DEVELOPMENT AND VALIDATION OF A STABILITY INDICATING RELATED SUBSTANCES OF BENZYDAMINE HYDROCHLORIDE AND CHLORHEXIDINE GLUCONATE BY RP-HPLC AND ITS DEGRADATION

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Abstract: ***A validated stability-indicating RP-HPLC method for benzydamine HCl and Chlorhexidine gluconate was developed by separating its degradation products on a C18 (150x4.6mm, 3.5µm) Waters symmetry column using 0.1% phosphoric acid in water and acetonitrile in simple gradient at a flow rate 1.0 ml/min. The column effluents were monitored by a photodiode array detector set at 230nm. The method was validated in terms of specificity, linearity, accuracy, precision, detection limit, quantification limit and robustness. Forced degradation of Benzydamine HCl and Chlorhexidine gluconate was carried out under acidic, basic, peroxide, reduction, thermal, photo and hydrolysis conditions. The proposed method is validated as per ICH Q2 (R1) guidelines.***

Index Terms- Benzydamine HCl, Chlorhexidine gluconate, RP-HPLC.

INTRODUCTION

Benzydamine HCl, Chlorhexidine gluconate used in conjunction with antibiotic [1] treatment, on the intensity of clinical signs and quality of life of patients with group A streptococcal tonsilopharyngitis [2].

Benzydamine hydrochloride is also known as Tantum Verde and branded in some countries as Difflam and Septabene available as the hydrochloride salt, is locally-acting nonsteroidal anti-inflammatory drug (NSAID) [3] with local anaesthetic [4] and analgesic [5] properties for pain relief and anti-inflammatory treatment of inflammatory conditions of the mouth and throat [6]. Benzydamine hydrochloride chemically 3-(1-benzyl-1H-indazol-3-yloxy)-N, N-dimethylpropan-1-amine. And their two impurities are Impurity-A chemically 3-D imethylaminopropyl-2-benzylaminobenzoate and second one is Impurity-C chemically 1-Benzyl-3-hydroxy-1H-indazole.

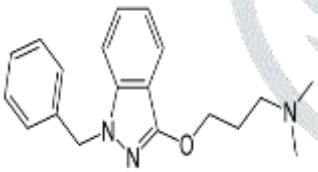

Fig.1: Structure of Benzydamine

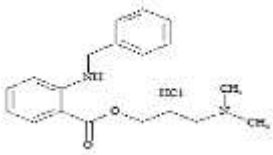

Fig 2: Structure for Imp-A

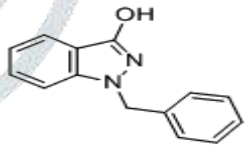

Fig 3: Structure for Imp-C

Chlorhexidine also known as Chlorhexidine gluconate (CHG) is a disinfectant [7] and antiseptic [8] that is used for skin disinfection [9] before surgery and to sterilize surgical instruments [10]. It may be used both to disinfect the skin of the patient and the hands of the health care providers [11]. It is also used for cleaning wounds [12], preventing dental plaque [13], treating yeast infections of the mouth [14], and to keep urinary catheters from blocking. It is used as a liquid or powder. Side effects may include skin irritation, teeth discoloration and allergic reactions. It may cause eye problems if direct contact occurs. Use in pregnancy appears to be safe [15]. Chlorhexidine chemically (1E)-2-[6-[[amino-(4-chloroanilino)methylidene]amino]hexyl]-1-[amino-(4-chloroanilino)methylidene]guanidine;(2R,3S,4R,5R)-2,3,4,5,6-pentahydroxyhexanoic acid. And the two impurities are Impurity-A chemically N-(4-Chlorophenyl)-N'-[6-[[(cyanoamino) iminomethyl] amino] hexyl] imidodicarbonimidic Diamide and second one is Impurity-B chemically 14-[(4-Chlorophenyl) amino]-3,12,14-triimino-2,4,11,13-tetraazatetradecanamide Dihydrochloride.

