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Development and Validation of Simple, Rapid and Sensitive High- Performance Liquid Chromatographic Method for the Determination of Butenafine Hydrochloride

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Authors' contributions

This work was carried out in collaboration among all authors. Author MJA designed the study, investigated and performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MMA and MKA managed the analyses of the study. Authors MFA, NA and SMAS did supervision and visualization of the project. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The current paper reports a simple, rapid, sensitive, accurate, and precise Reverse-phase high performance liquid chromatography (RP-HPLC) method with wide range of estimation to determine butenafine hydrochloride in nanosponges. This method has been validated as per ICH norms.
Study Design: Experimental design with influence of variables such as mobile phase composition, flow rate, temperature and wavelength on the chromatographic peaks.
Place and Duration of Study: Department of Pharmaceutics, College of Pharmacy, Prince Sattam Bin Abdulaziz University, Al-Kharj, Saudi Arabia between Jan 2020 and March 2020.

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Methodology: Separation was achieved by utilizing the most commonly used reverse phase column (C-18, 5 µm, 150 mm x 4.6 mm) set at 30ºC and quantified by UV detection at 280 nm after isocratic elution from a mobile phase (70:30 v/v of methanol: phosphate buffer pH 3.0) flowing at 1 ml/min.

Results: A sharp and symmetrical peak was observed at 4.08 ± 0.01 minutes. The low variation in peak area and retention time (1.12% and 0.29%, respectively) and a high number of theoretical plates (>2000) indicated this method's efficiency and suitability. The least square linear regression analysis (Y = 9265.5 X + 1961.4) showed excellent correlation (r^2 = 0.999 ± 0.0003) between concentration and peak area of butenafine hydrochloride through a wide concentration range of 1–50 µg/ml. The limits of detection and quantification (LOD and LOQ) were 0.18 µg/ml and 0.57 µg/ml, respectively. The assay or determinations were accurate, precise and reproducible with mean accuracy and mean relative standard deviation of precision of 101.53 ± 0.43% and 0.51 ± 0.11% respectively.

Conclusion: The developed RP-HPLC method was simple, sensitive, reproducible with wide range of estimation of butenafine hydrochloride in the nanosponges. The proposed method could be used for the analysis of butenafine hydrochloride in the conventional pharmaceutical formulations such as tablets, syrup, creams including novel formulations such as nanoparticles, nanosponges, nanoemulsions. The proposed method overcomes the specificity, sensitivity and reproducibility related issues of ultraviolet-visible spectroscopy.

Keywords: Butenafine; HPLC; chromatography; validation; accuracy; precision; nanosponge.

1. INTRODUCTION

Fungal infections are reported to affect over a billion of people worldwide [1]. These infections may be superficial, mucosal and systemic or invasive. The superficial fungal infections are caused by a group of fungi known as dermatophytes such as Trichophyton, Microsporum and Epidermophyton that affect skin, hair and nails etc [2]. The mucosal and systemic fungal infections are caused by fungi such as Candida, Aspergillus and Pneumocystis which affect almost every organ system. The superficial fungal infections may be mild to moderate while systemic fungal infection may be life threatening when left untreated. The invasive fungal infections are known to kill over 1.5 million people globally [3]. Treatment options for fungal infections may be broadly classified as topical and systemic antifungal agents to treat superficial and systemic fungal infection, respectively. Based on the chemical nature, commonly used antifungal agents include polyene derivatives (nystatin, amphotericin, etc.) which binds to ergosterol of fungal cell membrane and make it leaky; azole derivatives (imidazoles- clotrimazole, miconazole, ketoconazole etc., and triazoles-fluconazole, itraconazole, voriconazole etc.) that prevents conversion of lanosterol to ergosterol by inhibition of lanosterol 14α-demethylase; and allylamine derivatives (amorolfine, naftifine, and terbinafine) which inhibit squalene epoxidase [4].

