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Research Article

DEVELOPMENT AND VALIDATION OF A HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY FOR THE DETERMINATION OF TERBUTALINE SULFATE, BROMHEXINE HYDROCHLORIDE, AND ETOPHYLLINE IN PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

Objective: The study aimed to development and validation of simple, precise, and reliable high-performance thin-layer chromatography (HPTLC) for the determination of terbutaline sulfate (TBS), bromhexine hydrochloride (BRH), and etophylline (ETP) in pharmaceutical dosage form.

Methods: A simple, precise, rapid, and accurate HPTLC method was developed for the estimation of TBS, BRH, and ETP in pharmaceutical dosage form. Pre-coated silica gel G60 F254 aluminum sheet (10 cm^2×10 cm^2 and thickness 0.2 mm) was used as stationary phase while mobile phase consisting of benzene: methanol:glacial acetic acid 8:0.5:1.5 v/v/v detection at 275 nm. The present method had validated according to ICH guidelines.

Results: Migration distance found 80 mm at 275 nm. The retention factor found to be 0.24, 0.57, and 0.68, respectively. The detector response was linear in the concentration range of 60–210 ng/band, 2400–8400 ng/band, and 96–336 ng/band, respectively. The linear regression equation being Y=32.20x−562.9, Y=11.79x−1711, and Y=1.756x−5636, respectively. The limit of detection for TBS 0.677 µg, for BRH 8.123 µg, and for ETP 57.915 µg and limit of quantification to be 2.053, 24.617, and 175.5 µg, respectively, were found. The developed method validated by ICH guideline, i.e., accuracy, precision, robustness, specificity, and system suitability.

Conclusion: In this study, we had developed a simple, fast, and reliable HPTLC method for the determination of TBS, BRH, and ETP in pharmaceutical dosage form.

Keywords: Terbutaline sulfate, Bromhexine hydrochloride, Etophylline, High-performance thin-layer chromatography, ICH guidelines, Simultaneous estimation, Tablet.

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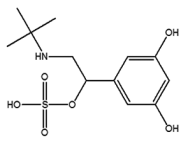
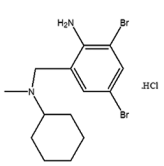
INTRODUCTION

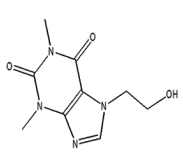
Terbutaline sulfate (TBS) is a selective beta-2 adrenergic agonist used as a bronchodilator and mucolytic; etophylline (ETP) is non-selective phosphor diesterase inhibitor and acts as cardiac stimulant and bronchodilator while bromhexine hydrochloride (BRH) is a mucolytic agent. Combination is used in the treatment of respiratory disorders associated with viscid or excessive mucus. Estimation of TBS and BRH is reported in Indian pharmacopoeia while ETP reported in European pharmacopoeia. Literature review revealed that the estimation of ternary mixture containing salbutamol sulfate, BRH, and ETP by thin-layer chromatography (TLC) method [1]. Determination of BRH in pharmaceuticals by TLC densitometry method [2], determination of TBS in human plasma by high-performance TLC (HPTLC) [3], stability-indicating HPTLC method for the determination of terbutaline sulfate in bulk and from submicronized dry powder inhalers [4], and development and validation of HPTLC method for the determination of BRH in human plasma [5,6] have been reported. In this work, an attempt made to develop economical and rapid HPTLC method for simultaneous estimation of TBS, BRH, and ETP in pharmaceutical dosage form.



Terbutaline sulphate



Bromhexine hydrochloride



Etophylline

EXPERIMENTAL

Instrumentation

Quantitative method has been developed using CAMAG HPTLC instrument. For estimation, Camag Linomat V (semiautomatic application, band application by spray-on technique) (2–500 µL), Hamilton syringe (100 µL), Camag TLC Scanner IV (scanning speed up to 100 mm/s, spectral range 190–800 nm), Camag Twin Trough Chamber (10 cm^2× 10 cm and 20 cm× 10 cm), dual-wavelength ultraviolet cabinet (dual-wavelength 254 and 366 nm), and Camag winCATS software V1.4.7 have used.

Chemicals and solvents

TBS, BRH, and ETP procured from Nirlife Healthcare, Nirma Ltd., Ahmedabad, India, as gratis samples. The solvents used methanol, benzene, and glacial acetic acid of analytical reagent (AR) grade purchased from Loba Chemicals, Mumbai, India. Commercial tablets of TBS 2.5 mg, BRH 4 mg, and ETP 100 mg purchased from local market.