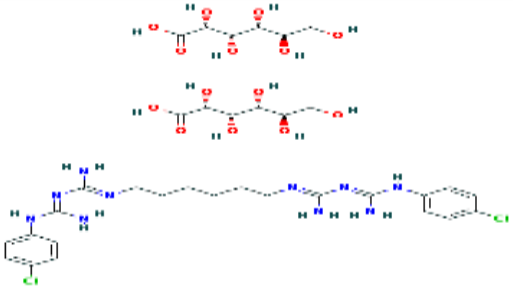

Fig 4: structure for Chlorhexidine Gluconate

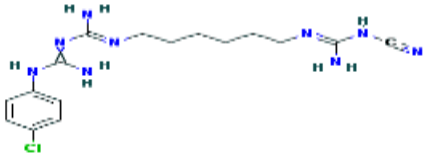

Fig 5: structure for Imp-A

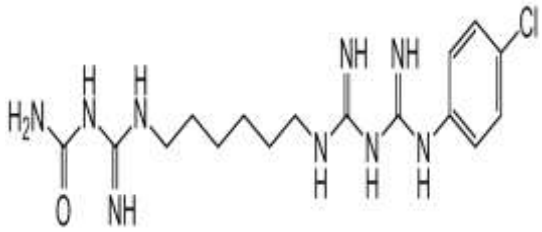

Fig 6: Structure for Imp-B

By the literature search there was no article published so far for the references. The purposed method was simple and economical sensitive for the estimation of Benzydamine HCl and Chlorhexidine gluconate.

MATERIALS AND REQUIREMENTS

Instrument:

HPLC, make: Waters alliance e-2695 chromatographic system consisting of quaternary pump, PDA detector-2996 and chromatographic software Empower-2.0 was used.

Reagents:

Acetonitrile (HPLC grade), Orthophospharic acid (HPLC grade), Water (HPLC grade).

Mobile Phase Preparation:

Mobile Phase-A: 1ml Orthophospharic acid is transferred into 1lt water. Filter through 0.45µ membrane filter and degas.

Mobile Phase-B: Acetonitrile

Diluent Preparation: Mix Mobile phase-A and Mobile phase-B in 50:50 v/v.

Optimization of mobile phase:

Different trails have done, different buffers and different mobile phases were used to develop the method. In all trails peaks are not separated properly. Finally for the proposed method all the peaks are separated and the entire suitability conditions are within the limit.

Gradient program:

Time (min)	Mobile Phase-A	Mobile Phase-B
0.00	80	20
5	50	50
7	20	80
10	20	80
12	80	20
17	80	20

Table 1: Gradient Program

Chromatographic conditions:

The chromatographic system was carried out in symmetry C18, (150x4.6mm, 3.5µm) column. Flow rate was maintained at 1.0ml/min injection volume is 10µl and sample and column temperatures are ambient. Wavelength detection is maintained at 230 nm.

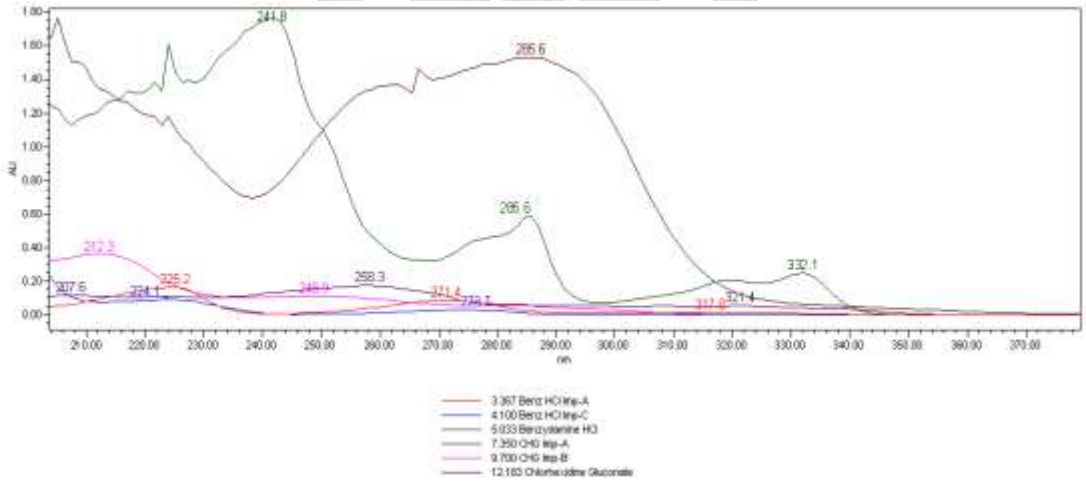

Fig 7: PDA Spectra for Benzydamine HCl and Chlorhexidine Gluconate

Standard Solution:

Weigh accurately 7.5mg of Benzydamine HCl and 6mg of Chlorhexidine Gluconate. These working standards were transferred into a 100ml volumetric flask, add 70ml of diluent sonicated for 10min to dissolve the contents make up to the mark with diluent. Further diluted 5ml of above solution to 50ml with diluent.

Sample Solution:

Transfer 10ml of sample into a 100ml volumetric flask diluted to volume with diluent. Filter through 0.45µ nylon syringe filter.

