Original Article

SIMULTANEOUS DETERMINATION OF BENZYDAMINE HYDROCHLORIDE, METHYLPARABEN AND PEPPERMINT OIL IN A SPRAY DOSAGE FORM BY GAS CHROMATOGRAPHY

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ABSTRACT

Objective: To develop and validate an analytical procedure for simultaneous determination of benzydamine hydrochloride, methylparaben and peppermint oil in a spray dosage form by gas chromatography method (GC).

Methods: The analytical method was conducted on Agilent 7890 gas chromatograph, equipped with HP-5 capillary column with helium as a mobile phase, split/splitless injector and flame ionization detector and an auto injector. Validation parameters, such as selectivity, linearity, precision, accuracy and, robustness were estimated.

Results: A method for simultaneous determination of benzydamine hydrochloride, methylparaben and peppermint oil in a spray dosage form by GC was developed. The retention time of menthol (marker substance of peppermint oil) methylparaben and benzydamine hydrochloride, was 5.0, 9.2, and 19.4 respectively. Relative standard deviation (RSD)% for precision was 0.24, 0.13 and 0.12 respectively. The linearity of the method for given analytes was estimated in a concentration range of 80-120% to a nominal concentration with the respective correlation coefficients of more than 0.999. Accuracy of the method was within 98-102% for all analytes.

Conclusion: The developed analytical procedure meets the acceptance criteria of validation parameters and can be used in quality control laboratories for determination of benzydamine hydrochloride, methylparaben and peppermint oil in a spray dosage form.

Keywords: Benzydamine hydrochloride, Methylparaben, Peppermint oil, Menthol, Gas chromatography, Method development, Validation

INTRODUCTION

Benzydamine hydrochloride is an active pharmaceutical substance, which is widely used for the dosage forms applied for the treatment of inflammatory diseases of the oral cavity. It exhibits analgesic and antipyretic properties [1, 2] and represented in finished dosage forms such as Tantum Verde, solution for oral treatment, Diflam, oral spray, Tantum Rosa, solution and many others.
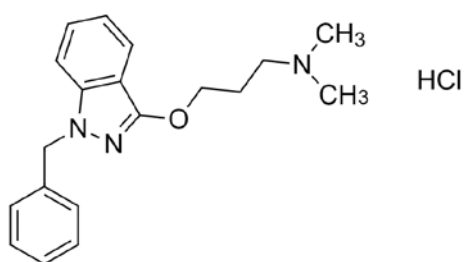

Fig. 1: Chemical structure of benzydamine hydrochloride

Methylparaben is a common antimicrobial preservative which is widely used in compositions with benzydamine hydrochloride for prevention growth of microbiological organisms in multiple used preparations, such as oral solutions, nasal and oral sprays and others [3-5].
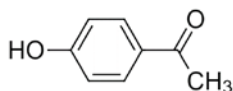

Fig. 2: Chemical structure of methylparaben

Peppermint oil is obtained from the leaves of the perennial herb, Mentha piperita L. and M. arvensis var. piperascens a member of the Labiatae family. It is a colorless, pale yellow or pale greenish-yellow liquid having characteristic odor and taste followed by a sensation of cold, freely soluble in ethanol (70%) [6, 7]. Peppermint oil is used for the treatment of digestive disorders and nervous system actions because of its antitumor and antimicrobial properties, chemoprevention potential, its renal actions, antiallergenic effects, and also for lessening cramping, digestive complaints, anorexia, nausea and diarrhea [8-11].

According to International Pharmacopea, pepermint oil contains: limonene (1.0-5.0%), cineole (3.5-14.0%), menthone (14.0-32.0%), menthofuran (1.0-9.0%), isomenthone (1.5-10.0%), menthyl acetate (2.8-10.0%), isopulegol (max. 0.2%),menthol (30.0-55.0%), pulegone (max. 4.0%) and carvone (max. 1.0%). The ratio of cineole content to limonene content should be minimum two [12].
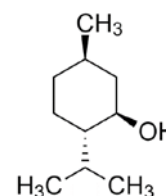

Fig. 3: Chemical structure of methol

Contemporary requirements of quality control laboratories match principles of green chemistry, which imply development, and further implementation of rapid analyzes for quality control [13-18]. Consequently, analytical method development of new drug products requires a thorough assessment of simultaneous determination of all substances to be analyzed by one injection, prioritizing rapid methods, which at the same time are less harmful to the

environment. Such strategy is vital for the economy of human resources, organic solvents, energy resources, and hence is considered to be a future of not only analytical laboratories, but of entire chemical manufacturing.