Selection of mobile phase

On the basis of various trial taken for optimized mobile phase for simultaneous estimation of TBS, BRH, and ETP, the mobile phase comprised phase benzene, methanol, and glacial (8:0.5:1.5 v/v/v) produces better separation of peak with resolution and reproducibility as shown in Fig. 1.

Preparation of the mobile phase and diluents

The mobile phase prepared by mixture of benzene: methanol:glacial acetic acid in the ratio of 8:0.5:1.5 v/v/v, respectively.

Preparation of system suitability solution (standard solution)

TBS stock solution (1 mg/mL)

100 mg of TBS transferred to 100 mL volumetric flask, dissolved, and diluted up to the mark with methanol to get TBS stock solution containing 1 mg/mL of TBS.

ETP stock solution (1 mg/mL)

Weigh 100 mg of ETP and transferred to 100 mL volumetric flask, dissolved, and diluted up to the mark with methanol to get ETP stock solution containing 1 mg/mL of ETP.

BRH stock solution (1 mg/mL)

Accurately weighed 100 mg of BRH transferred to 100 mL volumetric flask, dissolved, and diluted up to the mark with methanol to get BRH stock solution containing 1 mg/mL of BRH.

Preparation of working standard solution for calibration curve

The aliquot (0.075 mL) of TBS, (3 mL) ETP, and (0.12 mL) BRH stock solutions were transferred into 10 mL volumetric flask and diluted up to the mark with methanol to prepare mixture of working standard solution containing 7.5 µg/mL of TBS, 300 µg/mL of ETP, and 12 µg/mL of BRH.

Preparation of sample solutions

Twenty tablets precisely weighed and crushed. Weight equivalent to 2.5 mg of TBS, 100 mg of ETP, and 4 mg of BRH. The powder transferred to 100 mL volumetric flask. Powder dissolved in 20 mL of methanol and sonicate the solution for 45 min, filter it through Whatman filter paper No. 41 and diluted up to 100 mL with methanol to prepare solution containing 25 µg/mL of TBS, 1000 µg/mL of ETP, and 40 µg/mL BRH. Withdraw 3 mL solution from it, transferred to 10 mL to volumetric flask and dilute up to the mark with methanol to prepare solution containing 7.5 µg/mL of TBS, 300 µg/mL of ETP, and 12 µg/mL BRH (test solution).

Chromatographic condition

Working standard solution (12 µL) or sample solution for analysis of formulation (12 µL) was spotted on the pre-washed TLC plate and developed with mobile phase benzene: methanol:glacial acetic acid in the ratio of 8:0.5:1.5 v/v/v, respectively. Photometric measurements performed at 275 nm in absorbance/reflectance mode with Camag TLC Scanner-V using winCATS V 1.4.7 software.

METHODS

Working standard solution (12 µL) or sample solution for analysis of formulation (12 µL) spotted on the pre-washed TLC plate under nitrogen stream with semi-automatic spotter. Plate dried under IR lamp and developed in a Twin Trough Chamber previously saturated mobile phase benzene: methanol:glacial acetic acid in the ratio of 8:0.5:1.5 v/v/v, respectively. After development, the plate dried in oven. Photometric measurements performed at 275 nm in absorbance/reflectance mode with Camag TLC Scanner-V using winCATS V 1.4.7 software incorporating the track optimization option. Six replicates of each standard solution of TBS, ETP, and BRH were determined in the range of 60–210 ng/band, 2400–8400 ng/band, and 96–336 ng/band, respectively. Calibration graph plotted by the concentration of TBS, ETP, and BRH on X-axis and peak area on Y-axis and linearity curve is shown in Fig. 2. The amount of drug present in the sample had computed by calibration graph.

RESULTS AND DISCUSSION

The objective of the present work is to develop simple, precise, and reliable HPTLC method for the analysis of TBS, BRH, and ETP in bulk and pharmaceutical dosage forms. This was achieved using the most commonly employed Silica gel G60 F254 aluminum sheet (10 cm×10 cm and 10 cm×20 cm) column detection at 275 nm. The representative densitogram indicates TBS, BRH, and ETP.

Parameter fixation

In developing this method, a systemic study of the effects of various parameters was conducted by varying one parameter at a time and controlling all others. The following studies conducted for this purpose. Optimized chromatographic conditions of terbutaline sulfate, bromhexine hydrochloride, and etophylline parameters shown in (Table 1).