Impurity standard stock solution:
Weigh accurately each 10mg of all impurities into a 100ml volumetric flask. Add 70ml of diluent, sonicated to dissolve and make up.

Spiked Sample Solution:
Transfer 10ml of sample into a 100ml volumetric flask, add 70ml of diluent, and also add 1ml of impurity standard stock solution and makeup to the mark with diluent. Filter through 0.45µ syringe filter.

RESULTS AND DISCUSSION

Validation of proposed method

The method was validated for parameters like System suitability, Specificity, Linearity, LOD, LOQ, Precision, Accuracy, Robustness and Ruggedness as per ICH guidelines [16-17].

System Suitability

The HPLC system was stabilized for 60min to get a stable baseline. Six replicate injections of standard solution were injected. The results are summarized below table 2.

System Suitability parameter	Acceptance criteria	Drug Name	
		Benzydamine HCl	Chlorhexidine Gluconate
% RSD	NMT 2.0	0.86	0.75
USP Tailing	NMT 2.0	0.54	0.48
USP Plate Count	NLT 3000	7826	8478

Table 2: System Suitability Data

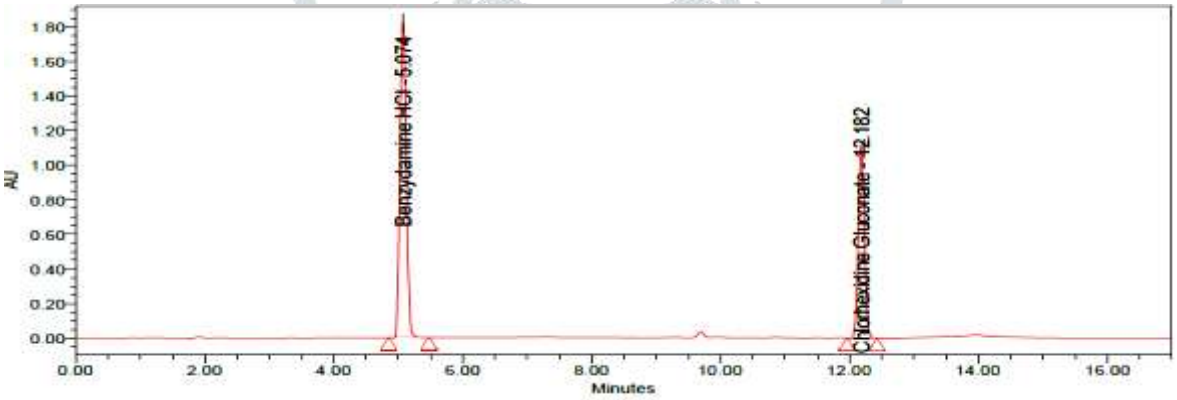

Fig. 8: Chromatogram for System suitability

Specificity

There is no interaction of peaks in blank and standard, sample, placebo chromatograms in the total runtime of chromatogram. Hence its proves that method is specific.