The aim of the current work was to develop a method for simultaneous determination of benzydamine hydrochloride, methylparaben and menthol (as a marker of peppermint oil), by gas chromatography, which could be applicable for modern requirements of quality control laboratories and perform a subsequent validation according to ICH Q2 requirements [19].

MATERIALS AND METHODS

Chemicals and reagents

Chloroform (GC grade) was supplied by Sigma Aldrich (Germany). Benzydamine hydrochloride reference standard and methylparaben reference standards were purchased as the Brittish Pharmacopeia standards. The menthol reference standard was purchased at Sigma Aldrich (Germany).

Spray formulation

Benzydamine dosage form, which contained peppermint oil and methylparaben was kindly provided by JSK "Farmak".

Instrument and software

Chromatography was carried out using Agilent 7890 gas chromatograph with flame ionization detector, autosampler, and injector with a split/splitless mode. A deactivated glass fiber was used as the insert of the liner. Separation was performed on HP-5 capillary column with geometry 30 m x 0.32 mm x 0.25 μm. Helium was chosen as a mobile phase. The flow rate of 1 ml per min was chosen for the method. Chemstation software was used for data acquisition.

Stock solution preparation

Standards of benzydamine hydrochloride (30 mg), and methylparaben (20 mg) were weighed and dissolved separately in 20 ml of water under slight heating in order to obtain a final concentration 1.5 mg/ml for Benzydamine hydrochloride and 1.0 mg/ml for methylparaben.

Standard of menthol was weighed and dissolved in 20 ml of isopropanol with a final concentration of 0.2 mg/ml.

Internal standard solution preparation

30 mg of benzydamine impurity A (internal standard) were weighed and dissolved in 20 ml of water to obtain a solution with 1.5 mg/ml concentration.

Standard solution preparation

5 ml of each stock solution and internal standard solution were transferred into a 50 ml flask, dissolved in 10 ml of chloroform and diluted to 50 ml with water. After an intensive shaking of the prepared solution, a chloroform layer was separated and used for analyses.

Test solution preparation

A dosage form of benzydamine hydrochloride, which contained methylparaben and peppermint oil was taken in the amount of 5 ml and transferred to a 50 ml volumetric flask. 5 ml of internal standard solution was added and 10 ml of chloroform was added. The obtained solution was diluted to 50 ml with water. After an intensive shaking of the prepared solution, a chloroform layer was separated and used for analyses.

Analytical method validation

Method validation was performed according to the ICH Q2 [19, 20] requirements for selectivity, linearity, precision, accuracy, system suitability, range, and robustness.

Selectivity

The selectivity of the method was demonstrated by injecting a test solution and a reference solution. The interference of placebo peaks to analyte peaks was assessed. The resolution of compounds to be analyzed was calculated to be not less than 1.5.

System suitability

A standard solution was injected into a gas chromatograph. The theoretical plate number, RSD% for the ratio of internal standard area to analyte area for each compound to be analyzed and peak symmetry was assessed.

Linearity

Linearity was estimated by injecting of standard solutions in triplicate with various concentration of analytes which were 80%, 90%, 100%, 110%, and 120% respectively to the nominal content in the dosage form (100%).

A calibration curve of linearity for Benzydamine hydrochloride was plotted by plotting its concentration ratio relative to the internal standard concentration in correspondence of their peak ratio. A calibration curve of linearity for methylparaben and menthol was made by plotting the respective concentrations of analytes in correspondence of their peak area.

The correlation coefficient (R^2) was calculated for each compound.

Precision

The precision of the method was estimated to check repeatability. Intra-day and inter-day precisions were studied on three concentration levels, by triplicate injection.

The coefficient of variation (RSD %) was calculated for each compound.

Accuracy

The accuracy of the procedure was studied by injecting a test solution after the addition of known amounts of a standard solution on concentration levels of 80, 100 and 120%. The amount of found concentration relative to the known concentration of analytes was calculated in %.

Robustness

Robustness was studied during the method development by changing parameters of the method (flow velocity, injection volume, the initial temperature of the column).

RESULTS

Method development

Method development for simultaneous determination of benzydamine hydrochloride, methylparaben and menthol was performed by variation and selection of optimal chromatographic parameters and sample preparation. For the method development, the initial conditions of the method for benzydamine hydrochloride assay form British Pharmacopeia were considered.