Mobile phase characteristics

Various experiments were conducted by changing composition of mobile phase and flow rate to achieve significant separation and resolution. For ideal separation of the drugs, AR grade benzene, methanol, and glacial acetic acid were used in the ratio of 8:0.5:1.5 (v/v/v) which were proved to be the best suitable of all the combinations since the chromatographic peak obtained was better resolved and free from tailing and fronting.

Linearity

A linearity study verifies that the sample solutions are in a concentration range where analytes response is linearly proportional to concentration. The linearity of response for the present method was determined by analyzing standard solution of TBS, ETP, and BRH which were determined in the range of 60–210 ng/band, 2400–8400 ng/band, and 96–336 ng/band, respectively (Fig. 3). The results have shown that the peak areas are linear within the concentration of analysis. The correlation coefficient was r^2=0.998, 0.999, and 0.997, respectively (Figs. 4-6). Result of linearity study shown in Table 2.

Accuracy

The accuracy calculated by standard addition method. A known amount of standard drug had added to the fixed amount of pre-analyzed standard solution. The percent recovery and percentage

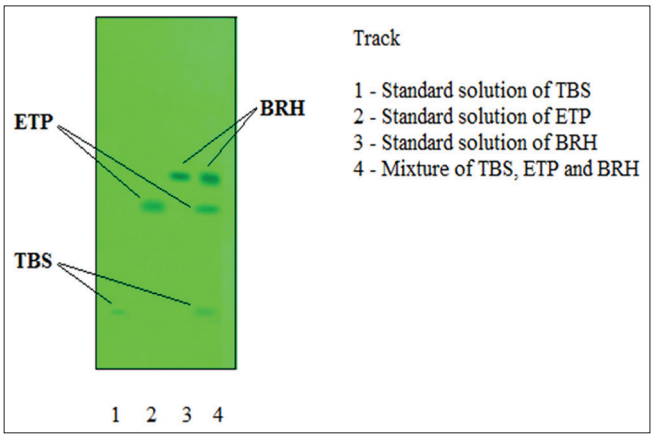


Fig. 1: Thin-layer chromatography plate of terbutaline sulfate, etophylline, and bromhexine hydrochloride

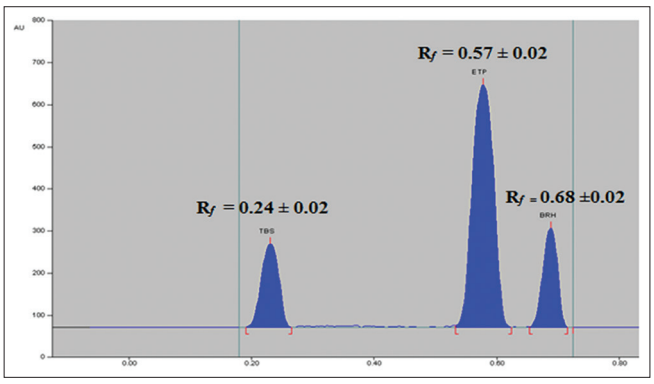


Fig. 2: Typical chromatogram of terbutaline sulfate R_f=0.24±0.02, ETP R_f=0.57±0.02, and BRH R_f=0.68±0.02

relative standard deviation (% RSD) was calculated, and the results are presented in Tables 3-5. Satisfactory recoveries for all three drugs have obtained by the proposed method, which indicate that proposed method is accurate.

Intraday precision
To study the intraday precision, three standard solutions (90, 120, and 150) of TBS, ETP, and BRH had spotted. The % RSD was calculated and it was found to be 0.31–0.40, 0.17–0.39, and 0.35–0.41 for TBS, ETP, and BRH, respectively, which are well within the acceptable criteria of not more than 2.0. Results are shown in Table 6.

Interday precision
To study the interday precision, standard solutions (90, 120, and 150) of TBS, ETP, and BRH have spotted. The % RSD was calculated and it was found to be 0.92–1.06, 0.86–1.37, and 1.24–1.42 for TBS, ETP, and BRH, respectively, which are well within the acceptable criteria of not more than 2.0. Results are shown in Table 7.