Butenafine hydrochloride is a novel synthetic small antifungal molecule chemically related with benzyl amine and naphthalene as shown in Fig. 1. It has a molecular formula of $C_{23}H_{27}N.HCl$ and molecular weight of 353.93 [5]. It is a potent and broad spectrum antifungal agent. It selectively inhibits fungal squalene epoxidase disabling synthesis of ergosterol, an important intermediate of fungal cell membrane synthesis [6]. It is used as an antifungal cream to treat ringworm (*Tinea corporis*), jock itch or ringworm of groin (*Tinea cruris*), and athlete's foot or ringworm of feet (*Tinea pedis*) [7]. Clinical trials of butenafine exhibited better efficacy than terbinafine, which is a chemically related antifungal drug with a similar mechanism of action [8,9].

This study developed and validated a simple, rapid, sensitive, accurate, and precise high-performance liquid chromatography (HPLC) method for the determination of butenafine hydrochloride in a newly developed butenafine loaded nano sponge. The UV-spectroscopic methods are rapid and simple; however, the lack of required sensitivity and reproducibility can become issue for spectroscopic analysis. Moreover, in ability of UV-spectroscopy to deal with interfering materials in the pharmaceutical formulations such as excipients, impurities, residual solvents, and degraded compounds are other challenges. There is a report of an UV-spectroscopic method for the determination of butenafine hydrochloride in pharmaceutical

formulation [10]. However, it exhibited low sensitivity and a narrow linearity range (10–60 µg/ml). There were no reports that the spectrum of formulation or the degradation samples ruled out the interference during analysis. The HPLC methods are considered a widely used technique to determine substances in pharmaceutical formulations and biological samples due to its high selectivity, sensitivity, accuracy, and reproducibility [11]. There are few HPLC methods available for the quantification of butenafine in dosage forms such as and creams; however, these methods have either low or short linearity ranges like 0.09–0.45 µg/ml [12] thus requiring multifold dilutions of test samples; or low sensitivity with narrow linearity ranges like 80–400 µg/ml [13] or 100–300 µg/ml [14], thus not suitable for the samples containing lower amounts of target compound.

In this paper, we report a simple, sensitive, accurate, and precise HPLC method for the determination of butenafine hydrochloride by utilizing the most commonly used reverse phase column (C-18, 5 µg, 150 mm x 4.6 mm) and an organic modifier (methanol). The method was validated as per ICH norms; thus, it is reproducible to determine if butenafine hydrochloride is present in any pharmaceutical formulations [15].

2. MATERIALS AND METHODS

2.1 Materials

Butenafine hydrochloride was purchased from Sigma Aldrich USA. HPLC grade solvents such as acetonitrile, methanol, orthophophoric acid and buffer component such as monobasic potassium phosphate was obtained from Panareac, Spain. Ultrapure water was obtained from Milli Q, Millipore.

2.2 Liquid Chromatography

The liquid chromatographic system was comprised of a separating module with efficient solvent and sample management system (Alliance e2695, Waters Co., MA, USA), column heater (Waters, alliance, 2695, Waters Co., MA, USA), and UV detector (Waters 2487). Empower Pro 2 (version 6.20) was employed for acquisition and data collection. Chromatographic separation was achieved on a C-18 reverse phase column Hypersil ODS (5 µm, 150 mm x 4.6 mm I.D, Thermo Fisher scientific, Waltham, MA, USA) maintained at 30ºC.

2.3 Calibration Standards and Quality Control Samples

An accurate amount of butenafine hydrochloride was dissolved in HPLC grade methanol to prepare a stock solution with a concentration of 100 µg/ml. The stock solution was then diluted to prepare working standards with concentration ranges from 1–50 µg/ml. Three quality control (QC) samples at three concentration levels were prepared to serve as lower quality control (LQC, 2 µg/ml), medium quality control (MQC, 20 µg/ml), and higher quality control (HQC, 40 µg/ml) samples.

