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Optimizing the Process of Bromhexine Determination in Tablets by Using the Response Surface Methodology

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ABSTRACT

Objective: *The study is to establish a method for the determination of bromhexine hydrochloride in tablets.*

Methods: *Bromhexine hydrochloride in the tablets were analyzed by a HIQ Sil C18 column (250 mm × 4.6 mm; 5 µm) with a mobile phase including methanol -0.09% acid orthophosphoric (60:40, v/v). The flow rate was 0.9 ml/min with the detection wavelength at 247 nm.*

Results: *This method was validated according to the International Conference on Harmonisation (ICH) Q2 (R1) guideline. Calibration graph was linear (r=0.9999, n=7) in concentration range of 2-40 µg/ml. The average recovery of bromhexine hydrochloride was 99.63%, and RSD was 1.01% (n=12).*

Conclusions: *The simple, fast, and reproducible RP-HPLC method has been successfully developed and validated. The proposed method is used for the quantitation of bromhexine in some products in the market.*

Keywords: Bromhexine; HPLC; Response surface methodology

INTRODUCTION

Bromhexine hydrochloride is an active ingredient used in the treatment of acute and chronic bronchopulmonary diseases associated with excessive mucus secretion. Clinically, bromhexine enhances mucus transport by reducing the mucus adhesion and activating the ciliated epithelium to transport the phlegm easily out of the respiratory tract [1].

There are currently over 80 brand name and more than 50 domestic companies producing pharmaceutical dosage forms containing this active ingredient. In Vietnam Pharmacopoeia V [2], the determination of bromhexine hydrochloride was studied by spectrophotometric method or potentiometric titration (for material). In addition, some authors have proposed other analytical methods such as the spectrophotometric method for derivatives of bromhexine [3], chromatography [4-6], combined form contains bromhexine [7-10]. However, up to now, no author from Vietnam has published the quantification process of bromhexine hydrochloride by liquid chromatography. Therefore, the purpose of literature research is to develop and validate the quantitative process of bromhexine

hydrochloride by liquid chromatography to contribute a technique for routine analysis of bromhexine hydrochloride in raw materials and pharmaceutical dosage forms.

MATERIALS AND METHODS

Instruments and chemicals

Bromhexine tablets 8 mg. HPLC Agilent 1260 system, PDA detector; Mettler Toledo analytical balance (0.01 mg); Arium® pro DI Ultrapure Water System (Sartorius-Germany), pH measurement (Metler Toledo), Potentiometric titration 888 Titrando (Metrohm-Sweden).

Methanol, acetonitrile (Merck-Germany), and other chemicals were chromatographic analytical grade. Bromhexine standard was supplied by The Institute of Drug Quality Control-Ho Chi Minh City (IDQC-HCMC).

Preparation of standard solution: Dissolve bromhexine standard in the mobile phase to obtain the concentration of 20 µg/ml; use the best in a day.

Preparation of sample solution: Twenty bromhexine tablets were ground to a fine powder and mix well. Weigh the amount of powder equivalent to the average weight of one 30 mg bromhexine tablet and transfer to a 100 ml volumetric flask. Add 35 ml of methanol in the flask, ultrasound for 15 minutes, and fill up to the mark with methanol. The liquid continuously passes through the filter paper, then 5 ml filtrate was diluted to 20 ml with mobile phase and filter through a 0.45 µm membrane for chromatographic injection.

Optimization of chromatographic conditions

The optimized conditions for the chromatographic procedure were obtained through two steps. Firstly, different factors of mobile phases were surveyed on a chromatographic a HIQ SilC18 column, including (i) a mixture of methanol and acetonitrile in various proportions, pH, and buffer solutions. Secondly, the response surface methodology was used to optimize three independent variables of chromatographic conditions: (x_1) concentration of organic solvents (methanol), (x_2) concentration of Orthophosphoric Acid (OPA) in mobile phase, and (x_3) flow rate. Each variable was assigned with three levels (-1), (0), and (+1) (Tables 1 and 2), and a total of twenty experimental runs with the different combinations of variables were presented in Table 1.

