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Development and Validation of Alternative Analytical Method for Determination of Related Substances of Benzydamine Hydrochloride in Oral Spray by HPLC

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Abstract: The aim of the present study was to develop and validate HPLC method for determination of related substances of benzydamine hydrochloride in a dosage form, containing benzydamine hydrochloride as an active substance. A new selective, specific and sensitive method was developed for determination of benzydamine degradation products in the formulation. Determination was carried out by reversed-phase HPLC using isocratic solvent elution. The method was validated and found to be precise, accurate and specific; the detector response was linear over 0,05% - 1.2% relative to the nominal concentration of benzydamine hydrochloride in the formulation. The developed HPLC method is useful for determination of benzydamine hydrochloride related substances in formulations and can be applied in Quality Control Laboratories.

Keywords: Benzydamine Hydrochloride, HPLC Method, Related Substances, Chromatographic Separation

1. Introduction

Recently, on the market, there are several drugs containing benzydamine hydrochloride, e.g.: nonsteroidal anti-inflammatory drug with local anaesthetic and analgesic properties for pain relief and mouth and throat inflammations treatment [1]. This is an effective drug without side effects [2], and the development of formulations based on it is a perspective way of pharmaceutical market progress.

Determination of related substances is a key indicator of quality for every dosage form. At first, development of such analytical procedure implies the appropriate method selection.

For the present, a few methods have been reported for determination of related substances of benzydamine hydrochloride. The British Pharmacopoeia describes a Thin Layer Chromatography method for determination of benzydamine hydrochloride impurities in finished drug products [3]. This method has a number of disadvantages associated primarily with complicated and ambiguous quantitative determination of impurities; and it`s selectivity could be unsatisfactory because of a complicated matrix of sample.

Some HPLC methods for determination of related substances have been described recently: the method, describing determination of benzydamine hydrochloride related impurities in collutory [4]. However, it declares mostly byproducts of synthesis of benzydamine hydrochloride and doesn`t show degradation studies. The separation is carried out using specific column at high pH rate (10,5). Such pH rate can`t be applied on most alkyl silyl phases due to the degradation of stationary phase. In addition, HPLC method with Fluorescent detection for determination of benzydamine hydrochloride and its N-oxide metabolite in biological fluids exists [5]. A photodegradation study of benzydamine is described [6]. There is also HPLC determination of 1-benzyl-1H-indazol-3-ol in benzydamine in pharmaceutical formulations [7].

Thus, development of the method for determination of benzydamine hydrochloride degradation products in the finished drug, taking into account the European regulatory documents requirements [8] and reliable method developing, appeared to be an unsolved problem yet. The major challenge

of this research was high variability of potential degradation products, which source could be either API or excipients, and therefore, interference between them in chromatographic procedure. Our task was to develop an appropriate method, to prove its validity and to show that all potential degradation products could be determined and quantified.

The present study describes development and validation of the method for determination of related impurities in the oral spray containing benzydamine hydrochloride by High Performance Liquid Chromatography (HPLC) using diode-array detector, which allows to quantify the content of impurities in the formulation. The developed method was validated according to the ICH Q2 requirements [9], and validation parameters were determined [10]. It has been shown, that the method is robust, specific, and selective in relation to benzydamine hydrochloride related substances.

2. Experimental

2.1. Chemicals and Reagents

Acetonitrile (HPLC grade) was supplied by Sigma Aldrich (Germany). Sodium perchlorate and perchloric acid were purchaced from Fluka Chemika Bio Chemika (Buchs, Switzerland). Benzydamine hydrochloride reference standard, impurity B and C reference standards were purchased as the Brittish pharmacopeia standards. Water (HPLC grade) was obtained from a Milli-Q plot water purification system (MA, USA) and was used for preparation of mobile phase buffers and reagent solutions.

2.2. Apparatus and Software

Chromatography was carried out using Shimadzu LC-20 liquid chromatograph with diode array detector, autosampler, degasser and quaternary pump. LC Solution software was used for the data acquisition.

2.3. Chromatographicconditions

Grace Alltima C18 column (250 mm * 4,6mm, particle size 5 microns) was used for the analyte separation. Mobile phase was prepared as follows: 3.0 g of sodium perchlorate were dissolved in 500 ml of water, then 1.0 ml of triethylamine was added, pH was adjusted to 3.0 with perchloric acid, and finally 500ml of acetonitrile were added. Flow rate was 1 ml / min, wavelength 320 nm; ambient column temperature.

Chromatography was carried out using 20 µl of both reference and test solutions.

The chromatographic system is considered to be suitable, if the following requirements are met (or under specific conditions):

-The resolution between peaks of benzydamine and impurity C for system suitability solution is at least 1.5;

-The effectiveness of the chromatographic system is at least 2000 theoretical plates.