Table 1: Optimized chromatographic conditions of terbutaline sulfate, bromhexine hydrochloride, and etophylline parameters

Parameters	Value
Mobile phase	Benzene: methanol: glacial acetic acid 8:0.5:1.5 v/v/v
Stationary phase	Pre-coated silica gel G60 F254 aluminum sheets 10 cm^2×10 cm^2, layer thickness 0.2 mm
Diluents	Methanol
Temperature	Room temperature
Chamber saturation time	15 min
Run distance	8 cm
Detection wavelength	275 nm
Retention factor	0.24, 0.57, and 0.68, respectively

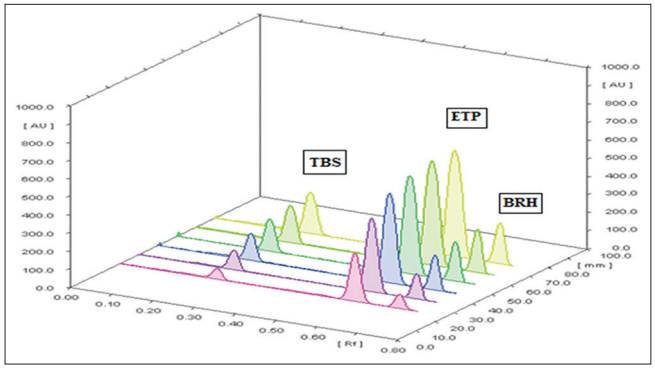


Fig. 3: Overlay linearity chromatogram of terbutaline sulfate, etophylline, and bromhexine hydrochloride 60–210 ng/band, 2400–8400 ng/band, and 96–336 ng/band, respectively

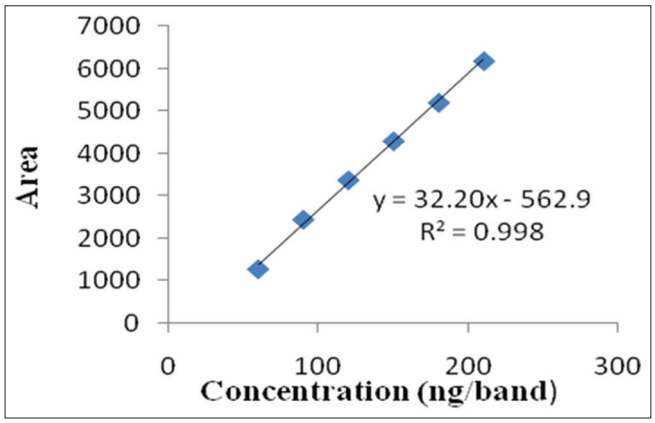


Fig. 4: Calibration curve for terbutaline sulfate

Specificity
The effect of a wide range of excipients and other additives normally presents in the combined dosage form of TBS, ETP, and BRH in the determinations under optimum conditions have performed. Chromatographic parameters maintained are specific for TBS, ETP, and BRH.

Limit of detection (LOD) and limit of quantification (LOQ)
The detection limit of the method has calculated by standard solutions of TBS, ETP, and BRH on TLC plate. Using the S/N method, the peak-to-peak noise around the analytes retention time is calculated, and subsequently, the concentration of the analytes that would yield a signal equal to certain value of noise-to-signal ratio is measured. An S/N ratio of 3 is generally accepted for estimating LOD and S/N ratio of 10 is used for estimating LOQ. This method commonly applied to analytical methods that exhibit baseline noise. The LOD for TBS, ETP, and BRH was found to be 0.667, 57.915, and 8.123 ng/band, respectively. The LOQ for TBS, ETP, and BRH was found to be 2.053, 175.500, and 24.617 ng/band, respectively.

Robustness
The HPTLC method has checked for robustness using Plackett–Burman (PB) design with 12 experiments [7-9]. Seven HPTLC conditions were screened: (a) Amount of benzene change in mobile phase composition, (b) change in amount of methanol in mobile phase, (c) change in amount of mobile phase volume, (d) saturation time change, (e) change in detection wavelength, (f) bandwidth change, and (g) change in

