drug and its dosage form can be avoided or minimized. Various regulatory authorities like ICH, USFDA, Canadian Drug and Health agency are even emphasizing on the purity requirements and the identification of impurities in Active Pharmaceutical Ingredient's (API's). A stability indicating hyphenated analytical techniques has been developed and validated for Benzonatate as per International Conference on Harmonization.The Benzonatate standard was exposed to acid hydrolysis, alkali hydrolysis , oxidation , photolytic and thermolytic degradation condition and separated using Column HiQSil C18 (250 ×4.6mm,5µ) Mobile Phase is Methanol (0.01%), Formic Acid (70:30v/v), Flow rate 1ml/min Column Temperature is Ambient Detector set at 308nm RT (min): 4.220 ± 0.787 Asymmetry: 1.12 Plates (N): 4136. This method was validated for linearity, precision, accuracy, ruggedness and robustness. Results obtained after validation study indicating that the proposed single method allowed analysis of degraded product formed of Benzonatateformed under the various stress conditions.

1. Introduction

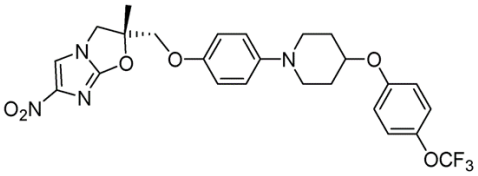

Figure 1: Structure of Benzonatate

Benzonatate is generally used as cough reliever. Benzonatate is an Antitussive that is cough suppressants. It functions by reducing the airways and lungs' natural reflex to cough.

Benzonatate act as non-opioid drug for the symptomatic relief of cough. [1][3] It improves cough associated with the various conditions like tuberculosis, asthama, bronchitis, pneumonia, emphysema. Benzonatate is used for suppression of hiccups. [2]. Benzonatate in the form of liquid capsule is used in the mouth to numb the oropharynx for awake incubation. Benzonatate act as a local anesthetic.[1] But, when the medicine is observed by the oral mucosa, there may be potentially fatal side effects include circulatory collapse circulatory collapse, hypersensitivity reactions and circulatory collapse.[1]Benzonatate is voltage gated sodium channel inhibitor.[7] It acts as a local anesthetic, after absorption.It acts as a local anesthetic by decreasing the sensitivity of vagal afferent fibers and stretch receptors in alveoli, bronchi and pleura in the lower airways and lung after absorption and circulation to the respiratory tract.[1][2][4]

2. Material and Instrument

Methanol (HPLC Grade),NaOH ,HCl, 30%H_2O_2 water with HPLC grade. Methanol , Sodium hydroxide , Hydrochloric acid, Hydrogen peroxide solution 30% w/v (H_2O_2) were brought from MERCK LABORATORIES PVT LTD, Mumbai.

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Instruments:

Analysis performed on HPLC instrument equipped with Borwin- PDA software (version 1.50) , Model PU 2080 Plus Intelligent HPLC pump, MX-2080-31 Solvent Mixing Module, BDS HypersilC18 column (250 × 4.6 mm, 5 µ), MD 2010 Plus Multi-wavelength PDA detector, Rheodyne sample injection port 2µl loop. Double beam UV-Visible spectrophotometer (Model JASCO V-730), Shimadzu (model AY-120) electronic weighting balance, Sonicator of Prama solutions laboratory, ELGA Lab (PUERELAB UHO-II) water purification system. Conductivity below 0.05 µS/cm, Photo stability chamber- Newtronic Electronic PH meter pH meter, Calibrated Glassware.

Preparation of Standard Stock Solution

An accurately weighed 100mg of Benzonatate was taken in 100ml volumetric flask and the volume was made to100ml with Methanol, to get standard stock solution of Benzonatate (1000 µg/ml). From the standard stock solution, working standard solution was prepared using methanol as final diluent.

Selection of Analytical Wavelength:A solution of 10µg/ml was separated from stock solution of Benzonatate (1000 µg/ml) and scanned over 200-400 nm in UV– Spectrophotometer. The maximum absorbance was shown at 308 nm. Hence 308nm selected as analytical wavelength and the UV spectrum is given in Fig 2.