Limits:

Impurity C: area of the peak corresponding to the response factor must not exceed 0.5 area of benzydamine hydrochloride on the chromatograme of reference solution (a) (0.5%);

Unspecified impurity: the peak area of each unspecified impurity should not exceed 0.2 area of benzydamine hydrochloride on chromatograme of reference solution (a) (0.2%); the sum of all impurities should not exceed 1.0%.

3. Results and Discussion

Development of HPLC method is a solution of the complex problem, which could be solved by selection of appropriate parameters of the chromatographic system. In HPLC method major variable parameters are column, pH of mobile phase, content of organic solvent, detection wavelength, and operating temperature.

The first step of HPLC method development is evaluation of analytes.

The studied formulation consisted of benzydamine hydrochloride, methylparaben, inorganic salts, and water for injection. So, benzydamine, it`s impurities and methylparaben were the only compounds that could be detected in UV light.

Benzydamine hydrochloride is a tertiary amin indazole derivative - {3-[(1-benzyl-1H-indazol-3-yl) oxy] propyl} dimethylamine (as hydrochloride salt). It`s pK is 9,26. This salt is based on weak base and strong acid.

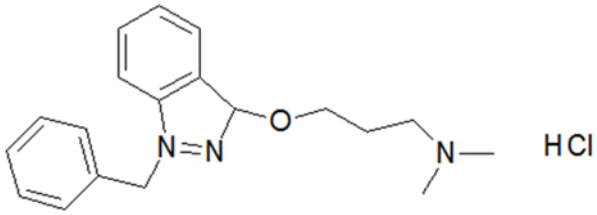

Figure 1. *Benzydamine hydrochloride.*

It exists in protonated form at pH of 7,2 and lower and deprotonated form at pH 11,4 and higher.

An identified impurity of benzydamine is 1-benzyl-1-H-indazol-3-ol (Impurity C); its pK is 7,9.

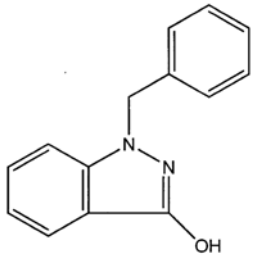

Figure 2. *1-benzyl-1-H-indazol-3-ol (Impurity C).*

From the theory of chromatography it is known, that ionized compounds don`t retain on a stationary phase [11] and consequently elute in void volume. The compound should stay in neutral form in order to retain on stationary phase [11]. Benzydamine and its impurity C exist in neutral

form at pH rate of 10 and higher. However, utilization of mobile phases with such pH is unacceptable for most of reversed phases because of alkyl groups hydrolysis. So, for mobile phase low pH rate was selected, where benzydamine and its impurities are protonated and exist in positively charged state.

It was considered to take a chaotropic anion of a buffer salt as a counter anion [12]. Perchloric and phosphate solutions were tested as mobile phase buffers. It has been shown, that phosphate buffer doesn't give sufficient resolution between impurity C and benzydamine, while it is reached with the use of perchlorate buffer (Figure 4).

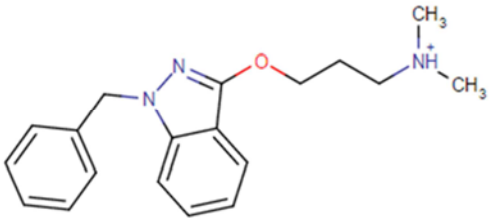

Figure 3. *Protonated benzydamine.*

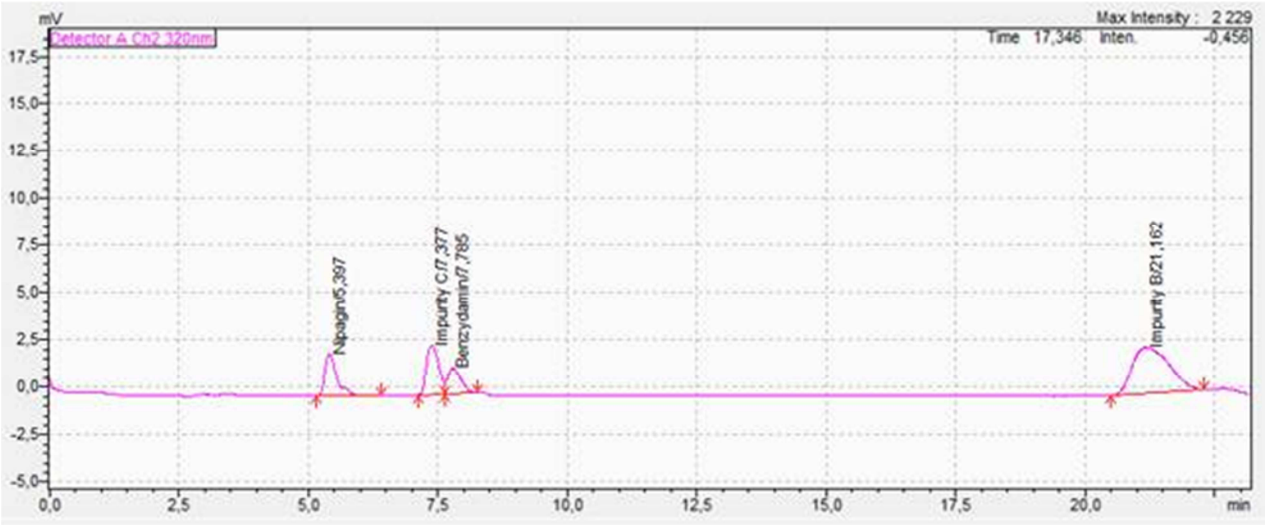

Figure 4. *A chromatogram, obtained with the use of phosphate buffer as a chaotropic salt.*