Selection of extraction conditions and internal standard

In the Pharmacopeia monograph, the chloroform extraction is carried out after an alkali treatment of the test solution. However, such treatment is inapplicable for the methylparaben extraction as it leads to the complete degradation of the preservative. Consequently, different pH values of test solution were prepared. The appropriate extraction was observed at neutral pH media (6.5-7.0). Chloroform was chosen as the organic solvent. As an internal standard for Benzydamine hydrochloride determination, an impurity A of Benzydamine was selected, which satisfies the mandatory requirements for the internal standard.

The chloroform layer was used for chromatography for the final analytical operation.

Selection of concentrations of test solutions

The initial concentrations of benzydamine hydrochloride, methylparaben, and menthol in the dosage form are 1.5, 1.0 and 0.22 mg/ml, respectively. It corresponds to a concentration of 0.15, 0.1

and 0.022 µg/µl during chromatography. No peak overloading was observed under such concentrations extent.

Screening of the stationary phase

The choice of a stationary phase is crucial for appropriate separation of analytes in gas chromatography. The polarity of the stationary phase plays a main role in compound retention in the column and hence on the elution order of analytes.

For the current separation, stationary phases with different polarity were tested, form very polar phases, such as DB-FFAP (PET bound to nitroterephthalic acid), to medium polarity phase DB-624 (6% cyanopropylphenyl/94% Dimethyl (poly) siloxane), and nonpolar phase HP-5 **(**5% Diphenyl/95% Dimethyl (poly) siloxane). The best resolution of compounds was observed on the nonpolar stationary phase. (fig. 4, 5). Hence, HP-5 column was selected for the method.
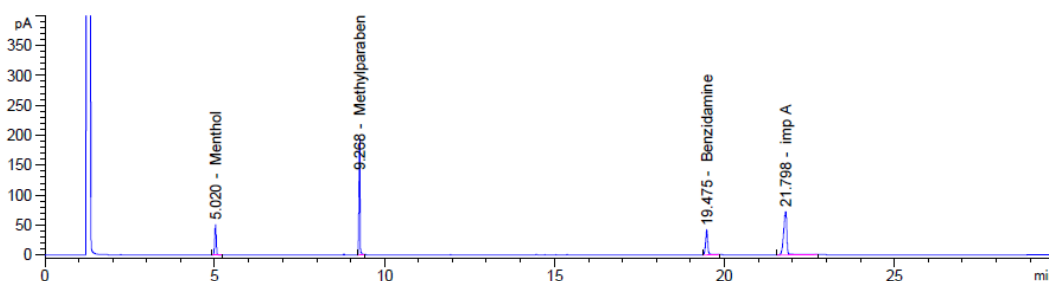

Fig. 4: Chromatogram of reference solution of benzydamine, menthol methylparaben and internal standard (impurity A of Benzydamine hydrochloride) on HP-5 column
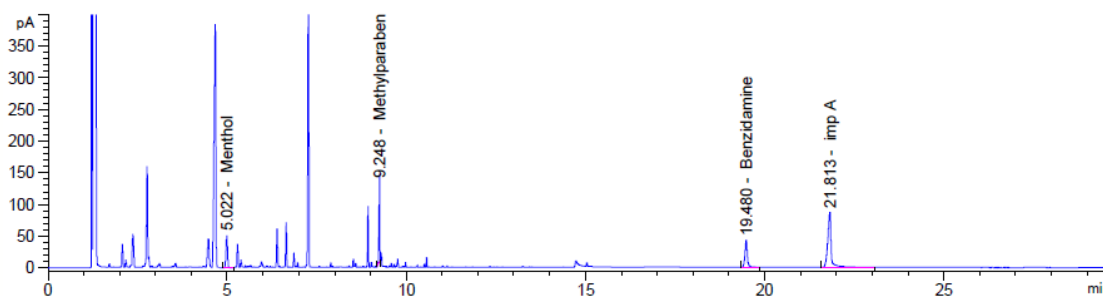

Fig. 5: Chromatogram of reference solution of benzydamine, menthol methylparaben and internal standard (impurity A of Benzydamine hydrochloride) on HP-5 column

Selection of the parameters for the injector and detector

To prevent the column overloading, 1 µl was chosen as an injection volume followed by 20:1 split ratio. Taking into account high boiling points of substances to be analyzed and their significant differences of boiling points, the temperature of 300 °C for injector was selected, which allowed reaching complete evaporation of the sample without potential discrimination nor degradation of substances to be analyzed.

The temperature of 320 °C for detector was chosen to prevent condensation of the sample on the detector after elution.