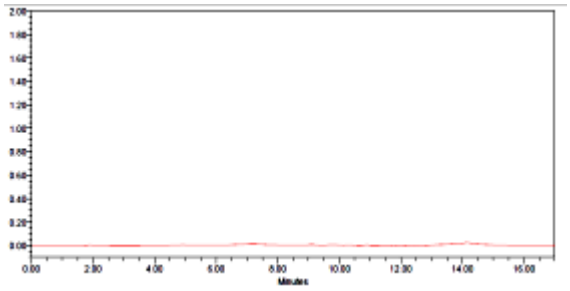

Fig 9: Chromatogram for Blank

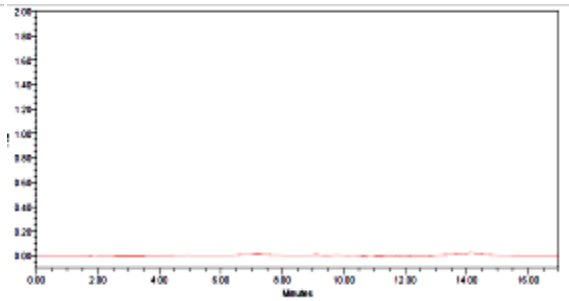

Fig 10: Chromatogram for Placebo

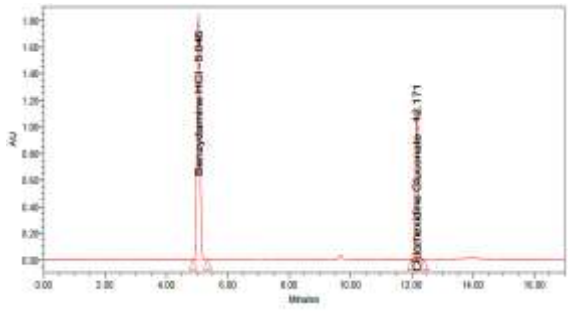

Fig 11: Chromatogram for Standard

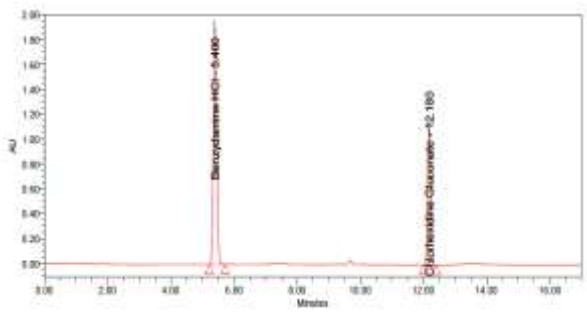

Fig 12: Chromatogram for Sample

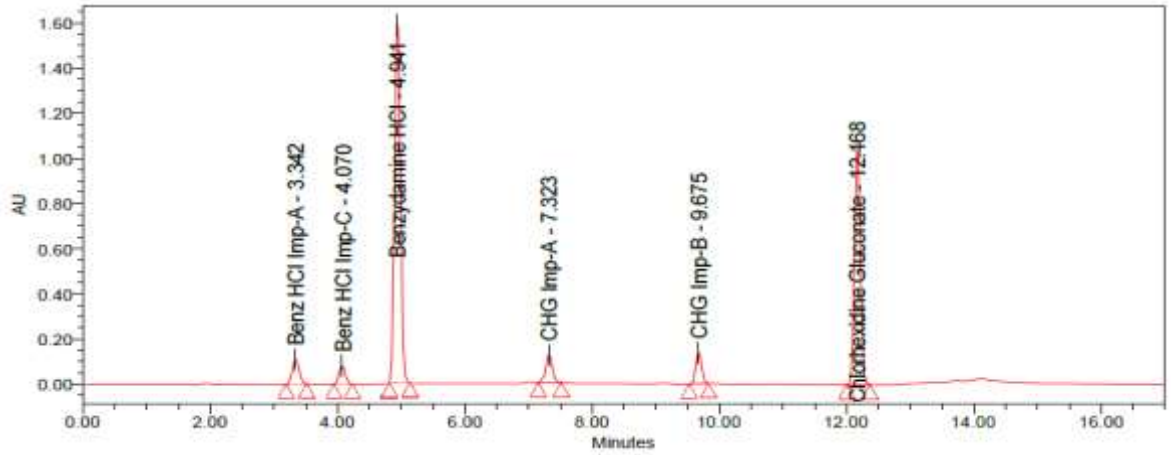

Fig 13: Chromatogram for spiked sample solution

Linearity

The linearity was observed in the concentration range of 7.5µg/ml to 112.5µg/ml for Benzydamine HCl. The regression equation is Y=129260X+289516 and correlation coefficient was found to be 0.9992. Impurity-A concentration range from 0.1µg/ml to 1.5µg/ml, regression equation is Y=809417X-6982.8 and correlation coefficient was found to be 0.9991. Impurity-C concentration range from 0.1µg/ml to 1.5µg/ml regression equation is Y=536869X+1541.9 and correlation coefficient was found to be 0.9997.

Chlorhexidine Gluconate concentration range from 6 µg/ml to 90µg/ml, regression equation is Y=145232X-108147 and correlation coefficient was found to be 0.9997. Impurity-A concentration range from 0.1µg/ml to 1.5µg/ml regression equation is Y=820772X+23154 and correlation coefficient was found to be 0.9991. Impurity-B concentration range from 0.1µg/ml to 1.5µg/ml, regression equation is Y=883459X+8740.2 and correlation coefficient was found to be 0.9998.