Ratio of organic solvent in mobile phase was chosen with respect to the optimal resolution of analytes. HETP of the column was about 14 units. The efficiency of separation was about 12000 theoretical plates.

Two columns were tested using octyl and octyldecyl silyl phases. The best performance was reached on C 18 sorbent.

The detection wave was chosen with respect to the maximum of benzydamine absorption at the wavelength 320 nm.

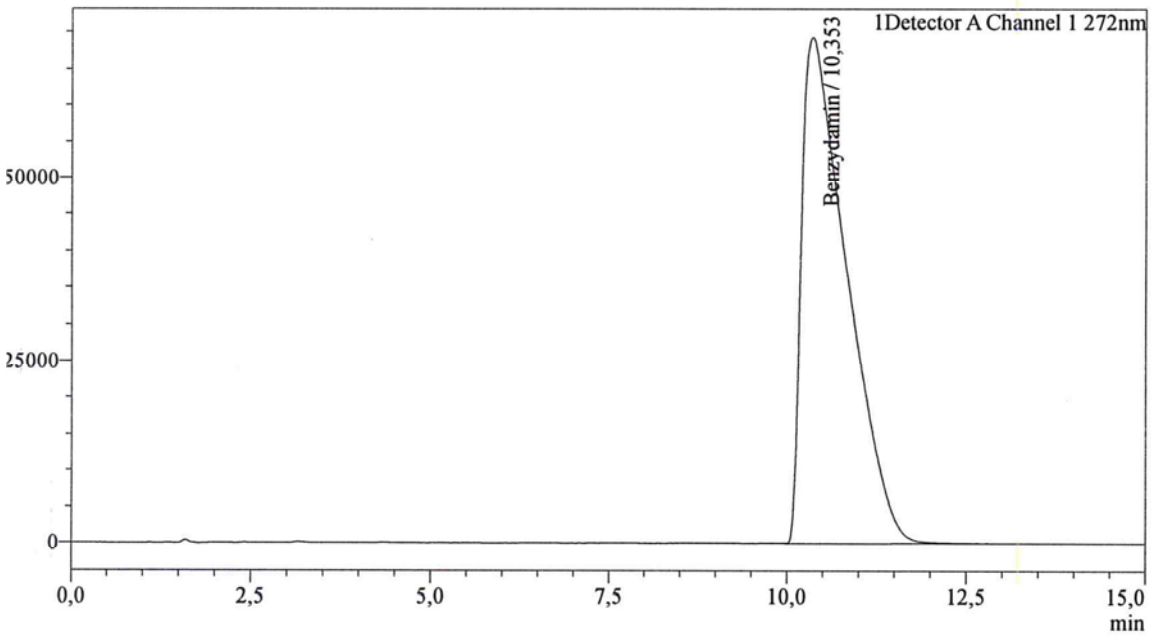

Figure 5. *Unsatisfactory symmetry for benzydamine.*

Triethylamine was added to the mobile phase in order to improve symmetry, by passivation of silanole groups of stationary phase. At this stage, a satisfactory resolution between analytes was reached (Figure 6).