2.4 Sample Preparation

The butenafine loaded nanosponges were weighed and dissolved in methanol with the help of an ultrasonicator. The obtained solution was appropriately diluted with the mobile phase. Ten ml of the prepared sample was injected in triplicate on the HPLC column for separation and evaluation for butenafine hydrochloride.

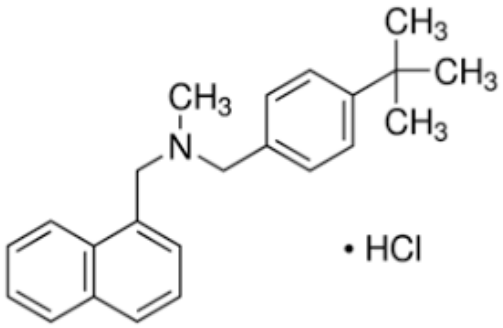

Fig. 1. Butenafine hydrochloride, chemical name- N-4-tert-butylbenzyl-N-methyl-1-naphthalenemethylamine hydrochloride

2.5 Method Development

The preliminary chromatographic parameters include a strong mobile phase (90% v/v of methanol and 10% v/v water) flowing at rate of 1 ml/min through a standard column set at an ambient temperature followed by UV detection at a wavelength of 254 nm to achieve a response after injecting 10 ml of a working standard of 10 µg/ml of butenafine hydrochloride in the methanol. Next, 10 mM monobasic potassium dihydrogen phosphate was added to the aqueous phase and acidified by using orthophosphoric acid to a pH of 3.0 to minimize peak tailing [16]. Further improvements in the size and shape of the peak was achieved by varying proportions of the organic phase, column temperature, and wavelength of detection. The optimized mobile phase consisted of 70 volumes of methanol and 30 volumes of 10 mM monobasic potassium dihydrogen phosphate buffer adjusted to a pH of 3.0 with orthophosphoric acid. The freshly prepared mobile phase was degassed by sonication and filtered by using a regenerated cellulose membrane filter (0.45-micron). Ten ml of calibrators, QC samples, or test samples were injected into the column. The isocratic separation and elution was achieved on C-18 column set at 30ºC from the mobile phase flowing at 1.0 ml/min. Drug peaks were detected by UV detector set at 280 nm.

2.6 Validation of Method

The assay method has been validated for parameters such as system suitability, linearity, sensitivity, accuracy and precision, as per ICH norms [15].

2.6.1 System suitability

The system suitability was first assessed by injecting and analyzing six replicates of butenafine hydrochloride at the lowest working standard of 1 µg/ml. The peak area of responses and retention times were recorded. The system and method was considered suitable if the relative standard deviation (% RSD) of the mean peak area and mean retention time was within ± 2%.

2.6.2 Linearity of assay method

The linearity of the assay method was determined by applying simple linear regression on the responses obtained after injecting 10 µl of working standard solutions within the range of 1–50 µg/ml of butenafine hydrochloride in triplicate. Calibration plots were constructed by plotting concentrations of calibration standards versus peak areas of the respective responses. A simple linear regression was applied and the correlation coefficient was calculated to evaluate the linearity of the plot.

2.6.3 Detection and quantitation limits

The limit of quantification (LOQ) and limit of detection (LOD) were calculated by using calibration line. LOQ and LOD were calculated as 3.3 σ/S and 10 σ /S, respectively, where σ is the standard deviation of intercept and S is slope of the line.

2.6.4 Accuracy

The accuracy of the method was determined by injecting quality control samples of butenafine hydrochloride at three levels (2, 20 and 40 µg/ml) in triplicate. Responses were evaluated and accuracy of method was established based on % recovery of quality control samples.