Table 1. Independence factors and corresponding levels

No.	Name Variable	Unit	Code	Range Values			Responses	
				−1	0	+1	t_R	Symm.
1	MeOH	% (v/v)	x_1	55	60	65		
2	OPA	% (v/v)	x_2	0.02	0.2	0.12		
3	Flow	ml/min	x_3	0.8	1.0	1.2	Y_1	Y_2
Note: MeOH- Methanol; OPA- Orthophosphoric Acid, Flow- Flow rate; and t_R- Retention Time; Symm- Symmetry								

The second-order polynomial function was established to clarify the mathematic relationship between responses (y) and three independent variables (x_i) as the following function (1).

$$y = f(x) = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad (1)$$

Where y are the predicted responses, x_1, x_2, x_3 are independent variables, b_0 is the y-intercept, b_1, b_2, b_3 are the linear coefficients, b_{11}, b_{22}, b_{33} are second-order coefficients, and b_{12}, b_{13}, b_{23} are interaction regression coefficients among

three variables. The Analysis of Variance (ANOVA) using Design-Expert version 7.0.0 (State Ease, Inc.) was carried out to determine the appropriate values of responses.

Other chromatographic conditions: HPLC Agilent 1260 system (Gremany) with PDA detector, detection wavelength at 247 nm, column temperature was maintained at 40°C, and the injection volume was 20 µL. HPLC system was set up with HIQ SilC18 column (250 mm x 4.6 mm; 5 µm) as a stationary phase, the corresponding precolumn. Isometric elution with a mixture of MeOH and 0.09% OPA solution (60:40, v/v) at 0.9 ml/min. The observed amount of bromhexine recorded by HPLC system in comparison with the label was calculated as the equation:

$$X(\%) = \frac{S_t}{S_c} \times C_C \times D \times \frac{m_{TB}}{m_c} \times \frac{100}{H}$$

Where S_c, S_t: Peak area of bromhexine in standard and sample solution; C: Concentration of standard solution (mg/ml); D: Dilution factor; mtb: Mean amount of Tablet (g); mc: sample weight (g); H: the labeled amount (H=8 mg).

Validation of analytical procedure

The analytical method was validated according to ICH guidelines [11] for system suitability, selectivity, linearity, precision, accuracy, Limit of Detection (LOD), and Limit of Quantitation (LOQ).

Data analysis: Experimental data were analyzed by ANOVA statistical technique on Microsoft Office Excel software and presented in the equation $\overline{X} \pm SD$. Hypothesis was tested based on t-test Student. The difference was statistically significant when P-value<0.05.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

High-performance liquid chromatography is a separation technique with many advantages: fast, sensitive, repeatable, and high accuracy method. Thus this is considered one of the leading techniques in pharmaceutical quality control. However, the HPLC method is affected by factors of stationary phase (normal phase, reverse phase, particle size, manufacturer); Mobile phase (components of the organic solvent, buffer solution, pH); System (manufacturer, injection, flow rate, column temperature, detector); Environment (location, temperature, humidity) and the analyst. Initially, the screening of various variables that influence the responses was designed according to the Plackett-Burman matrix with 05 input variables in 12 experiments, the experimental results are summarized in Table 2.

Table 2. The results of screening chromatographic variables according to Plackett-Burman design

Factor	% Contribution			
	Y_1	Y_2	Y_3	Y_4
Methanol (%)	(-)50.43[a]	(-)5.40[b]	(-)18.27[a]	(-)67.63[a]
OPA (%)	(+)23.03[a]	(+)0.15[b]	(+)7.61[a]	(-)7.55[b]
Flow rate (ml/min)	(-)6.93[b]	(+)11.48[b]	(-)8.28[a]	(-)1.24[b]
Sample injection volume (µL)	(+)0.10[b]	(+)45.32[a]	(+)29.04[a]	(-)5.88[b]
Column temperature (°C)	(-)0.49[b]	(-)12.11[a]	(-)8.70[a]	(+)13.00[a]

Note: Signal of bromhexine: Y_1-The theoretical plates number; Y_2-Peak area; Y_3-Symmetry; Y_4-Retention time; (-) Negative effects, (+) Positive effects; and a-significant ($P \leq 0.05$); b-not significant ($P > 0.05$).