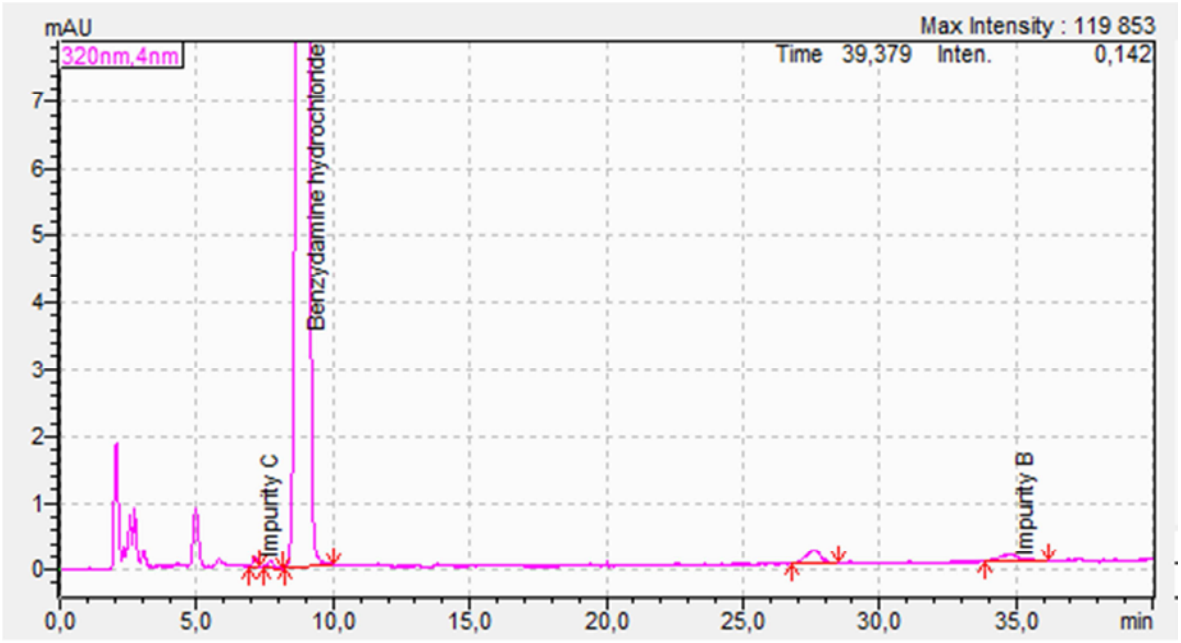

Figure 6. *Satisfactory symmetry for benzydamine.*

3.1. Forsed Degradation Studies of the Drug Product

Forced degradation study of the preparation helps to identify possible degradation products, to test the method for specificity, and to prove the absence of peak co-elution.

"Benzydamine hydrochloride oral spray 1.5 mg / ml" formulation sample was subjected to the stress test to assess the possibility of interference between benzydamine hydrochloride, impurity C, and possible unidentified products of degradation. The influence of alkali, acid, oxidation, photolysis, temperature was studied. Blank and placebo solutions were injected to prove the nature of degradation products.

Degradation studies have shown that benzydamine hydrochloride is stable in alkali medium, under temperature and photolysis treatment. A significant degradation of benzidamine hydrochloride occurred under H_2O_2 (oxidation) and acid treatment with formation of unknown product and impurity C, respectively (Figure 7). The unknown degradation product was studied using LC-MS. It was determined that the unknown impurity was benzydamine N-oxide, with corresponding mass 326, 2 m/z (Figure 8).