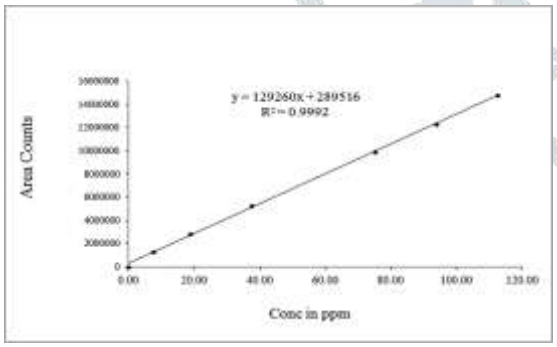

Fig 14: Linearity Plot for Benzydamine HCl

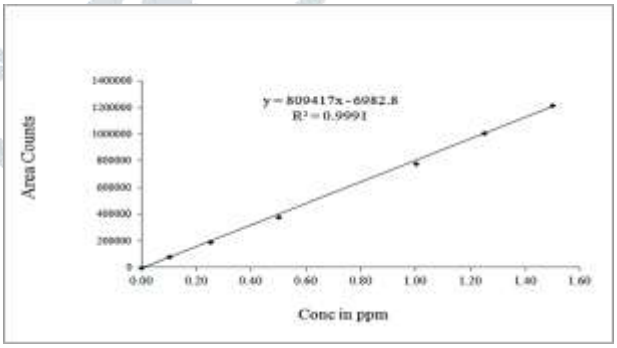

Fig 15: Linearity Plot for Benz HCl Imp-A

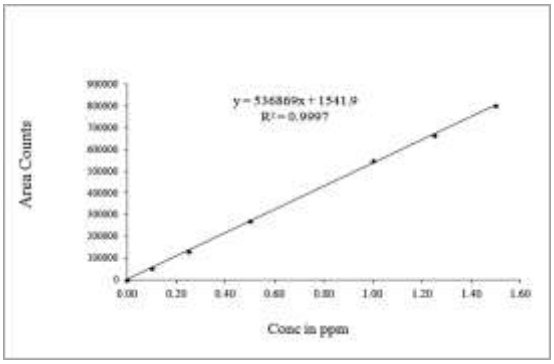

Fig 16: Linearity Plot for Benz HCl Imp-C

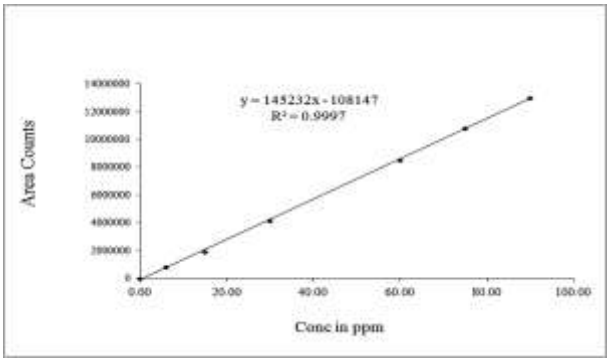

Fig 17: Linearity Plot for Chlorhexidine Gluconate

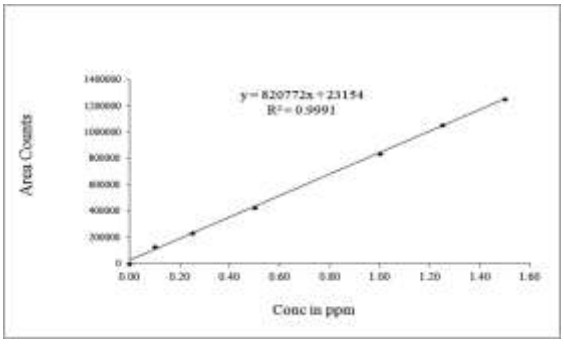

Fig 18: Linearity Plot for CHG Imp-A

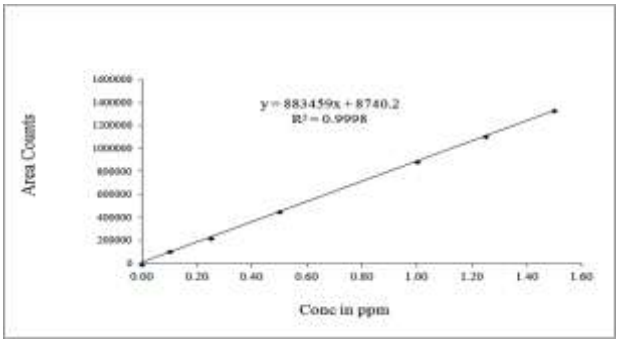

Fig 19: Linearity Plot for CHG Imp-B

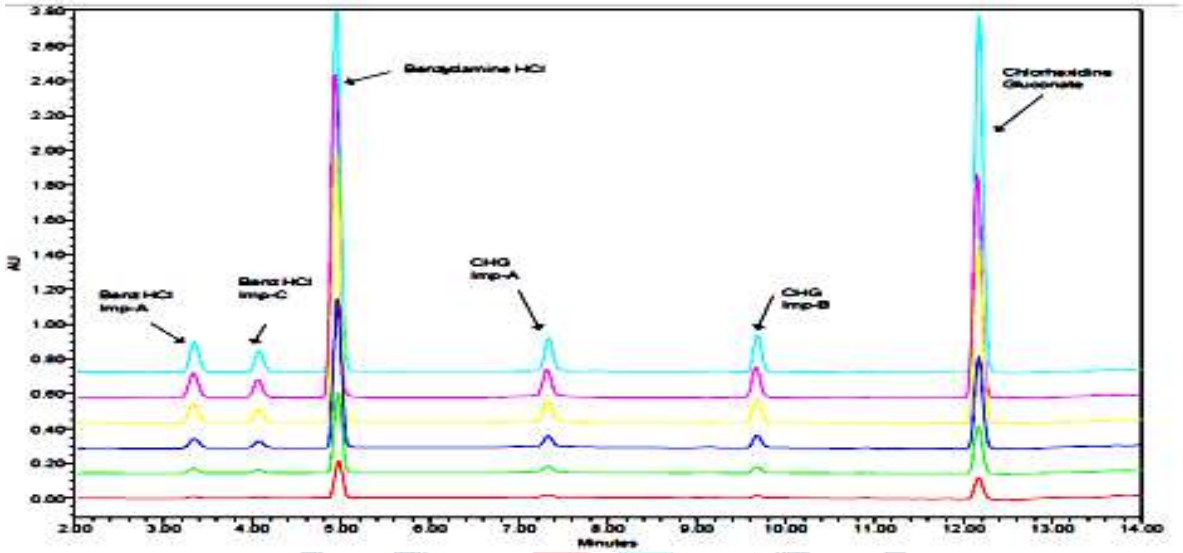

Fig 20: Overlay chromatogram for Linearity

Accuracy

The accuracy of the related substances test procedure was determined by spiking of Benzydamine HCl and Chlorhexidine gluconate impurities stock solution to test the sample. So that the concentration of the impurity would be 0.5% of the test concentration as per the test method. Injecting samples in triplicate at 50%, 100% and 150% of the target concentration. The recovery results should be NLT 95.0% and NMT 105.0%.

S.No.	% Level	% Recovery	Avg. %Recovery
1	50	100.36	100.18
2		100.14	
3		100.05	
4	100	100.57	100.86