After surveying, three independent factors including MeOH content (%), (x_1), concentration of OPA (%, v/v), (x_2), and flow rate (ml/min), (x_3) was planned experiments according to Box-Behnken design in the range of 55-65%, 0.02-0.12%, and 0.8-1.2 ml/min for x_1, x_2, and x_3, respectively. The important responses are time retention (y_1) and symmetry factor (y_2). The results of designed experiments are shown in Table 3.

Table 3. RSM for planning experiments and data collected

No.	Independent variables			Retention Time (Y_1)		Symmetry factor (Y_2)	
	X_1	X_2	X_3	Actual values	Predicted value	Actual values	Predicted value
1	0.0	0.5	-0.5	4.61	4.60	1.00	1.02
2	-1.0	-1.0	-10	6.52	6.43	0.99	1.01
3	0.0	0.0	1.0	3.43	3.42	1.02	1.02
4	-1.0	-1.0	1.0	4.28	4.19	0.99	0.99
5	1.0	1.0	-1.0	4.12	4.1	0.89	0.9
6	0.0	-1.0	-1.0	4.68	4.87	0.92	0.92
7	1.0	-1.0	1.0	2.61	2.61	0.89	0.89
8	1.0	0.0	0.0	3.23	3.26	0.95	0.95
9	1.0	1.0	1.0	2.73	2.75	0.97	0.96
10	1.0	-1.0	1.0	2.61	2.61	0.89	0.89
11	1.0	-1.0	-1.0	3.94	3.91	0.83	0.83
12	-1.0	1.0	-1.0	6.92	6.9	1.07	1.07
13	-1.0	1.0	0.0	5.36	5.5	1.08	1.08
14	-1.0	-1.0	1.0	4.02	4.19	1.00	0.99
15	-1.0	0.0	-1.0	6.85	6.84	1.11	1.09
16	1.0	1.0	1.0	2.73	2.75	0.96	0.96
17	1.0	-1.0	-1.0	3.91	3.91	0.84	0.83
18	1.0	1.0	-1.0	4.12	4.1	0.92	0.9
19	-1.0	1.0	1.0	4.74	4.63	1.04	1.05
20	0.0	-1.0	0.0	3.89	3.72	0.95	0.94

Analysis of variance (ANOVA) calculates that P-value is lower than 0.05. R^2 coefficient is good (>0.9) and the confidence level is 95% (Tables 1a and 1b). RSD <2% demonstrated the precision for both models of responses. Besides, both models show that the predictive power is high compatibility since all points relating to actual and predicted values are located close to the 45-degree line, and the remainder also presents a random distribution ($\sigma = \pm 3$) (Figure 1).

The Design-Expert software predicts that the optimal mixture of the mobile phase is MeOH and 0.09% OPA (60:40, v/v), and the flow rate is 0.9 ml/min (D=1.0) (Figure 2). In order to validate the prediction accuracy of the mathematical model, verification experiments were carried out under the optimal conditions, the chromatographic parameters of the sample solution (Figure 3) meet all chromatographic requirements (Table 4).

Table 4. ANOVA analysis for experimental model

No.	Time retention (R_t), (y_1)				Symmetry factor, (y_2)			
	Mean square	F-value	P-value	ANOVA	Mean square	F-value	P-value	ANOVA
Model	3.6451	245.77	<0.0001	Std. Dev.: 0.012; C.V. %: 2.86; Mean=4.27; Adj R: 0.999; Adeq Precision: 49.857; Lack of Fit: 0.1195 (not significant)	0.0125	64.37	<0.0001	Std. Dev.: 0.014; C.V. %: 2.86; Mean=4.27; Adj R: 0.999; Adeq Precision: 49.857; Lack of Fit: 0.1195 (not significant)
x_1	18.8194	1268.89	<0.0001		0.0713	368.41	<0.0001	
x_2	0.3476	23.44	0.0007		0.0161	83.14	<0.0001	
x_3	12.3848	835.04	<0.0001		0.0015	7.85	0.0187	
x_1x_2	0.0687	4.63	0.0569		0.0000	0.17	0.6889	
x_1x_3	0.6993	47.15	<0.0001		0.0048	24.84	0.0006	
x_2x_3	0.0011	0.07	0.7901		0.0000	0.15	0.7065	
x_1^2	0.2226	15.01	0.0031		0.0000	0.13	0.7228	
x_2^2	0.0725	4.89	0.0515		0.0047	24.31	0.0006	
x_3^2	0.1553	10.47	0.0089		0.0003	1.73	0.2184	