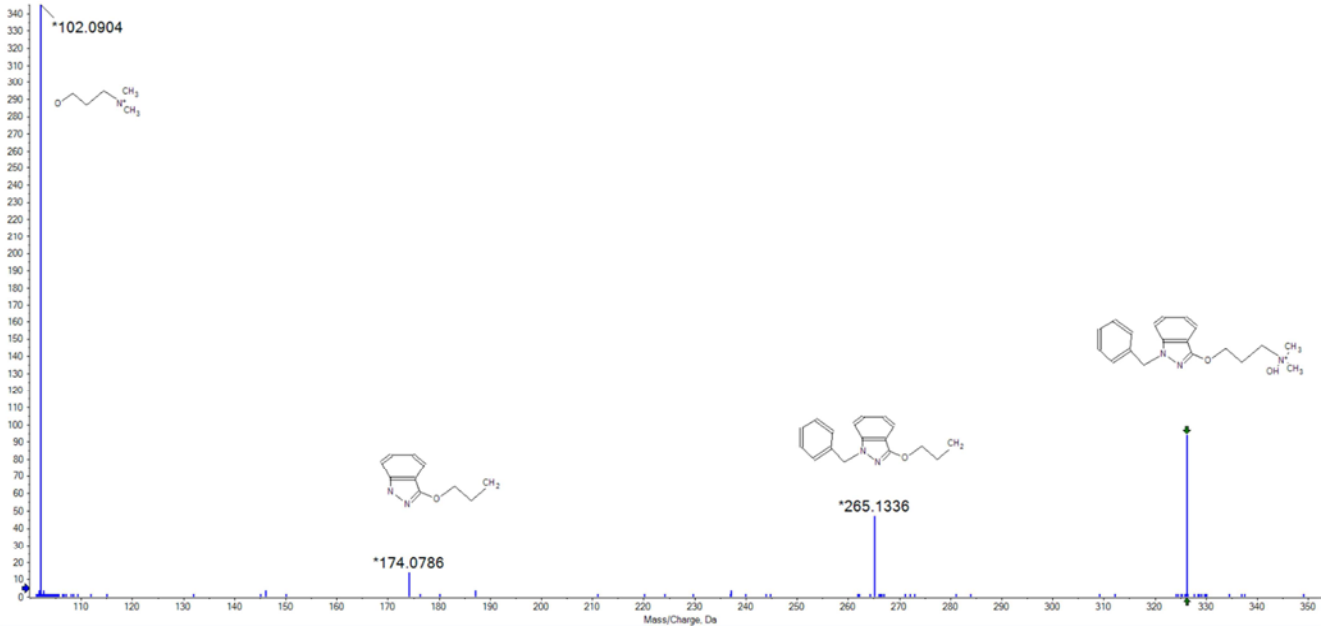

Figure 7. *MS-spectra of benzydamine N-oxide.*

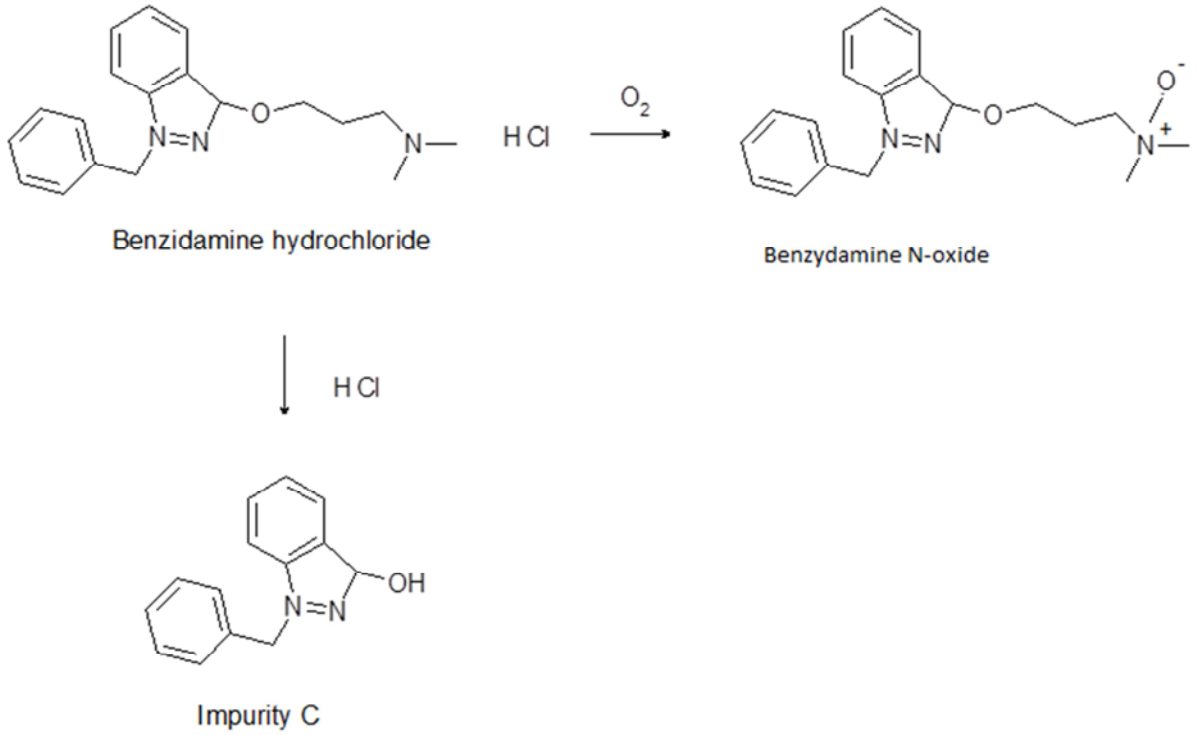

Figure 8. *Scheme of benzydamine degradation.*

The purity factor, obtained for API peak for all formulation samples, subjected to stress test was more than 990,00. All individual impurities, formed under stress tests didn't interfere with each other and with benzydamine peak. The method was recognized as specific for all potential benzydamine degradation products (Figure 9).