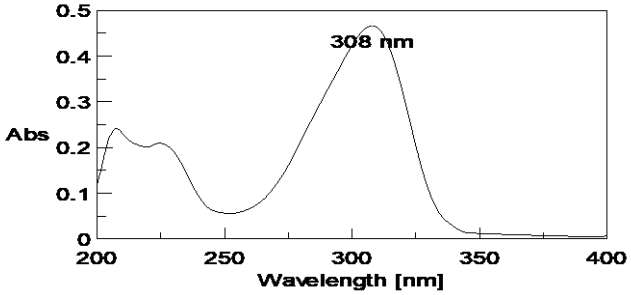
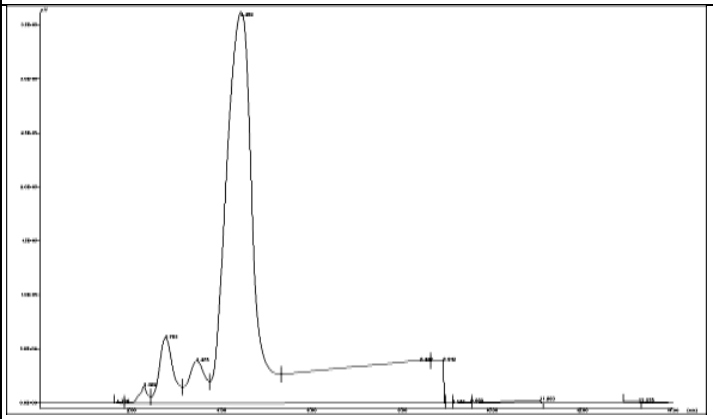


Figure 2: UV of Benzonatate in methanol (10 µg/ml)

Mobile Phase Optimisation: Few mobile phases were tried to achieve optimum chromatographic condition. Ammonium acetate buffer and methanol mobile phase system was initially tried but did not get a considerable number of theoretical plates as well as peak shape. Methanol (0.01%) and Formic acid (70:30%) was tried and has obtained considerable theoretical plates and appropriate peak shape, with appropriate system suitability parameters

Table 1: Trials of mobile phase for Benzonatate

Sr. No.	Column and M.P.	Observation	Chromatogram (308 nm)
1.	Ammonium acetate buffer: Methanol (30:70v/v)	Proper Peak Shape was not found.	

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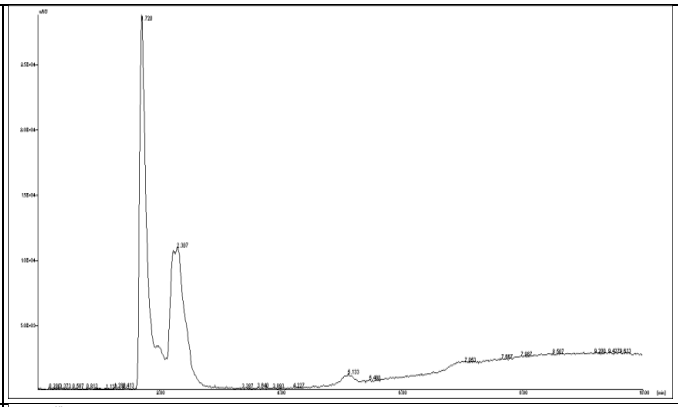
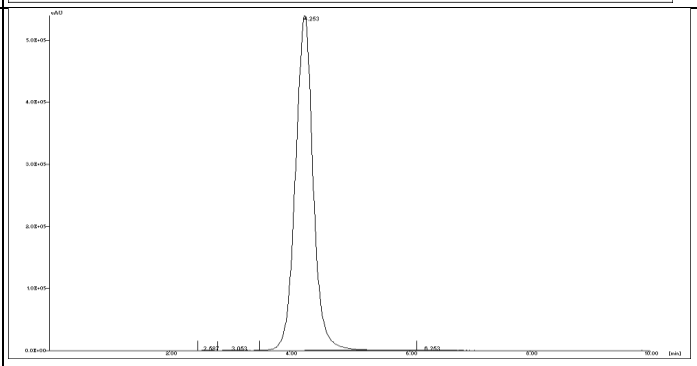
2.	Metahnol : water (50:50v/v)	Proper Peak Shape was not found.	
3.	Methanol: 0.01%Formic acid (70:30v/v)	R.T 4.253Good shape of peak	