5		100.98	
6		101.02	
7	150	99.89	100.10
8		100.26	
9		100.14	

Table 3: Accuracy results for Benzydamine HCl

S.No.	% Level	% Recovery	Avg. %Recovery
1	50	100.54	100.24
2		100.02	
3		100.16	
4	100	101.23	100.68
5		100.56	
6		100.24	
7	150	100.36	100.23
8		100.13	
9		100.21	

Table 4: Acc results for Chlorhexidine Gluconate

S.No.	% Level	% Recovery	Avg. %Recovery
1	50	100.12	100.11
2		100.08	
3		100.14	
4	100	100.54	100.44
5		100.36	
6		100.42	
7	150	100.25	100.10
8		100.63	
9		100.75	

Table 5: Acc results for Benzydamine HCl Imp-A

S.No.	% Level	% Recovery	Avg. %Recovery
1	50	100.26	100.36
2		100.54	
3		100.28	
4	100	100.12	100.36
5		100.82	
6		100.15	
7	150	100.24	100.46
8		100.78	
9		100.35	

Table 6: Acc results for Benzydamine HCl Imp-C

S.No.	% Level	% Recovery	Avg. %Recovery
1	50	100.54	100.31
2		100.16	
3		100.22	
4	100	100.14	100.37
5		100.23	
6		100.74	
7	150	100.16	100.18
8		100.13	
9		100.25	

Table 7: Accuracy results for CHG Impurity-A

S.No.	% Level	% Recovery	Avg. %Recovery
1	50	100.42	100.17
2		100.21	
3		99.87	
4	100	100.14	100.31
5		100.28	
6		100.51	
7	150	100.76	100.47
8		100.36	
9		100.28	