Note: significant $P \leq 0.05$; not significant $P > 0.05$

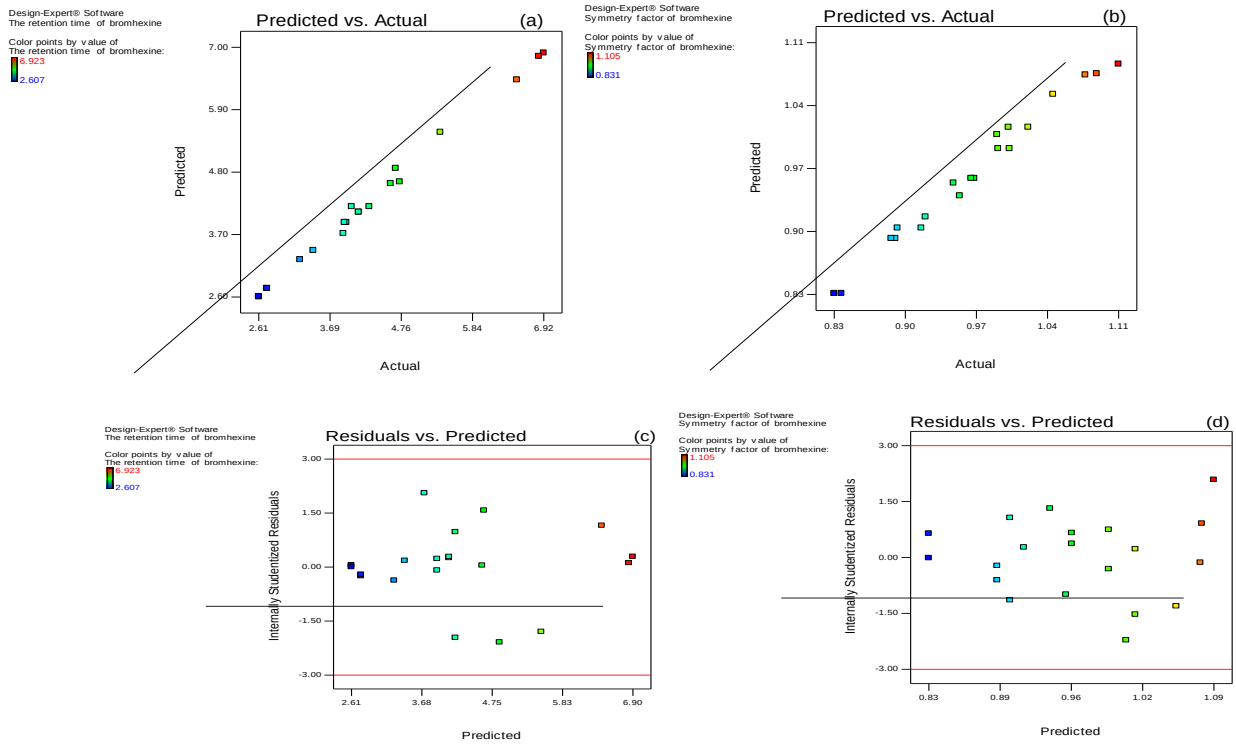

Figure 1. Actual and predicted values (a, b); Random distribution of experimental values (b, d)

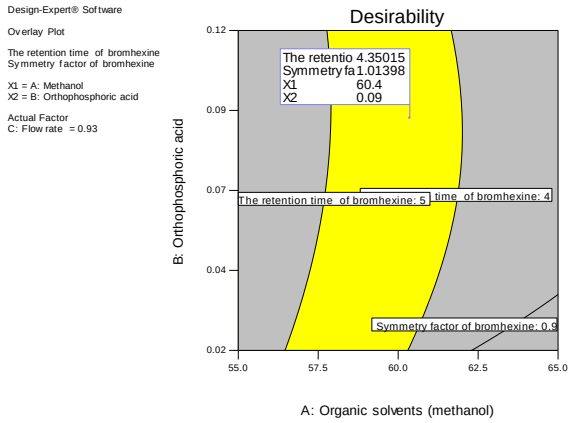

Figure 2. The three-dimensional response surface of desirability for the optimization of the experimental model