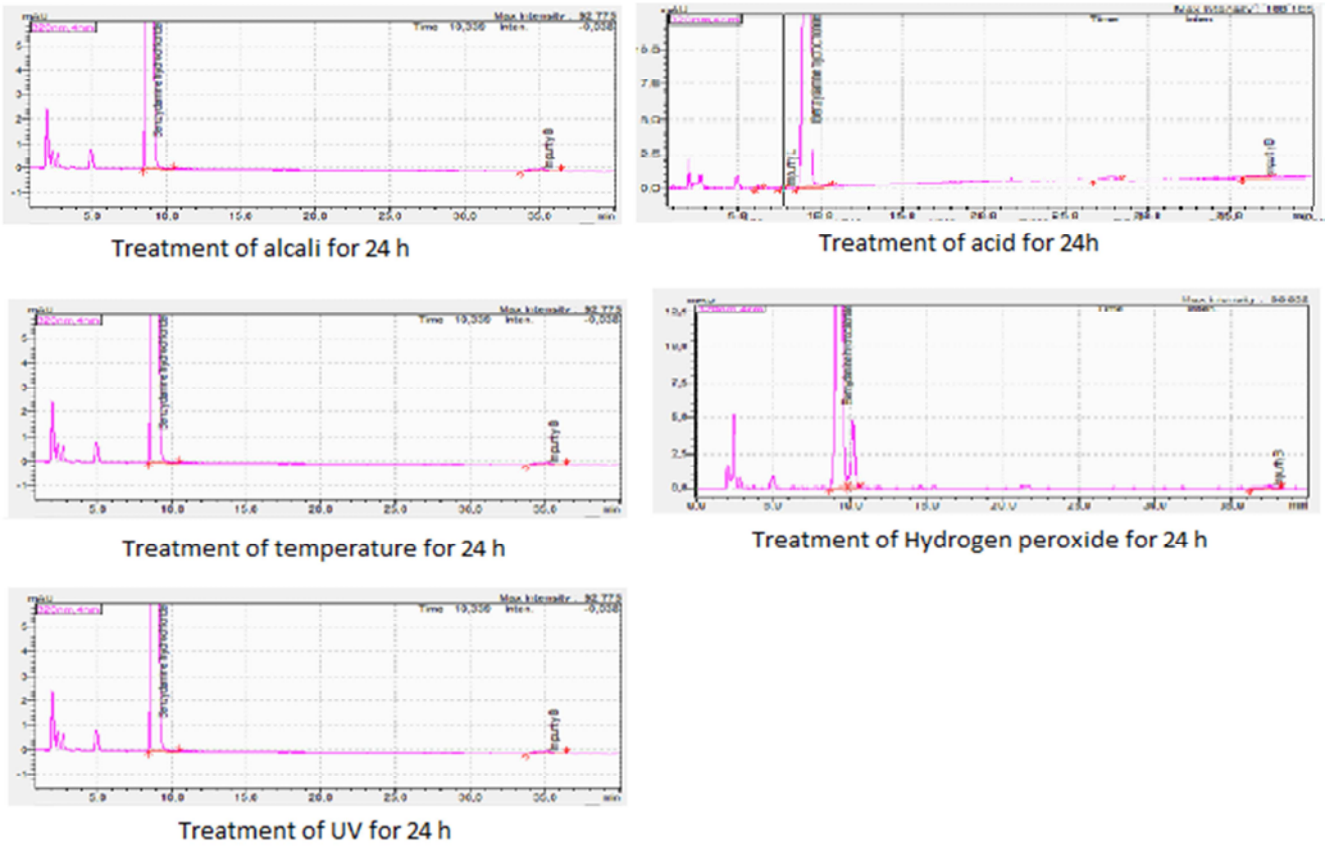

Figure 9. *Chromatograms of benzydamine forced degradation test.*

3.2. Validation of the HPLC Method

Developed HPLC method was validated according to the ICH requirements [9]. It was tested for linearity, detection and quantitation limits, precision, accuracy, robustness, and specificity.

3.2.1. Specificity

The specificity of HPLC method could be established by evaluating resolution between peaks, peak purity test, and the absence of interference between peaks of matrix.

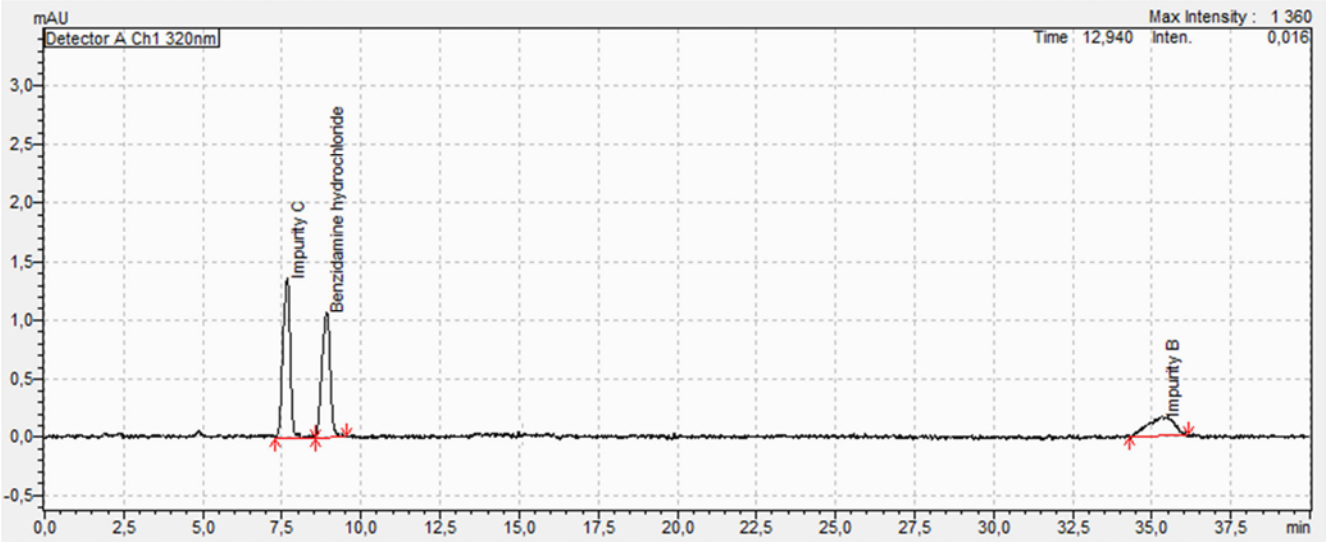

Figure 10. *A chromatogram of system suitability solution.*

It has been shown, that resolution between peaks of impurity C and benzydamine hydrochloride in the chromatogram of system suitability solution is 3.48.