Table 8: Accuracy results for CHG Impurity-B

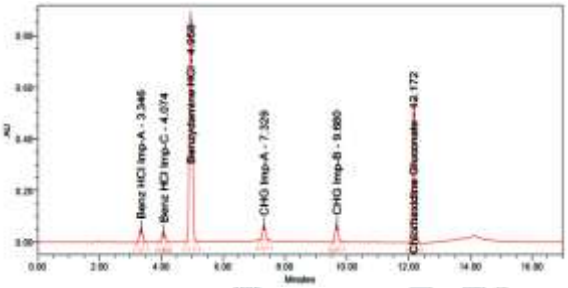

Fig 21: Chromatogram for Accuracy 50%

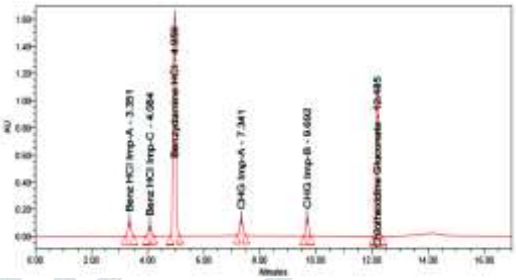

Fig 22: Chromatogram for Accuracy 100%

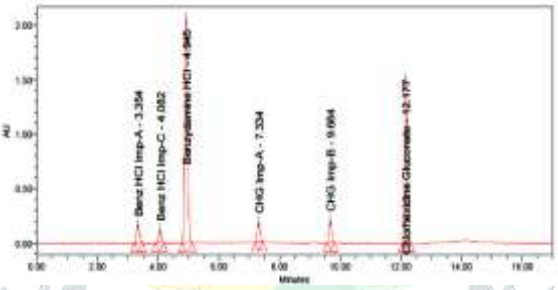

Fig 23: Chromatogram for Accuracy 150%

Precision

Precision of the test method was determined by injecting test preparation and tested through the complete analytical procedure from sample preparation to the final result. Repeatability assessed using a minimum of 6 determinations and calculated % relative standard deviation of impurities. The results were given in Table 9,10. Related substances results meet the specification limits.

Sample No.	% of Related Substances		
	Spiked Impurities	Total Impurities	% Purity (100-Total Imp.)
1	1.26	0.24	99.76
2	1.23	0.27	99.73
3	1.25	0.25	99.75
4	1.28	0.22	99.78
5	1.27	0.23	99.77
6	1.24	0.26	99.74
Average	1.26	0.25	99.76
%RSD	1.49	7.64	0.02

Table 9: Precision results for Benzydamine HCl

Sample No.	% of Related Substances		
	Spiked Impurities	Total Impurities	% Purity (100-Total Imp.)

1	2.46	0.54	99.46
2	2.43	0.57	99.43
3	2.45	0.55	99.45
4	2.49	0.51	99.49
5	2.51	0.49	99.51
6	2.48	0.52	99.48
Average	2.47	0.53	99.47
%RSD	1.17	5.47	0.03

Table 10: Precision results for Chlorhexidine Gluconate

Intermediate Precision

Six replicates of a sample solution were analysed on a different day, different analyst and different instrument. Peak areas were calculated which were used to calculate mean, % RSD values. The results are given below table 11, 12.

Sample No.	% of Related Substances		
	Spiked Impurities	Total Impurities	% Purity (100-Total Imp.)
1	1.27	0.23	99.77
2	1.24	0.26	99.74
3	1.28	0.22	99.78
4	1.25	0.25	99.75
5	1.27	0.23	99.77
6	1.25	0.25	99.75
Average	1.26	0.25	99.76
%RSD	1.23	7.64	0.016

Table 11: Precision results for Benzydamine HCl

Sample No.	% of Related Substances		
	Spiked Impurities	Total Impurities	% Purity (100-Total Imp.)
1	2.46	0.52	99.48
2	2.43	0.55	99.45
3	2.45	0.54	99.46
4	2.49	0.56	99.44
5	2.51	0.55	99.45
6	2.48	0.54	99.46
Average	2.47	0.54	99.46
%RSD	1.17	2.51	0.01

Table 12: Precision results for Chlorhexidine Gluconate

LOD and LOQ

LOD and LOQ were separately determined by calibration curve method [18]. LOD and LOQ of the compound were determined by injecting progressively lower concentrations of standard solutions using developed RP-HPLC method. The LOD concentrations for Benzydamine HCl their impurities-A,C are 0.075,0.001,0.001µg/ml their s/n values are 3,5,7 and Chlorhexidine gluconate their impurities-A,B are 0.12,0.001,0.001 µg/ml their s/n values 4,6,8. The LOQ concentration for Benzydamine HCl their impurities-A, C are 0.75, 0.01, 0.01 µg/ml their s/n values are 22, 25, 27 and Chlorhexidine gluconate their impurities-A, B are 1.12, 0.01, 0.01 µg/ml their s/n values are 24, 26, 28.