Optimized Chromatographic Condition: Column: HiQSil C18 (250 ×4.6mm,5µ) Mobile Phase is Methanol: 0.01% Formic Acid (70:30v/v) Flow rate : 1ml/minColumn Temperature:AmbientDetector set at 308nmRT (min): 4.220 ± 0.787Asymmetry: 1.12Plates (N): 4136

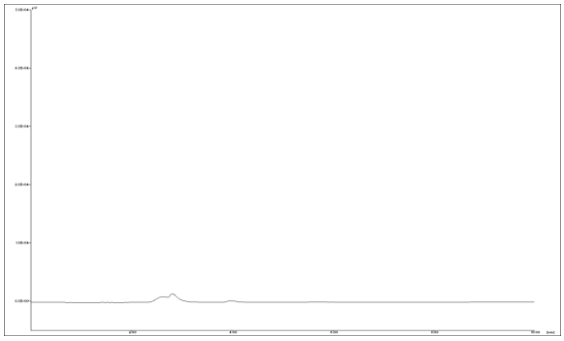


Figure. 3: Chromatogram of mobile phase blank

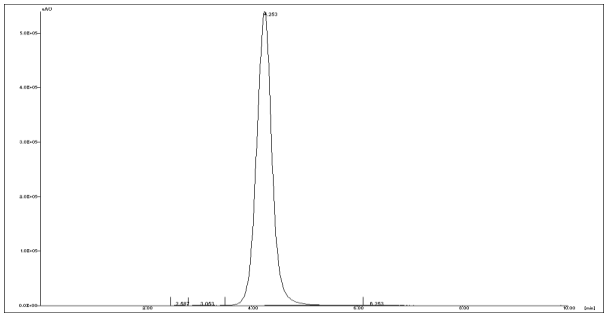


Figure 4: Chromatogram of Benzonatate

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Validation of Analytical Method: [8-9]

In the development and validation of proposed analytical method involves factors like specificity, linearity, precision, accuracy, and assay, limit of detection and limit of quantitation. It is validated according to ICH Q2 guidelines.

Specificity:

Specificity was checked by injecting blank, placebo and comparing the peaks observed in sample solution with standard solution. No interferenceswere observed. Observed peak in sample solution matched standard peak of Benzonatate showed that, the method is specific. Also Specificity was assessed using peak purity profiling studies. More than 997 was discovered for the max purity values. The results demonstrate that no additional degradation product peak, contaminant, or matrix interfered with the studies.(Table 2)

Table 2: Peak purity of Benzonatate

Drug	Purity tail	Purity front
Benzonatate	998.24	997.67

System suitability:

System suitability was evaluated by retention time, theoretical plates, asymmetric factor parameters and the method indicated good performance of thesystem as depicted in Table 3

Table 3: System suitability parameters

Concentration µg/ml	RT (min) ± RSD	Area	Plates	Asymmetry
10	4.220 ± 0.787	447151.08	4136	1.07

Linearity:

Linearity is directly proportional to concentration of the analyte in the sample. A solution containing 100 g/ml of methanol was prepared from the normal stock solution (1000 g/ml) of Benzonatate. Also, a range of solutions with six different concentrations were made using this solution. By examining six solutions with concentrations ranging from 5 to 30 g/ml, the linearity was ascertained, and the calibration curve's equation, y = 45152x–6566.5, was discovered. Table 6.4 for Benzonatate contains the results that were attained. To create the calibration curve, the peak area of drug was plotted against the corresponding concentration as shown in Fig. 4 and overlay of Linearity Range is given in Fig. 5.

Table 4: Results of Linearity for Benzonatate

Replicates	Concentration (µg/ml)					
	5	10	15	20	25	30
	Peak Area					
1	224415.60	447151.08	666585.74	868633.62	1125851.42	1338118.44
2	226639.50	443971.50	669945.20	873910.20	1127309.60	1344543.10

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3	227761.50	444912.50	678826.90	860698.80	1126195.20	1371349.20
4	226639.50	443971.50	669945.20	883910.20	1127309.60	1357454.10
5	223461.50	444912.10	668826.90	880698.80	1146195.20	1361349.20
6	227456.50	447818.90	667713.90	884903.20	1121858.40	1358100.70
Mean	226062.35	445456.20	670307.31	875459.14	1129119.90	1355152.46
SD	1579.72	1497.54	3991.93	8707.46	7853.73	10934.96
% RSD	0.70	0.34	0.60	0.99	0.70	0.81