2.6.5 Precision

The precision of the method was determined by injecting quality control samples of butenafine hydrochloride at three levels (2, 20, and 40 µg/ml) in triplicate during the same day (intraday or intra-assay precision) and at different days (inter-day, inter-assay or intermediate precision). The intra-assay precision or repeatability was evaluated by calculating relative standard deviations (RSD) of the responses observed on day 1 and day 2; whereas, intermediate precision was evaluated by calculating the overall RSD on day 1 and day 2 together.

3. RESULTS AND DISCUSSION

3.1 Optimization of Method

The method was optimized by varying several parameters sequentially and observing the responses. For instance, the composition of the mobile phase such as the proportion of organic phase, buffer and pH condition of aqueous phase, flow rate, column temperature and detection wavelength varied to optimize the size (sensitivity) and shape of chromatographic peak during the development phase. Table 1 shows the optimum chromatographic settings.

Table 1. Optimum chromatographic setting for separation of butenafine hydrochloride

Parameters	Observations
Mobile phase	30 volumes of 10 mM KH_2PO_4 buffer pH 3.0 adjusted with H_3PO_3 70 volume of HPLC grade methanol.
Column	C 18 (150 x 4.6 mm, particle size 5 µm)
Temperature	30ºC
Mobile phase flow	1 ml/minute
Injection volume	10 µl
Detector	UV
Method	Isocratic
Wavelength	280 nm

3.2 Validation of Method

3.2.1 System suitability

The system suitability test of instruments and methods was first done before analysis as it is considered an important test for all chromatographic methods. The system suitability test is performed to verify that the system is suitable for analysis. The equipment, electronics, analytical operations, and samples to be analyzed are all considered as parts of system [17]. The repeatability of peak response (precision of peak area, peak height, and retention time), resolution factor, capacity factor, tailing factor, and column efficiency are some commonly used system suitability tests. System suitability tests verify that the chromatographic systems provide acceptable and reproducible results to ensure the reliability of chromatographic data. As per USP, system suitability tests must meet to a predefined standard before any sample analysis is performed [18]. In the case of system suitability failures, all the analytical data should be rejected. Table 2 presents the results of system suitability parameters. The variation in peak area and retention time was found as 1.12% and 0.29%, respectively. Furthermore, the number of theoretical plates was > 2000, which indicates the efficiency of the column and the suitability of the system (Table 2).

3.2.2 Linearity

The calibration curves were prepared in triplicate by plotting peak area against concentration. Table 3 shows the mean calibration data, which is the calibration standards and corresponding responses (Mean ± SD, n=3) along with its relative standard deviation (% RSD). The %RSD of the responses was less than 1.00, which indicates an excellent reproducibility of the chromatograms. The responses with % RSD less than 2 are considered as reproducible [19].

The linearity of the method was evaluated by simple linear regression analysis. The method was found linear within the range of 1–50 µg/ml with an excellent correlation (r2 = 0.999 ± 0.0003) as shown in Fig. 2. Table 4 shows the data for simple linear regression analyses of standard plots (n=3) such as linearity range, linearity equation, coefficient of correlation, slope, and intercept.

Table 2. System suitability of the chromatographic method

Sample	[a] AUP	[b] RT (min)	[c] $W_{0.5}$ (min)	$RT/W_{0.5}$	[d] N
1	18839	4.069	0.200	20.345	2295.181
2	18788	4.092	0.200	20.460	2321.201
3	18508	4.09	0.200	20.450	2318.933
4	18692	4.094	0.200	20.470	2323.471
5	18947	4.073	0.200	20.365	2299.696
6	19120	4.068	0.200	20.340	2294.053
Mean	18815.67	4.081	0.200	20.405	2308.756
[e] SD	210.29	0.012	0.000	0.061	13.838
[f] RSD	1.119	0.299709	0.000	0.299	0.599

a Area of peak, b Retention time, c Peak width at 50 % of peak height, d Number of theoretical plates calculated by using formula N= 5.545 (RT/W0.5)2, e Standard deviation, f Relative standard deviation