Retention time of benzydamine hydrochloride in the test solution chromatogram coincides with retention time of benzydamine hydrochloride in the system suitability chromatogram. In the test solution chromatogram, benzydamine hydrochloride retention time coincides with retention time of the peak in system sutability chromatogram. In placebo solution chromatogram, no peaks, retention time of which coincides with retention time of benzydamine hydrochloride or impurities B and C, are observed.

3.2.2. Linearity

Linearity for unspecified impurities, and impurity C was studied in the concentration range of 0.05% - 1.2% relative to the nominal concentration of benzydamine hydrochloride in the formulation. 7 model solutions were prepared with benzydamine hydrochloride concentration of: 0.05%, 0.1%, 0.2%, 0.5%, 0.7%, 1.0%, 1.2% with respect to the nominal concentration of benzydamine hydrochloride in the formulation. Each solution was injected in triplicate.

The determined content of benzydamine hydrochloride was plotted versus theoretical concentration of benzydamine hydrochloride in the test solution. According to the experimental data plotted, in the coordinates "The determined content of benzydamine hydrochloride vs prepared relative concentration of benzydamine, i.e.: Y = B • Xi + A (Figure 11). The graph of theoretical concentration of impurity C versus found concentration was plotted in a similar way. (Table 1).

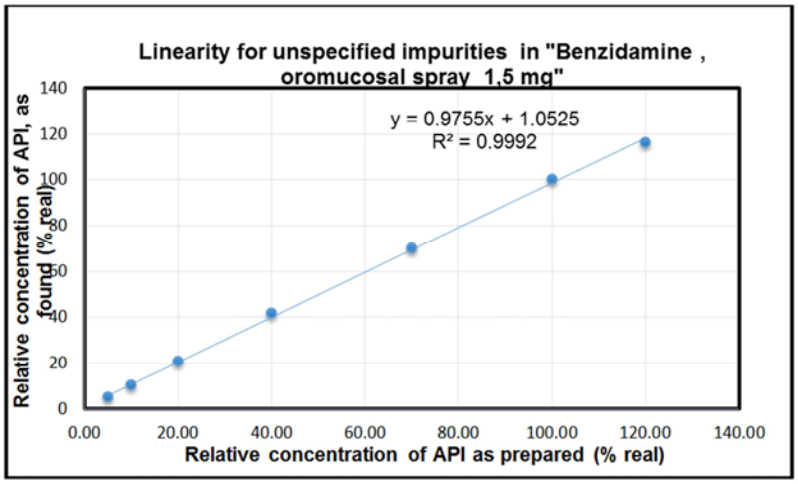

Figure 11. *Graph of determined benzydamine hydrochloride content versus theoretical content.*

Table 1. *Estimated data for impurity C and unspecified impurity.*

Ci, % (theoretical)	Unspecified impurity		Impurity C	
	Si/S*100%, % (found)	RRF,%	Si/S*100%, % (found)	RRF,%
5,00	4,91	98,20	5,10	101,91
10,00	10,31	103,13	10,02	100,19
20,00	20,58	102,92	20,95	104,75
50,00	41,53	103,83	51,41	102,82
70,00	69,81	99,74	69,39	99,13
100,00	100,00	100,00	100,00	100,00
120,00	116,23	96,88	120,73	100,61
mean		100,68		101,34
RSD, %		2,66		1,92

Linearity parameters for the method have been estimated (Table 2).

Table 2. *Linearity parameters for the method.*

Parameter	Symbol	Acceptance criteria	Obtained value for unspecified impurity	Obtained value for impurity C
Correlation coefficient	R	0,990	0,9996	0,9999
Intercept	A	≤ 5	1,0525	0,3880
Slope	B	---	0,9755	0,9997
Standard deviation	S_A	---	0,8097	0,4627
Relative standard deviation of response factors of every individual concentration level	---	< 7	2,66	1,92

3.2.3. Determination of the Limit of Detection (LOD) and the Limit of Quantification (LOQ)

Limit of detection and limit of quantitation of impurity C, and benzydamine hydrochloride (Table 3) were calculated on the basis of results of linear regression, using the standard deviation value of intercept and slope of the calibration curve by the formulas: $\mathrm{LOD} = \frac{3,3 \cdot S_A}{B}$; $\mathrm{LOQ} = \frac{10 \cdot S_A}{B}$.