Selected injector and detector parameters allowed to obtain an appropriate sensitivity and reproducibility of the method.

Chromatography temperature program selection

The current method implies a simultaneous separation of 3 different substances with various chemical and physical properties. In this case, isothermal separation cannot secure an appropriate time of analysis which is important for routine quality control. Consequently, different temperature gradient programs were tested

to obtain a sufficient separation of all compounds, as well as to reach a proper time of a single procedure.

It was finally discovered that menthol elutes at 220 °C temperature within 5 min under the proposed chromatographic system and methylparaben within 10 min. After the elution of methylparaben, a temperature increase of 20 °C/min up to 270 °C was performed. The 18 min exposure under 270 °C allows eluting both benzydamine and internal standard.

Method validation

Selectivity

The selectivity of the method was demonstrated by the resolution of analytes and internal standard peak with any other peaks of placebo (Fig.5). No co-elution was observed, hence the method has sufficient selectivity for determining compounds.

System suitability

The system suitability parameters of the method are given in table 1. Given data is mean for 3 injections.

Table 1: System suitability of the developed method (n=3)

Compound	Retention time, min	RSD% for the ratio of ISTD area/analyte area	Theoretical plate number	Tailing factor
Menthol	5.0	0.24	64108	1.14
Methylparaben	9.2	0.25	406197	1.72
Benzydamine hydrochloride	19.5	0.12	436177	1.04

n is number of injections

Acceptance criteria

The relative standard deviation for three parallel injections for each analyte shouldn`t exceed 2.0 %Theoretical plate number for each compound shouldn`t be less than 30000. Peak tailing factor for each compound is not more than 2.0 for each compound.

The maximum RSD % for the peak area ratio is 0.25 for three parallel injections. The theoretical plate number for menthol is 64108, which is twice more than the system suitability threshold for the method. Peak tailing factor is less than 1.8 for every compound. Hence, system suitability is within the method requirements.

Linearity and range

The results of the method linearity are given in table 2.

The obtained results for linearity show that the method is linear for determined compounds. The correlation coefficient for menthol, methylparaben, and benzydamine was found to be 0.9991; 0.9996 and 0.9997 respectively. The linearity is proved to be acceptable for all compounds in the range of 80-120% of nominal concentration. Consequently, the method is linear for determined compounds.

Precision

Results of method precision are given in table 3 and 4.

Table 2: Linearity parameters of the method (n=3)

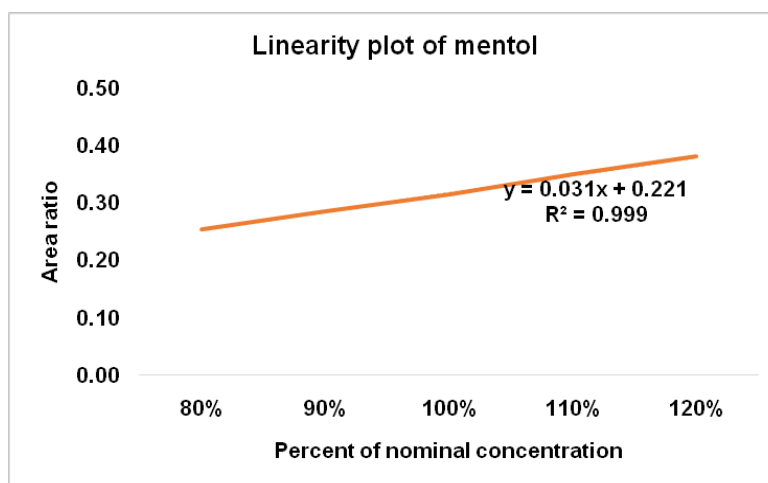| Concentration level%	Mentol (peak area ratio vs internal standard)	Methylparaben (peak area ratio vs internal standard)	Benzydamine (peak area ratio vs internal standard)
80%	0.25	0.71	0.32
90%	0.28	0.80	0.36
100%	0.31	0.89	0.39
110%	0.35	0.97	0.43
120%	0.38	1.07	0.47
Linearity equation	y = 0.0319x+0.221	y = 0.0888x+0.623	y = 0.0393x+0.2765
Correlation coefficient	0.9991	0.9996	0.9997