Table 3. Calibration data of butenafine hydrochloride

Concentration (µg/ml)	Mean peak area ± SD[a] (n=3)	% RSD[b]
1	10040 ± 57	0.33
2	18821 ± 149	0.79
5	48577 ± 36	0.07
10	93565 ± 128	0.14
20	190554 ± 290	0.15
30	264557 ± 2669	1.01
40	375914 ± 1268	0.34
50	470614 ± 2890	0.61

a Standard deviation, bRelative standard deviation

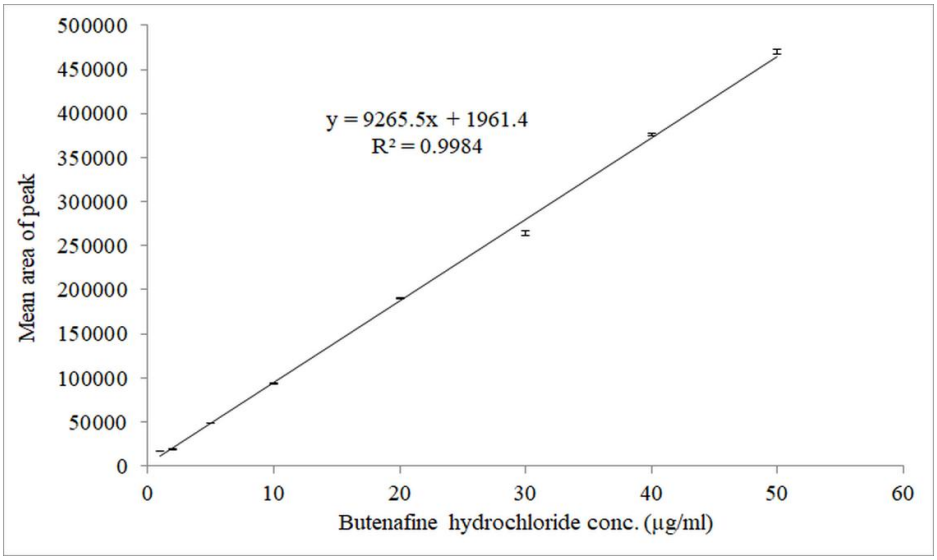

Fig. 2. Calibration plot of butenafine hydrochloride prepared by plotting concentration (1-50 µg/ml) against mean area of peaks (n=3) obtained by Waters, Alliance after separation on reverse phase column (C-18, 5 µm, 150 mm X 4.6 mm) set at 30 ºC and detection by UV detector set at 280 nm after isocratic elution from a mobile phase containing 70:30 v/v of methanol: phosphate buffer, pH 3.0, flowing at 1 ml/min

Table 4. Linear regression data for calibration plot (n=3)

Parameters	Observations
Linearity range	1-50 µg/ml
Regression equation	$y^a = 9265.5\ x^b + 1961.4$
Correlation coefficient ± SD [c]	0.999 ± 0.0003
Slope ± SD [c]	9265.54 ± 32.65
Intercept ± SD [c]	1961.27 ± 529.83

[a]Peak area, [b]Concentration of standard (µg/ml), [c]Standard deviation

3.2.3 Limit of detection (LOD) and limit of quantitation (LOQ)

The chromatograms of samples containing very small amount of analyte may exhibit response at the retention time of analyte either due to analyte itself or due to baseline noise (fluctuating baseline). Thus, it is very important to establish the LOD and LOQ. The LOD and LOQ are the lowest amount of analyte that can be detected or quantified by the method with defined accuracy and precision. There are several methods to establish these limits namely, visual method, signal to noise method and standard deviation

method. In this validation, the LOD and LOQ were determined by using standard deviation method because it the easiest one and the quickest one. The LOD and LOQ were calculated as 0.18 and 0.57 µg/ml respectively. The LOD of the method indicates that if the analyte concentration in the sample is less than this limit then the responses observed should not be attributed to presence of analyte with certainty. It may be either due to baseline noise only or due to traces of analyte and baseline noise altogether. The LOQ of the method is 0.57 µg/ml which is good as it corresponds to the lower level of the calibration standard [20].