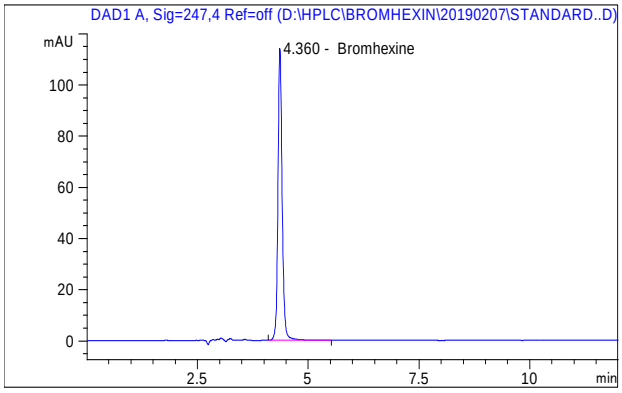

Figure 3. HPLC chromatogram at optimized conditions:C18 column (4.6 mm x 250 mm; 5 µm); mobile phase including MeOH and 0.09% OPA solution (60:40, v/v), flow rate at 0.9 ml/min

Method validation

System suitability: The injection of standard solution at the concentration of 20 µg/ml was replicated six times into HPLC system. The recorded data show the sharp and symmetrical peaks (USP Tailing 0.8-1.5) and the high theoretical plates number of each substance (N>3000). The method demonstrated to produce excellent repeatability with RSD values (%) of retention time (t_R), and peak area (S) is not more than 2% shows the suitability of the analytical system. The results are presented in Table 5.

Table 5. Results of system suitability validation (n=6)

No.	Chromatographic parameters of bromhexine peak				
	t_R (min)	S (mAU*S)	k'	USP Tailling	N
1	4.373	431.859	0.514	1.057	12426
2	4.371	431.869	0.514	1.047	12426
3	4.378	431.806	0.516	1.027	12341

4	4.375	431.811	0.514	1.066	12383
5	4.373	430.877	0.514	1.066	12407
6	4.385	429.991	0.518	1.016	12388
TB	4.376	431.369	0.515	1.047	12395
S.D	0.005	0.777	0.002	0.021	32
%RSD	0.0012	0.0018	0.0032	0.0168	0.0026

Selectivity: Selectivity has expressed the ability to distinguish the peak of analyzed substance with impurities or other substances peaks. Sample without and with the standard addition, standard solution, and placebo (a mixture of lactose, corn starch, povidone, sodium starch glycolate, magnesium stearate) were analyzed. The results are shown in Figures 4 and 5.

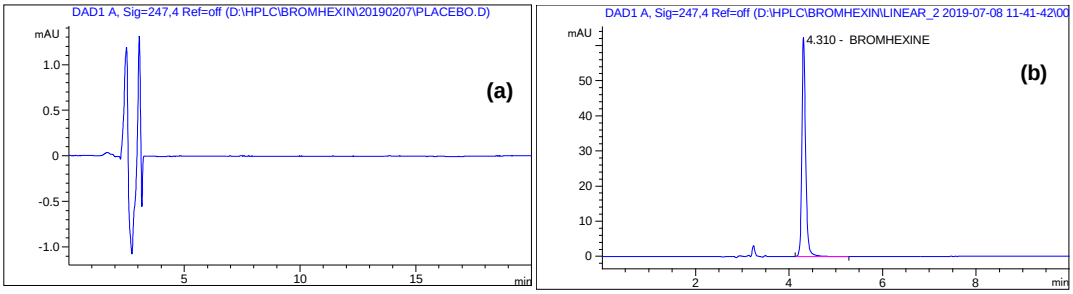

Figure 4. Chromatogram of (a) placebo; (b) standard; (c) sample, and (d) sample with standard addition