n is number of injections
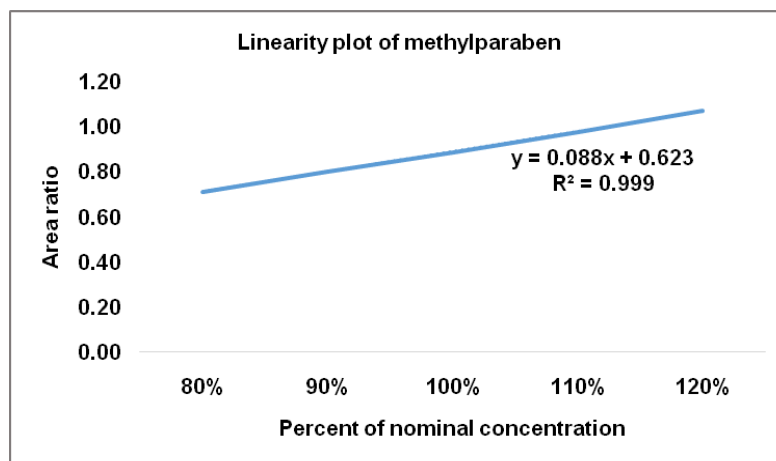

Fig. 6: Linearity plot for menthol

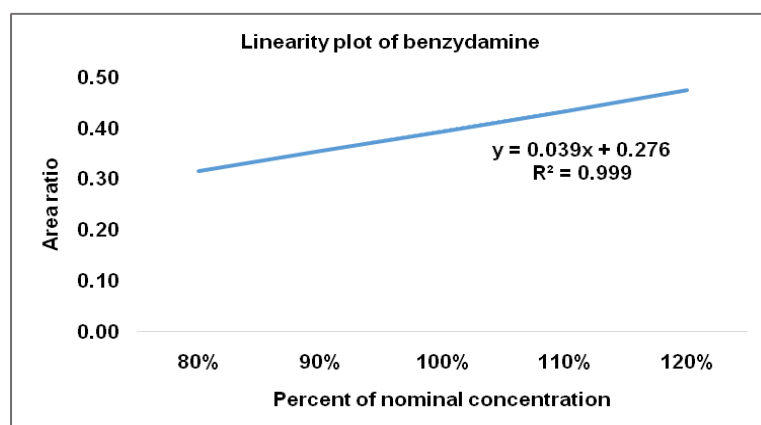**Fig. 7: Linearity plot for methylparaben**

Fig. 8: Linearity plot for benzydamine

Table 3: Intra-day precision of the method (n=3)

Relative concentration%	Menthol (peak area ratio vs internal standard), n=3, mean±SD	Methylparaben (peak area ratio vs internal standard), n=3, mean±SD	Benzydamine(peak area ratio vs internal standard), n=3, mean±SD
90%	0.28±0.02	0.80±0.02	0.36±0.02
100%	0.31±0.02	0.89±0.01	0.39±0.01
110%	0.35±0.03	0.97±0.01	0.43±0.02
RSD%	0.24%	0.13%	0.12%

n is number of injections, SD is standard deviation, % RSD is percent relative standard deviation

Table 4: Inter-day precision of the method (n=3)

Relative concentration %	Menthol (peak area ratio vs internal standard), mean±SD	Methylparaben (peak area ratio vs internal standard), mean±SD	Benzydamine (peak area ratio vs internal standard), mean±SD
90%	0.27±0.02	0.78±0.01	0.35±0.01
100%	0.30±0.01	0.86±0.01	0.38±0.02
110%	0.34±0.02	0.96±0.02	0.42±0.01
RSD%	0.21%	0.14%	0.11%

n is number of injections, SD is standard deviation, % RSD is percent relative standard deviation, The developed method is shown to have sufficient precision. The RSD % for intra-day precision for menthol, methylparaben and benzydamine peaks was 0.24, 0.13 and 0.12 respectively. For inter-day precision, RSD % was 0.21, 0.14 and 0.11 respectively. Obtained results indicate appropriate precision for the developed method.

Table 5: Accuracy for benzydamine hydrochloride (n=3)

Level%	Amount spiked,%	Amount recovered,%	% Recovery	%RSD
80	79.3	80.2	101.13	0.95
80	80.1	79.5	99.25	
80	79.6	79.9	100.38	
100	99.8	100.7	100.90	0.91
100	100.1	99.3	99.20	
100	100.3	100.9	100.60	
120	120.0	121.3	101.08	0.99
120	120.2	119.3	99.25	
120	119.8	120.8	100.83	

n is number of injections

Table 6: Accuracy for methylparabene (n=3)