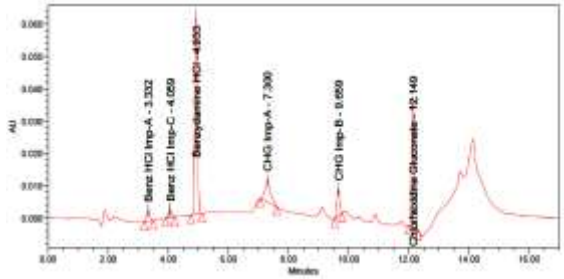

Fig 24: Chromatogram for LOD

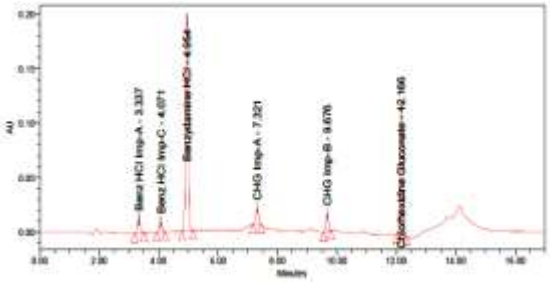

Fig 25: Chromatogram for LOQ

Robustness

The robustness of the method was evaluated by analyzing the system suitability standards and evaluating system suitability parameter data after varying the HPLC pump flow rate (±0.2 ml) and organic solvent content (±10%). The alterations caused a significant change in peak area R.S.D (%), USP tailing factor and retention times.

Parameter name	% RSD for Purity	
	Benzydamine HCl	Chlorhexidine gluconate
Flow (0.8 ml/min)	0.89	0.56
Flow (1.2ml/min)	0.47	1.02
Organic solvent (+20%)	0.56	0.34
Organic solvent (-20%)	1.05	0.27

Table 13: Robustness data

Stability

The stability of Benzydamine HCl and Chlorhexidine gluconate in solution was determined by sample solution stability initial to 24hr at different time intervals at room temperature and 2-8°C. There is no significant deviation of purity. Difference between initial to 24hr ±5.0%

Stability	Purity of Benzydamine HCl	% of Deviation	Purity of Chlorhexidine gluconate	% of Deviation
Initial	99.76	0.00	99.52	0.00
6Hr	99.72	0.04	99.48	0.04
12Hr	99.68	0.08	99.42	0.10
18Hr	99.64	0.12	99.36	0.16
24Hr	99.58	0.18	99.30	0.22

Table 14: Results for Solution Stability

Forced Degradation

The Benzydamine HCl and Chlorhexidine gluconate sample was subjected into various forced degradation conditions to effect partial degradation of the drug. Forced degradation studies were performed to show the method is suitable for degraded products. Moreover, the studies provide information about the conditions in which the drug is unstable so that measures can be taken during formulation to avoid potential instabilities [19].

Acid Degradation:

10ml of sample transferred into a 100ml volumetric flask add 10ml of 0.1N HCl heat for 15min at 60ºC after that add 10ml of 0.1N NaOH then makeup to mark with diluent. Then the solution is filter through 0.45µ nylon syringe filter.

Alkali Degradation:

10ml of sample transferred into a 100ml volumetric flask add 10ml of 0.1N NaOH heat for 15min at 60ºC after that add 10ml of 0.1N HCl then makeup to the mark with diluent. Then the solution is filter through 0.45µ nylon syringe filter.

Peroxide Degradation:

10ml of sample transferred into a 100ml volumetric flask add 5ml of 10%H_2O_2 heat for 30min at 60ºC then cool to makeup with diluent. Filter the solution with 0.45µ nylon syringe filter.

Reduction Degradation:

10ml of sample transferred into a 100ml volumetric flask add 10ml of 10% sodium bicarbonate solution heat for 15min at 60ºC then cool to makeup with diluent. Filter the solution with 0.45µ nylon syringe filter.

Thermal Degradation:

The sample drug solution was placed in oven at 105ºC for 6Hr. The resultant solution was injected into HPLC system.

Photolytic Degradation:

The sample solution was exposed into sunlight for 6hr. The sample was injected into HPLC system

Degradation Condition	% of Purity	
	Benzydamine HCl	Chlorhexidine gluconate
Acid Degradation	94.36	92.14
Alkali Degradation	93.17	90.54
Peroxide Degradation	92.44	89.17
Reduction Degradation	95.21	94.36
Thermal Degradation	94.18	92.77
Photolytic Degradation	90.16	84.36

Table 15: Results for Forced Degradation

Conclusion

The developed method gave good resolution between Benzydamine HCl and Chlorhexidine gluconate and their 4-impurities with short runtime (17min), high efficiency and complies with modified SST specifications of USP. The use of C18 column in the present work has shown better elution of analytes with good resolution, improved plate count, tailing. So the C18 column can be used to achieve high specificity in shorter time of analysis of Benzydamine HCl and Chlorhexidine gluconate as per ICH Q3A (R2) [20] guidelines. The proposed method was found to be simple, precise, accurate, linear, robust and rapid for simultaneous determination and quantification of Benzydamine HCl and Chlorhexidine. The sample recovery was in good agreement with their respective label claims suggested non-interference in the estimation. Hence, the method can be easily and conveniently adopted for routine analysis of Benzydamine HCl and Chlorhexidine in combined dosage form.

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