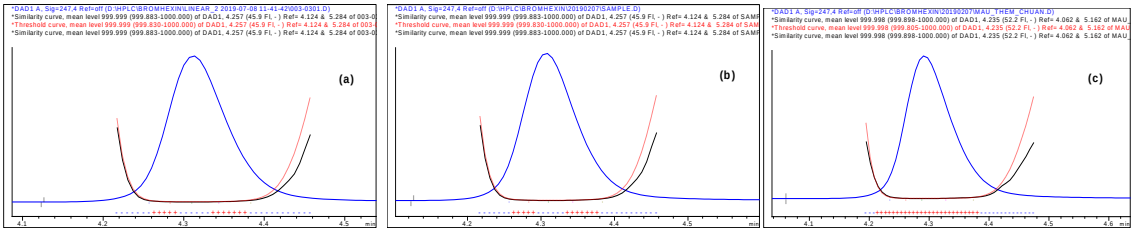

Figure 5. Graphs show the purity of bromhexine peaks of (a) standard, (b, c) sample without and with the standard addition, respectively

Peaks of analyte and other substances are separated on chromatograms of separate peaks (Figure 4); the retention time of bromhexine is found at 4.3 minutes (Figure 4b-d). Meanwhile, no peak appeared at the retention of bromhexine on the chromatogram of blank (Figure 4a). The purity of analyte peaks in the samples with or without the standard addition, standard solution were evaluated by Chemstation-Agilent software. The results showing bromhexine signals are over 99.9% pure (Figure 5a-5c) demonstrate a specific method for quantitative analysis of bromhexine.

Accuracy, precision, linearity, limit of detection (LOD) and limit of quantitation (LOQ): The linearity is set up in the bromhexine concentration range of 2-40 µg/ml. From chromatographic data obtained, the regression correlation between the bromhexine concentration and peak area at the wavelength of 247 nm is established in the following equation: y=15.199x-1.145; R2=0.9999. Thus, the linear concentration range of the method is: 2-40 µg/ml; Detection limit (LOD) is 0.12 µg/ml; Quantitative limit (LOQ) is 0.39 µg/ml. Data is shown in Figures 6 and 7.

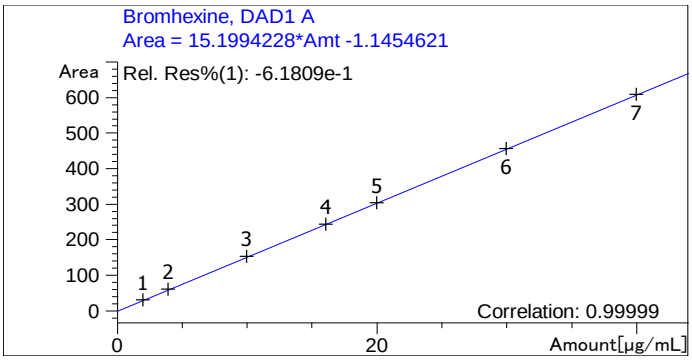

Figure 6. The graph shows the correlation between concentration and peak area of bromhexine

The accuracy of the analytical method is determined by the method of standard addition. In the current study, nine spiked samples at three different concentration levels of 80%, 100%, and 120%, respectively equivalent to 16 µg/ml, 20 µg/ml and 24 µg/ml, respectively in comparison with the quantitative concentration (20 µg/ml) of bromhexine were prepared. Each concentration level was replicated three samples, and each sample was injected 1 time into HPLC system. The recovery percentage was found in the range of 99.5-99.8%, which lies well inside the acceptable criteria of 98-102% (ICH guidelines 2005).

The precision of the method assessed the distribution of data values by analyzing samples intra-day and inter-day. The repeatability expressed by percent Relative Standard Deviations (RSD) of the analyte concentration is 1.03% (n=6). The % RSD value of intermediate precision was found to be 0.99% (n=12). Thus, the analytical procedure meets the requirement of overall precision (RSD<2%). The percent RSD values of bromhexine concentration of inter-day and intra-day precision are reported in Table 6 and Figure 7.