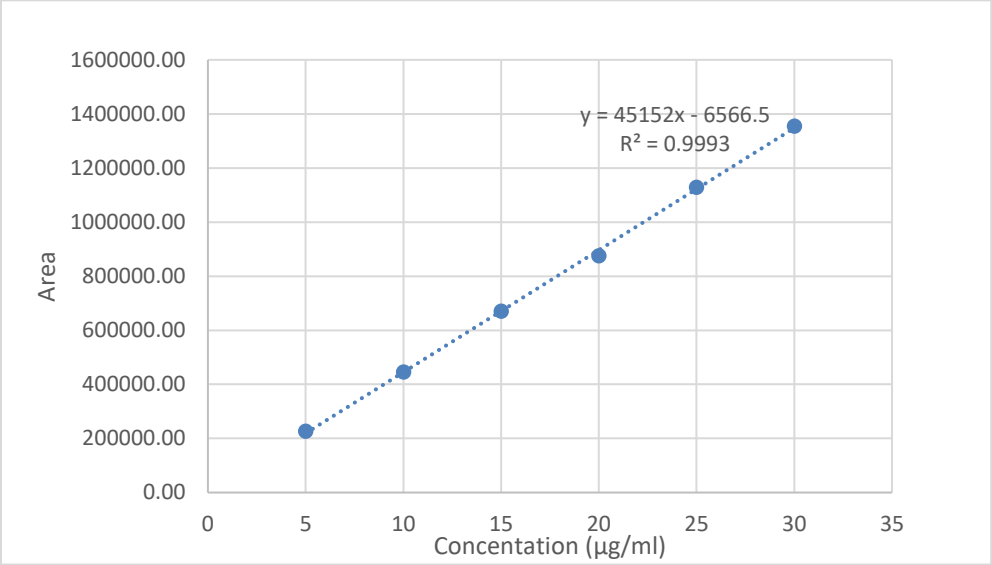

Figure 6: CalibrationCurve of Benzonatate (5-30µg/ml)

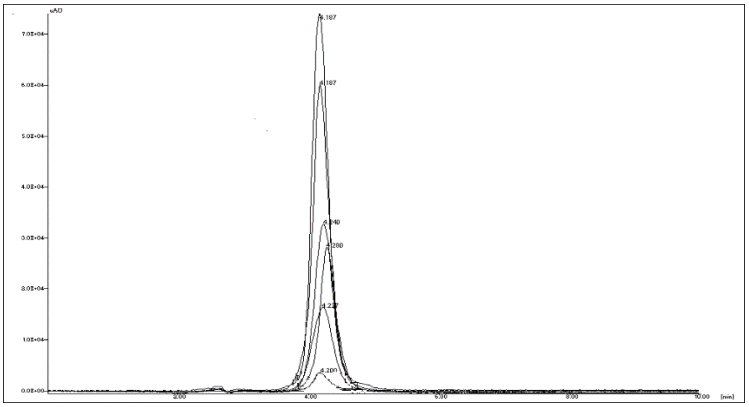

Figure 6: Overlay of Linearity Range (5-30 µg/ml)

Precision:

By conducting intraday and interday variation investigations, the method's precision was shown. In intraday studies, the percentage RSD was computed after the same-day analysis of three replicates of three distinct concentrations. In interday investigations, the

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% RSD was computed after the analysis of 3 replicates of 3 distinct concentrations over the course of 3 days. Tables 5(a) and 5(b) display the findings of the interday and intraday precision experiments, respectively.

Table 5 (a): Results of Intraday Precision of Benzonatate

Concentration	Area	Practical Concentration (µg/ml)	% Drug Content	Mean ± SD	%RSD
10	445512.825	10.012	100.124	99.868 ± 0.468	0.469
10	446165.612	10.027	100.268		
10	441390.546	9.921	99.211		
20	888685.642	19.828	99.138	99.466 ± 0.389	0.391
20	889683.436	19.850	99.248		
20	896586.878	20.003	100.013		
30	1351667.265	30.081	100.271	101.512 ± 0.352	0.350
30	1361665.274	30.303	101.009		
30	1351456.289	30.077	100.256		