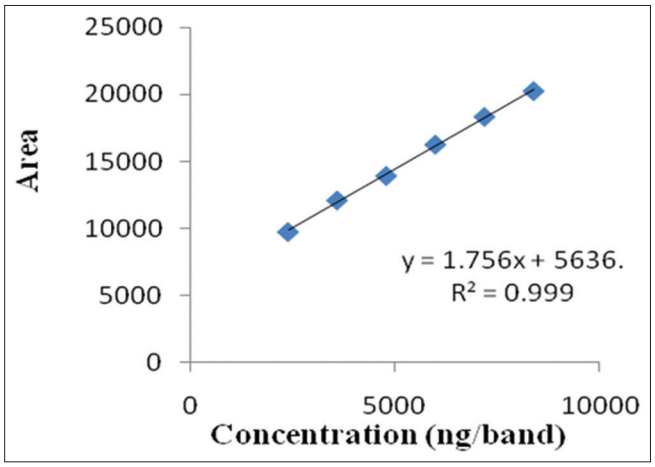


Fig. 5: Calibration curve for etophylline

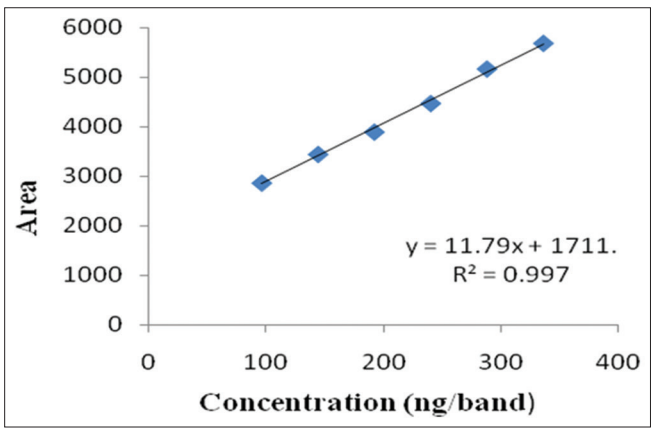


Fig. 6: Calibration curve for bromhexine hydrochloride

Table 2: Linearity data of TBS, ETP, and BRH (n=6)

TBS		ETP		BRH	
Concentration (ng/band)	**Mean area**	**Concentration (ng/band)**	**Mean area**	**Concentration (ng/band)**	**Mean area**
60	1258.18	2400	9748.56	96	2871.68
90	2429.26	3600	12,115.45	144	3449.66
120	3361.26	4800	13,945.43	192	3899.13
150	4284.71	6000	16,284.33	240	4474.71
180	5194.9	7200	18,363.55	288	5168.75
210	6176.75	8400	20,287.65	336	5687.30

TBS: Terbutaline sulfate, ETP: Etophylline, BRH: Bromhexine hydrochloride

Table 3: Accuracy data of terbutaline sulfate (n=3)

% level	Sample concentration (ng/band)	Standard added (ng/band)	Total concentration (ng/band)	Amount found (ng/band)	% amount found	Mean±SD	% RSD
80	90	72	162	160.21	98.89	99.17±0.27	0.273
			162	161.08	99.43		
			162	160.73	99.21		
100	90	90	180	180.11	100.82	100.47±0.30	0.302
			180	180.06	100.37		
			180	180.08	100.24		
120	90	108	198	196.78	99.38	99.75±0.36	0.366
			198	198.23	100.11		
			198	197.54	99.76		

RSD: Relative standard deviation, SD: Standard deviation

Table 4: Accuracy data of etophylline (n=3)

% level	Sample concentration (ng/band)	Standard added (ng/band)	Total concentration (ng/band)	Amount found (ng/band)	% amount found	Mean area±SD	% RSD
80	3600	2880	6480	6463.20	99.19	99.45±0.42	0.424
				6467.84	99.23		
				6471.93	99.94		
100	3600	3600	7200	7123.76	98.93	98.92±0.23	0.237
				7139.63	99.15		
				7105.25	98.68		
120	3600	4320	7920	7810.53	98.61	99.36±0.66	0.671
				7890.74	99.62		
				7910.21	99.87		

RSD: Relative standard deviation, SD: Standard deviation

Table 5: Accuracy data of bromhexine hydrochloride (n=3)

% level	Sample concentration (ng/band)	Standard added (ng/band)	Total concentration (ng/band)	Amount found (ng/band)	% amount found	Mean area±SD	% RSD
80	144	115	259	256.42	98.84	99.73±1.23	1.241
				262.36	101.15		
				257.15	99.22		
100	144	144	288	285.34	98.95	99.64±0.69	0.697
				289.47	100.34		
				287.62	99.65		
120	144	173	317	315.39	99.36	99.78±0.48	0.484
				316.61	99.68		
				318.12	100.31		

RSD: Relative standard deviation, SD: Standard deviation

Table 6: Intraday precision data for TBS, ETP, and BRH (n=3)