Table 3. *Limit of detection and limit of quantification.*

Parameter	benzydamine hydrochloride, %	impurity C,%
LOD	0,0274	0,0153
LOQ	0,0830	0,0463

It has been shown, that unspecified impurity can be quantified at the concentration of 0.05% relative to the concentration of benzydamine hydrochloride in the test solution; impurity C can be quantified at the concentration of 0.030% relative to the concentration of benzydamine hydrochloride in the test solution.

3.2.4. Precision

Precision characteristics involve repeatability test, system suitability and intra-laboratory precision.

Solution for system suitability was injected in triplicate. Repeatability was assessed by triplicate injection of reference solution and 6 test solutions prepared from one model sample of the remedy. Intermediate precision was studied for two days. Three test solutions and one reference solution were prepared with two analysts in two different days. Results of precision studies are shown in Table 4.

Table 4. *Results of precision studies.*

Criteria for system suitability					Criteria for system repeatability Relative standard deviation of six obtained results (RSD)		Intra-laboratory precision	
Area of benzyd amine hydro chloride	RSD\ (%)	Symmetry of benzyd amine hydro chloride	Number Of theo retical plates of benzyd amine hydro chloride	Resolu tion between benzyd amine hydro chloride and impurity C	Obtained value	Acceptance criteria	Difference of average results, % Impurity C	
19293							By days	0,34
19269	1,43	1,005	7980	3,48	0,87%	≤ 10,0%	Analyst 1	1,81
19801							Analyst 2	1,12

3.2.5. Accuracy

The accuracy was assessed using nine model solutions for the unspecified impurities and 9 model solutions of impurity C.

Solutions with known concentration of impurities were injected in triplicate for each solution.

The acceptance criteria for the unspecified impurities: Recoveries (Z) should be in the range of 80.0% -120.0%. RSD for each concentration level should be less than 15.0%.

The acceptance criteria for impurity C: Recovery rate (Z) must be within 85.0% - 115.0%. RSD for each concentration level should be less than 10.0%. Results for accuracy studies

are shown in table 5.

Table 5. *Criteria for Accuracy.*

	Unspecified impurity			Impurity C		
Solution	**Recovery, Z (%)**	**Average recovery Z**	**RSD, %**	**Recovery, Z (%)**	**Average recovery Z**	**RSD, %**
M 1-1	102,93			102,18		
M 1-2	97,63	101,09	2,91	97,16	98,95	2,74
M 1-3	102,70			97,51		
M 2-1	97,64			94,62		
M 2-2	96,92	98,78	2,66	93,97	96,42	3,82
M 2-3	101,78			100,66		
M 3-1	102,04			92,84		
M 3-2	103,77	100,83	3,67	92,21	95,07	4,65
M 3-3	96,67			100,17		
Mean		100,23	2,92		96,81	3,75

3.2.6. Robustness

Robustness of the method was shown by studying the reliability of the chromatographic procedure and stability of test solutions.

Table 6. *Stability of solutions in time.*

Time of storage	**Deviation of area rate of impurity C S (TS) / S (RS), %**
Control solution	-
48 hours	0,56%

Acceptance criteria:

Variability (%), calculated for the peak area ratios S (TS) / S (RS) of benzydamine hydrochloride and examined impurities should not exceed 5%.

i. Reliability of chromatographic procedure

1. Flow rate

Three injections of reference and test solution were made with mobile phase flow rate 1,0 ml/min, 0,9 ml/min, and 1,1 ml/min.

Table 7. *The influence of flow rate (or Flow rate influence).*

Flow rate	**Deviation of area rate of impurity C S (test solution) / S (reference solution) relative to control flow rate, %**
1,0 ml/min	-
0,9 ml/min	0,39%
1,1 ml/min	1,48%

Acceptance criteria:

Variability (%), calculated for the peak area ratios S (test solution) / S (reference solution) of benzydamine hydrochloride and impurity C relative to the control conditions should not exceed 5%.

ii. The pH of the mobile phase

Three injections of reference and test solution were made with mobile phase pH change.

Table 8. *Effect of mobile phase pH.*

pH of mobile phase	**Deviation of area rate of impurity C S (test solution) / S (reference solution) relative to control pH, %**
pH 3	-
pH 2	1,44%
pH 4	1,79%

Acceptance criteria:

Variability (%), calculated for the peak area ratios S (test solution) / S (reference solution) of benzydamine hydrochloride and impurity C relative to the control conditions should not exceed 5%.

4. Conclusion

This paper describes development and validation of HPLC method for determination of related substances of benzydamine hydrochloride in the formulation (Development and validation of HPLC method for determination of related substances of benzydamine hydrochloride in the formulation are described in the article). Validation of the method was carried out according to the European regulatory requirements.

It has been shown, that the method meets the validated requirements and can be applied for determination and quantification of potential degradation products in complex formulations of benzydamine hydrochloride.

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