Table 5 (b): Results of Interday Precision

Concentration	Area	Obtained Concentration (µg/ml)	%Drug Content	Mean±SD	%RSD
10	443751.078	9.973	99.734	100.124 ± 0.295	0.295
10	446971.535	10.045	100.447		
10	445818.923	10.019	100.192		
20	888633.612	19.826	99.132	99.883 ± 0.531	0.532
20	898910.263	20.054	100.270		
20	898698.841	20.049	100.246		
30	1338118.439	29.781	99.271	99.411 ± 0.252	0.254
30	1344815.344	29.930	99.765		
30	1337118.552	29.759	99.197		

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Limit of detection (LOQ) and limit of quantitation (LOQ)

The smallest amount of analyte in a sample that can be quantitatively determined with the necessary precision and accuracy is known as the quantitative limit of an analytical method, according to ICH. The lowest concentration of analyte in a sample that can be quantitatively identified with the necessary precision and accuracy is the limit of detection of an analytical method. The smallest amount of analyte in the sample that can be detected but not necessarily quantitated as an accurate value is the limit of detection of an analytical procedure.

Formula for calculation of LOD and LOQ.

$$LOD = \frac{3.3\ \sigma}{S} \qquad LOQ = \frac{10\ \sigma}{S}$$

Ϭ = S.D. of the response at lowest concentration or standard deviation of Y intercept;

S = Average of slope of the calibration curve.

LOD of Benzonatate = 0.271µg/ml

LOQ of Benzonatate = 0.821µg/ml

Assay:

Benzonatate containing 100mg drug was transferred into 100ml volumetric flask with methanol. The solution prepared was found to be clear having the strength 1000µg/ml. 10ml of this solution was diluted to 100ml with methanol to get 100 µg/ml solution. This solution is further diluted to 10ml with mobile phase which gives 10µg/ml solution, which was injected to system and chromatograph was recorded. The procedure was followed for six times. Percentage drug recovered obtained is shown in Table6.

Sr. No.	Peak Area	Amount Recovered (µg/ml)	% Recovery (µg/ml)	Mean ± % RSD
1	448467.124	10.078	100.778	100.191 ± 0.837
2	449276.136	10.096	100.957	
3	440648.602	9.905	99.047	
4	440368.845	9.898	98.985	
5	447561.336	10.058	100.578	
6	448561.347	10.080	100.799	

Table 6: Results of Assay studies

Accuracy:

Accuracy was determined by method of standard addition. Calculated amount of API to be analyzed was added to the marketed formulation of Benzonatate. In the assay solution pure API was spiked at 50%, 100%, 150% level. The 3 replicates of 3 concentrations were evaluated to calculated % recovery. The obtained results are summarized in table 7.

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Table 7: Result of Accuracy Studies

Level	Conc. Of Sample Solution (µg/ml)	Conc. Of Standard solution spiked (µg/ml)	Area	Amount recovered (µg/ml)	% Recovery	% Recovery (Mean ± %RSD)
50 %	10	5	671790.412	15.024	100.159	100.682 ± 0.373
			676559.145	15.129	100.863	
			677655.902	15.154	101.025	
100 %	10	10	898691.925	20.049	100.246	99.597 ± 0.460
			889909.735	19.855	99.273	
			889908.314	19.855	99.273	
150 %	10	15	1111858.488	24.770	99.081	99.967 ± 0.724
			1121858.265	24.992	99.967	
			1131866.784	25.213	100.853	

Robustness:

Robustness is employed to perform the study in situations where the mobile phase composition (+2ml composition), detecting wavelength (+1nm), and flow rate (+0.05ml/min) were altered. The effects on the area were then observed. The method's robustness was confirmed by deliberate variation of the analytical parameters, which revealed that areas of interest peaks were unaffected by minor changes in the parameters.

Table 8: Robustness Study

%RSD found for Robustness study (Peak Area)								
MP composition (+ 2ml composition)			**Detection wavelength (±1nm)**			**Flow rate (±0.5ml/min)**		
72:28	70:30	68:32	307	308	309	0.95	1	1.05
0.532	0.380	0.501	0.565	0.170	0.292	0.647	0.462	0.571

Summary of results of validation parameters

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Table 9: Summary of results of Validation parameters