TBS			ETP			BRH		
Concentration (ng/band)	**Mean area±SD***	**% RSD**	**Concentration (ng/band)**	**Mean area±SD***	**% RSD**	**Concentration (ng/band)**	**Mean area±SD***	**% RSD**
90	2467.32±3.32	0.401	3600	12,187.23±16.94	0.293	144	3471.23±15.37	0.352
120	3392.65±3.24	0.362	4800	13,921.42±17.32	0.173	192	3831.63±13.64	0.412
150	4238.53±4.62	0.314	6000	16,195.53±47.32	0.391	240	4421.85±17.74	0.385

TBS: Terbutaline sulfate, ETP: Etophylline, BRH: Bromhexine hydrochloride, RSD: Relative standard deviation, SD: Standard deviation

Table 7: Interday precision data for TBS, ETP, and BRH (n=3)

TBS			ETP			BRH		
Concentration (ng/band)	Mean area±SD*	% RSD	Concentration (ng/band)	Mean area±SD*	% RSD	Concentration (ng/band)	Mean area±SD*	% RSD
90	2445.13±26.16	1.061	3600	12,183.63±122.43	1.026	144	3442.41±48.41	1.241
120	3372.53±23.52	0.972	4800	13,921.36±95.32	0.862	192	3801.31±72.43	1.426
150	4214.86±27.32	0.921	6000	16,181.42±130.54	1.374	240	4417.31±68.32	1.352

TBS: Terbutaline sulfate, ETP: Etophylline, BRH: Bromhexine hydrochloride, RSD: Relative standard deviation, SD: Standard deviation

Table 8: Twelve experiments Plackett–Burman design to examine the seven high-performance thin-layer chromatography factors

Experiment	Factors						
	A	B	C	D	E	F	G
1	−1	1	1	−1	1	1	1
2	1	−1	−1	−1	1	−1	1
3	1	−1	1	1	−1	1	1
4	1	1	−1	1	1	1	−1
5	1	1	−1	−1	−1	1	−1
6	−1	−1	1	−1	1	1	−1
7	−1	1	−1	1	1	−1	1
8	−1	1	1	1	−1	−1	−1
9	1	−1	1	1	1	−1	−1
10	1	1	1	−1	−1	−1	1
11	−1	−1	−1	−1	−1	−1	−1
12	−1	−1	−1	1	−1	1	1

Table 9: P value for robustness study

Factor	p-value		
	TBS	ETP	BRH
A	0.2997	0.2848	0.5915
B	0.2180	0.6445	0.3684
C	0.4668	0.2006	0.2264
D	0.2721	0.2076	0.4362
E	0.7468	0.2983	0.2905
F	0.7386	0.5709	0.5708
G	0.9604	0.2752	0.7165

TBS: Terbutaline sulfate, ETP: Etophylline, BRH: Bromhexine hydrochloride

Table 10: Quantification of formulation

Amount spotted (ng/band)			Amount found (ng/band)			% assay		
TBS	ETP	BRH	TBS	ETP	BRH	TBS	ETP	BRH
90	3600	144	89.22	3622.31	142.90	99.13	100.61	99.23
90	3600	144	89.34	3617.82	142.85	99.26	100.49	99.20
90	3600	144	89.77	3667.42	143.11	99.74	101.87	99.38
Mean			89.44	3635.85	142.96	99.37	100.99	99.27
SD			0.289	27.432	0.137	0.321	0.764	0.096
% RSD			0.323	0.754	0.102	0.323	0.766	0.101

TBS: Terbutaline sulfate, ETP: Etophylline, BRH: Bromhexine hydrochloride, RSD: Relative standard deviation, SD: Standard deviation

solvent run distance (Table 8). The selection of factors was based on observations during method development and own experience. Each factor has studied at two levels. The limits of the factors studied have selected according to error ranges, which would commonly found in an analytical laboratory.