Level%	Amount spiked,%	Amount recovered,%	% Recovery	% RSD
80	78.6	79.9	101.65	1.69
80	81.1	82.5	101.73	
80	79.8	78.8	98.75	
100	99.5	100.3	100.80	0.90
100	100.3	99.3	99.00	
100	101.1	100.9	99.80	
120	121.4	121.3	99.92	1.30
120	120.7	119.3	98.84	
120	119.1	120.8	101.43	

n is number of injections

Table 7: Accuracy for menthol (n=3)

Level%	Amount spiked,%	Amount recovered,%	% Recovery	%RSD
80	82.3	80.7	98.06	0.23
80	81.6	80.2	98.28	
80	80.4	79.2	98.51	
100	99.5	100.3	100.80	1.25
100	100.3	98.9	98.60	
100	101.1	99.8	98.71	
120	121.4	121.9	100.41	1.58
120	120.7	119.2	98.76	
120	119.1	121.4	101.93	

n is number of injections, Obtained results show sufficient accuracy of the method for all analytes. Recovery for menthol, methylparaben, and benzydamine was within 98-102%. The maximum RSD % for analytes was 1.58, 1.69 and 0.99 respectively.

Table 8: Initial column temperature

Column temperature, °C	220	215	225
	Benzydamine hydrochloride		
The difference between peak area ratio relative to initial method temperature, %	—	0.34	0.26
	Methylparaben		
The difference between peak area ratio relative to initial method temperature, %	—	0.77	0.53
	Menthol		
The difference between peak area ratio relative to initial method temperature, %	—	0.94	0.81

Table 10: Results of flow rate influence

Flow rate, ml/min	Difference between benzydamine hydrochloride peak areas relative to final method conditions, %	Difference between methylparaben peak areas relative to final method conditions, %	Difference between menthol peak areas relative to final method conditions, %
1.0	-	-	-
0.9	0.19	0.33	0.28
1.1	0.28	0.21	0.41

Table 11: Results of injection volume influence

Injection volume µl	Difference between Benzydamine hydrochloride vs internal standard peak area ratio relative to final method conditions, %	Difference between methylparaben vs internal standard peak area ratio relative to final method conditions, %	Difference between Menthol vs internal standard peak area ratio relative to final method conditions, %
1.0	-	-	-
0.9	0.09	0.11	0.12
1.1	0.06	0.10	0.11

It was shown, that a slight change of method parameters (±10%) didn`t influence on quantitative determination of compounds to be analyzed, so the method is reliable under normal usage.

Evaluation of accuracy

Results of method accuracy are given in tables 5-7.

Robustness

Robustness of the analytical procedure was studied during the method development, which included the variation of method parameters, such as flow velocity, injection volume, and initial column temperature.

Results of robustness study are given in tables 8-10.

DISCUSSION

The developed GC method for simultaneous determination of benzydamine hydrochloride, methylparaben and menthol is simple, fast, precise, accurate, specific, robust and consistent with green chemistry approach.

In previous researches determination of benzydamine and methylparaben had been described separately and predominantly by high performance liquid chromatography method or UV spectrophotometric method [21-25]. Determination of menthol had been described by GC-MS method [26].

In the current study, the GC method with flame ionization detection was implemented which allowed achieving a satisfactory selectivity for all compounds.

The method development was based on the selection of extraction solvent, with subsequent gas chromatography. The internal standard was chosen for the method for decreasing of quantification uncertainty. Method parameters were varied to achieve an appropriate separation and selectivity. Finally, an HP-5, 30 m capillary column was chosen for the separation as it exhibited the best selectivity and effectiveness of the chromatographic system. The total time of a single run under proposed method conditions is 30 min, which is much less then it would be in case of separate evaluation of components.

Validation of the method was carried out according to ICH Q2 guidance. All validated parameters were within the acceptance criteria. The method is linear for all compounds with a correlation coefficient of more than 0.999.

The maximum RSD% for three parallel injections was less than 0.3% for each compound. The accuracy for all compounds was within 98%-102%. The method is robust and will not be affected by minor

changes of method parameters, which has been shown by robustness study.

CONCLUSION

This paper describes the development of a method for simultaneous determination of menthol, methylparaben and benzydamine hydrochloride in a dosage form of benzydamine hydrochloride.

The essence of the method matches the approaches of green chemistry, in terms of a simultaneous determination of three compounds, time and cost-effective separation. The method is proved to be precise and selective for all compounds.

The method demonstrates appropriate performance and is fit for utilization in quality control and research laboratories.

AUTHORS CONTRIBUTIONS

All the authors have contributed equally

CONFLICT OF INTERESTS

Declared none

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