Sr. No.	**Parameters of Validation**	**Benzonatate Results**
1.	Linearity Equation R^2 Range	y= 45152x -6566.5 R^2= 0.9993 5-30 µg/ml
2.	Precision	(%RSD)
	Intraday	0.350 – 0.469
	Interday	0.254 – 0.532
3.	Assay	100.191 ± 0.837
4.	Accuracy	Mean ± %RSD
	50	100.682 ± 0.373
	100	99.597 ± 0.460
	150	99.967 ± 0.724
5.	LOD	0.271 µg/ml
6.	LOQ	0.821µg/ml
7.	Specificity	Specific
8.	Robustness	Robust

3. Forced Degradation Studies

Acid Degradation:

Sample was prepared by addition 1ml of 2N HCl to 1ml of stock solution (1000µg/ml) of Benzonatate. The solution was placed at room temperature for about 2 hours. The solution was then neutralized and the volume was made to 10ml with methanol and further diluted with mobile phase to get 100µg/ml solution which was injected to system. Chromatogram is shown in Fig.7.

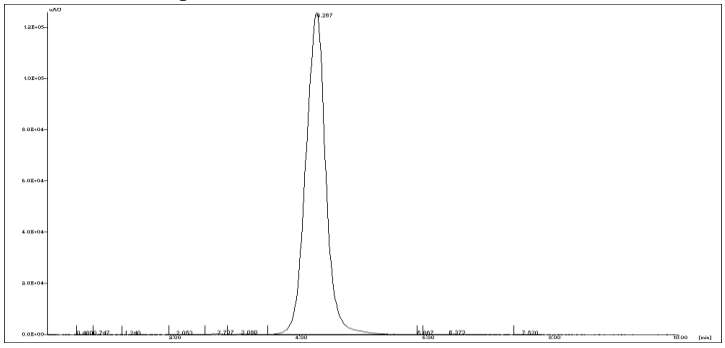

Figure 7: Chromatogarm of Acid Degradation (100µg/ml)

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Alkali Degradation:

Sample was made by adding 1 ml of 1NaOH to 1 ml stock solution (1000 µg/ml) of Benzonatate. The prepared solution was placed at room temperature for about 2 hours. Solution was the neutralized and volume was then made to 10ml with methanol and further diluted with mobile phase to get 100 µg/ml solutions which was injected to system. Shown in Chromatogram Fig. 8.

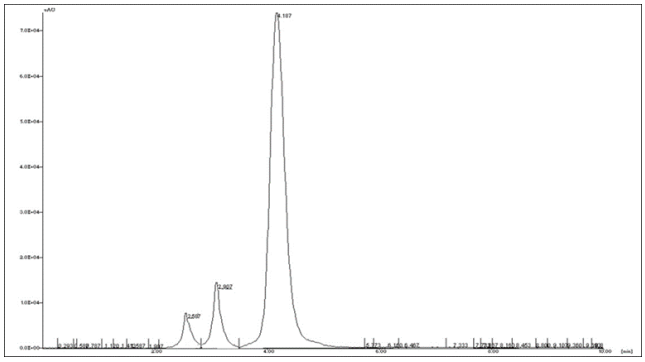

Fig. 8 Chromatogram of Alkali Degradation (100µg/ml)

Hydrogen-Peroxide Induced Degradation:

Sample was made buy addition of 1 ml of 3% w/v H_2O_2 to 1ml stock solution (1000 µg/ml) of Benzonatate . The prepared solution was kept at room temperature for about 2 hours. Volume was made to 10ml with methanol and further diluted with mobile phase to get 100µg/ml solutions was injected to system. Chromatogram is shown in fig.9.

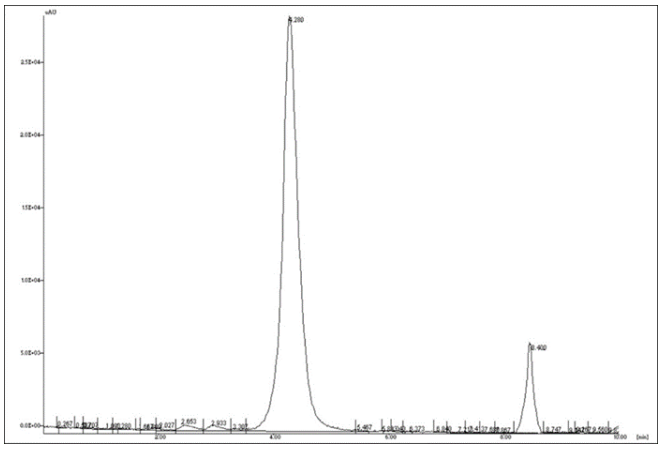

Fig 9: Chromatogram hydrogen peroxide degradation (100 µg/ml)

Thermal InducedDegradation:

The Bulk drug was exposed to 100°C temperature in hot air oven for 2 hours. The sample was cooled to room temperature and then 10mg of the power was dissolved in methanol to 10ml. One ml was diluted to 10ml with mobile phase to get 100µg/ml solution which was injected to system. Chromatogram is shown in Fig,10.