The Design-Expert 9 software has used to set up the experimental designs for HPTLC method. The area for TBS, ETP, and BRH has observed at each experiment designed for HPTLC method. Combined

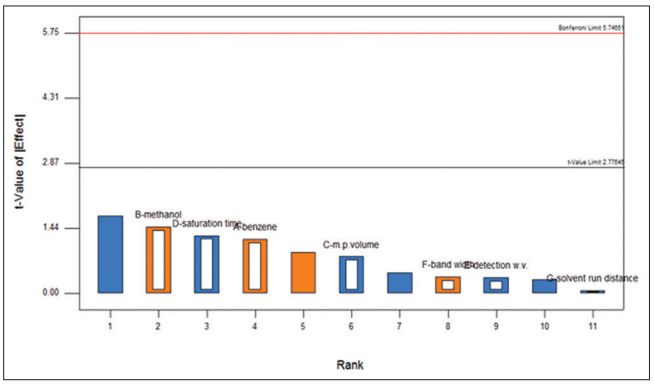


Fig. 7: Pareto chart for terbutaline sulfate

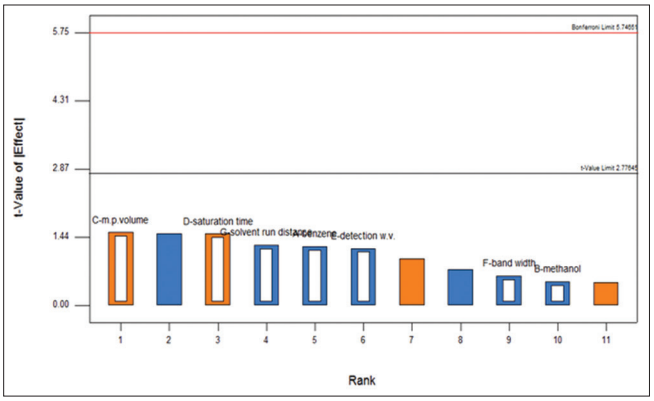


Fig.8: Pareto chart for etophylline

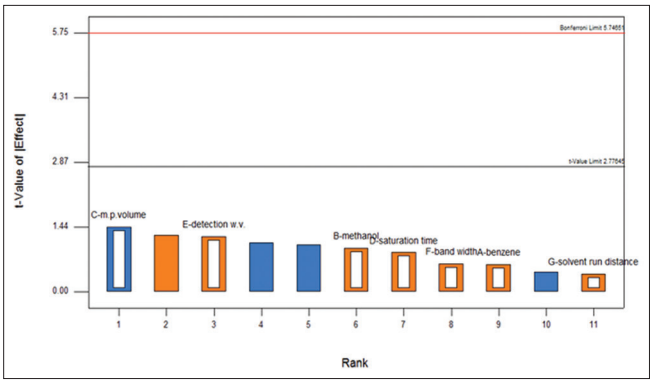


Fig. 9: Pareto chart for bromhexine hydrochloride

standard solution of TBS (90 ng/band), ETP (3600 ng/band), and BRH (144 ng/band) was analyzed at each design experiment. The experiment has repeated 3 times. The experiments were executed in random order.

The obtained graphs are presented in Figs. 7-9. It may be more suitable for viewing the Pareto chart, which has the significant

effects selected. The Pareto graph (Figs. 6-8) consists of bars with a length proportional to the absolute value of the estimated effect, divided by the pseudo standard error (PSE) known by Lenth (Lenth's PSE). All main effects and interaction terms found to be statistically insignificant as absolute values of main effects are below the critical t-value.

$p<0.05$ that means that factor is significantly effect on the response. However, here, no one is more than 0.05 means that method is robust as shown in Table 9.

CONCLUSION

In this study, a simple, accurate, rapid, reliable, and robust HPTLC method was developed and validated as per ICH guideline for the simultaneous determination of TBS, ETP, and BRH in combined pharmaceutical formulations. The result of formulation was found accurate and percentage assay obtained nearly 100% W/W for marketed formulation (as shown Table 10). Since the proposed methods has the lowest LOQ values for TBS, ETP, and BRH 2.053, 175.500, and 24.617 ng/band, respectively. The HPTLC method found robust as robustness has checked using PB design with 12 experiments. From the results obtained, we concluded that the suggested methods showed high sensitivity, specificity, accuracy, and reproducibility. Moreover, the method was simple and inexpensive, and this could be useful for the routine quality control of TBS, ETP, and BRH in pharmaceutical formulations.

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AUTHORS' CONTRIBUTIONS

Mehul Patel and Jigisha Chauhan have conceived of the presented idea. Both have developed the theory and performed the experiments. Mehul Patel has verified and validated the analytical methods. Mehul Patel encouraged Jigisha Chauhan to the development of analytical methods and interpreted the findings of this work. All authors discussed the results and contributed to the final manuscript.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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