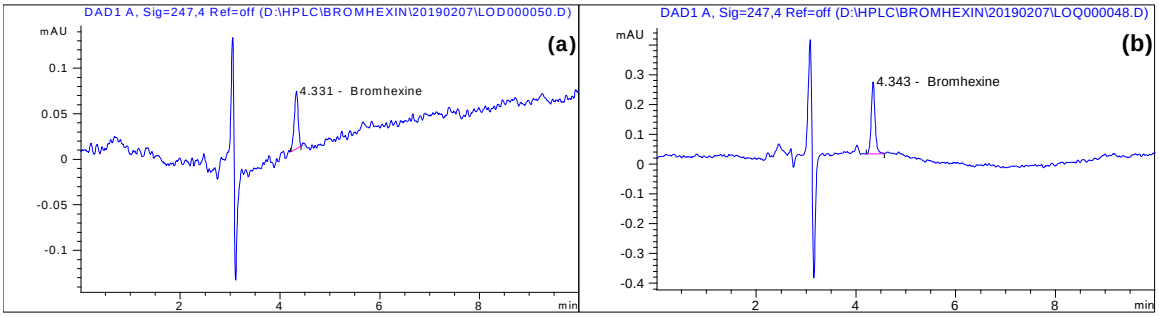

Figure 7. Chromatograms of samples at the concentration of (a) LOD 0.12 µg/ml; (b) LOQ 0.39 µg/ml

Table 6: Data showing linearity, LOD, LOQ, precision, and accuracy of analytical method

Linear range			2 - 40 (*µg/ml*)	
Regression equation; Correlation coefficient (R)			y = 15.199x - 1.145; R = 0.9999	
Limit of detection (LOD)			0.12 (*µg/ml*)	
Limit of quantitation (LOQ)			0.39 (*µg/ml*)	
Precision	Intra-day (n=6)		Inter-day (n = 12)	
	Accuracy (%) $\overline{X} \pm SD$	RSD (%)	Accuracy (%) $\overline{X} \pm SD$	RSD (%)
	99.45 ± 1.03	1.03	98.96 ± 0.98	0.99
Accuracy (n = 3)	Concentration	Concentration found	% Recovery $\overline{X} \pm SD$	

		$\overline{X} \pm SD$	
	16 (*µg/ml*)	15.99 ± 0.03	99.8 ± 0.32
	20 (*µg/ml*)	19.98 ± 0.05	99.6 ± 0.53
	24 (*µg/ml*)	24.03 ± 0.02	99.5 ± 0.51

Validation results demonstrate that the process of determining bromhexine by HPLC method with PDA detector meets all requirements of selectivity, linearity, accuracy, and precision, LOD, and LOQ according to ICH guidelines. So, the validation study leads to the acceptance of a newly developed method in routine qualitative process for bromhexine in tablets.

Comparison of the proposed method with the existing process in Vietnamese Pharmacopoeia V: Six samples from different products were determined following the established method and the quantitative process suggested in Vietnamese Pharmacopoeia V. The results are shown in Table 7.

Table 7. Determination results of 8 mg bromhexine tablets by two methods

	The ratio of found value and labeled value		ANOVA analysis
No.	**Vietnamese Pharmacopoeia V**	**Proposed method**	
1	97.41	98.88	F<Fcrit (4.76<4.96); P-value (0.054>0.05); Df =10.
2	100.18	98.53	
3	99.63	98.81	
4	99.64	98.09	
5	99.67	97.56	
6	100.14	98.67	
Mean	99.45	98.42	
%RSD	1.03%	0.52%	

According to ANOVA analysis, the content of bromhexine compared to the labeled content was determined by two methods: (i) newly developed HPLC method and (ii) the suggested method in Vietnamese Pharmacopoeia V. The results showing no statistically significant differences with a confidence level at 95% suggests a close fit of the developed method to the process mentioned in Vietnamese Pharmacopoeia V.

CONCLUSION

A quantitative method of bromhexine in tablets by HPLC system was developed and optimized using Response surface methodology software, and the influence level was analyzed by ANOVA analysis. The procedure was validated according to ICH guidelines with criteria including selectivity, inter-day and intra-day precision with RSD of <2%, and accuracy of 99.5-99.8% in the linear range of 2-40 µg/ml.

The procedure was applied to determine the concentration of 8 mg bromhexine tablets and compare the results with the method suggested in the Vietnamese Pharmacopoeia V. ANOVA analysis showed that the results were not significantly different (P>0.05) between 2 methods.

From the achieved results, this study can be developed for application in controlling the content of bromhexine in material and tablet products.

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