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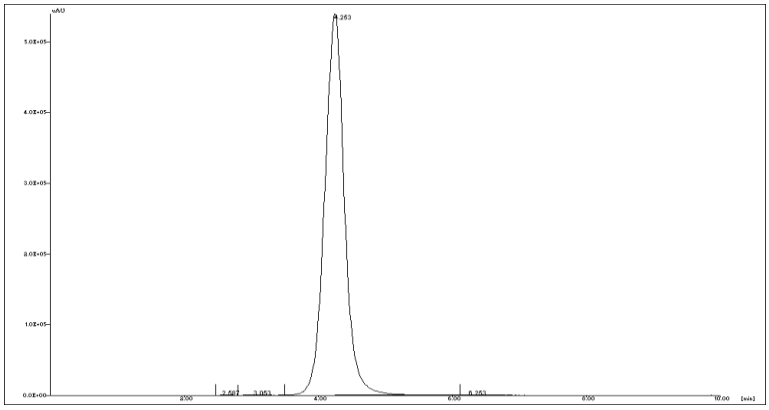

Fig 10: Chromatogram thermal induced degradation (30µg/ml)

Photolytic Degradation:

Sample was exposed to UV light for not less than 200 watt hrs/sqmt followed by white fluorescent light of illumination for not less than 1.2 million lux hours. After exposure 10mg of power was weighed and dissolved in methanol to 10ml. From this final dilution of concentration 100µg/ml was prepared and injected to get chromatogram shown in Fig.11.

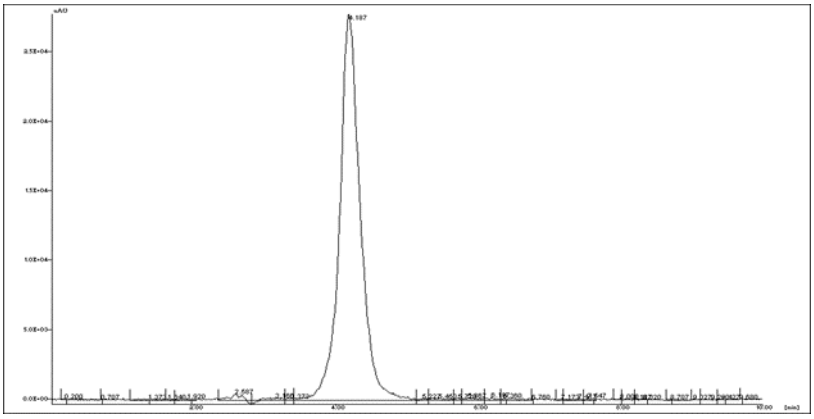

Fig 11: Chromatogram UV Degradation (100µg/ml)

Table 10: Summary of stressed degradation

SN	Parameter and Condition	% Recovery	% Degradation	RT of degraded products
1	Acid Degradation (2N HCl for 2 hr)	96.58	3.42	
2	Alkaline Degradation (1N NaOH for 2hr)	86.46	13.54	DP1- 2.587 min DP2 – 2.907 min
3	Oxidative Degradation (30% H_2O_2 for 2 hr)	82.95	17.05	DP3 – 8.400 min

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4	Thermal Degradation (100^0C for 2 hr)	98.73	1.27	--
5	Photo degradation UV light 200 watt hr/square meter followed by fluorescence light of NLT 1.2million Lux-Hr)	99.24	0.76	--

4. Conclusion

The development and validation of the devised HPLC technique showed that it is suitable for the analysis of Benzonatate. It isaccurate,easy, quick, precise, specific and stability-indicating. The approach is reliable enough to replicate precise and accurate results under various chromatographic circumstances. The uniformity and absence of interferences with the peak of interest were confirmed by degradation experiments. The created approach can be applied to standard Benzonatate analysis.

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