3.2.4 Accuracy of determination

The accuracy of the determinations used in this study were evaluated by calculating the percentage of recoveries of 9 quality control samples of butenafine hydrochloride. The quality control samples at three different concentration levels, i.e. low, mid, and high (2, 20, and 40 µg/ml, respectively) were freshly prepared in triplicate. Table 5 presents the responses of the recovery study. The mean percentage of recovery of the samples at low, mid, and high levels were 100.97 ± 0.80, 102.23 ± 0.16, and 100.84 ± 0.37, respectively. The percentage of recoveries of all 9 samples was between 100.28–102.24 %, while the overall recovery was found to be 101.53 ± 0.43%, which indicates the accuracy of our method.

3.2.5 Precision of assay

The precision of the assay was evaluated on two levels: 1. intra-day (intra-assay precision or repeatability); and 2. inter-day (inter-assay precision or intermediate precision). The quality control samples at low, mid, and high concentrations (2, 20, and 40 µg/ml, respectively) were freshly prepared in triplicate and analyzed. Table 6 presents the responses as peak area, mean area, standard deviation, and relative standard deviation. The samples at higher concentration levels exhibited better precision as compared to samples with lower concentrations. The intra-assay precision or repeatability of quality control samples at different levels ranged from 0.15–0.79 % on day-1 and varied slightly on day-2 and ranged from 0.20–1.22 %. The intermediate precision was found to be 0.51 %. These results suggest that the method is highly precise and may be reproduced precisely in any lab as the precision at different levels was less than 2%.

3.3 Determination of Butenafine Hydrochloride Loaded in Nanosponges

The determination of butenafine hydrochloride loaded in nanosponges was done by the assay method used in this paper. Fig. 3 depicts the typical chromatogram of butenafine hydrochloride in nanosponges. The retention time of butenafine hydrochloride in the nanosponge samples was the same as that observed in the standard and quality control samples. Moreover, the peak was sharp, symmetrical, and well resolved; and there was no interference with any excipients of the formulation.

Table 5. Accuracy of the method (n = 3 x 3)

Quality control samples	Nominal concentration (µg/ml)	Concentration found (µg/ml)	Accuracy (% recovery)	Mean accuracy (± SD[a])	% RSD[b]
LQC	2	2.02	101.12		
	2	2.01	100.60	100.97 ± 0.80	0.79
	2	2.04	102.18		
MQC	20	20.49	102.43		
	20	20.50	102.51	102.23 ± 0.16	0.15
	20	20.55	102.73		
HQC	40	40.60	101.50		
	40	40.47	101.17	100.83 ± 0.34	0.34
	40	40.33	100.81		
		Over accuracy of the method		101.53	0.43

[a] *Standard deviation,* [b]*Relative standard deviation*

Table 6. Precision of the method (n = 3 x 3)

Samples and parameters	Intraday precision (repeatability-day 1)			Intraday precision (repeatability-day 2)		
	[a] LQC	[b] MQC	[c] HQC	[a] LQC	[b] MQC	[c] HQC
N1	18788	190314	377160	18839	190166	377278
N2	18692	190472	375956	18508	190739	375859
N3	18985	190877	374626	18947	190879	374682
MEAN	18821.67	190554.3	375914	18764.67	190594.7	375939.7
[d] SD	149.37	290.39	1267.52	228.75	377.78	1299.88
[e] RSD (Repeatability)	0.79	0.15	0.33	1.22	0.20	0.35
Mean repeatability	0.43 ± 0.33			0.59 ± 0.55		
RSD (Intermediate precision)	0.51 ± 0.11					

[a] Lower quality control sample, [b] Middle quality control sample, [c] Higher quality control sample, [d] Standard deviation, [e] Relative standard deviation

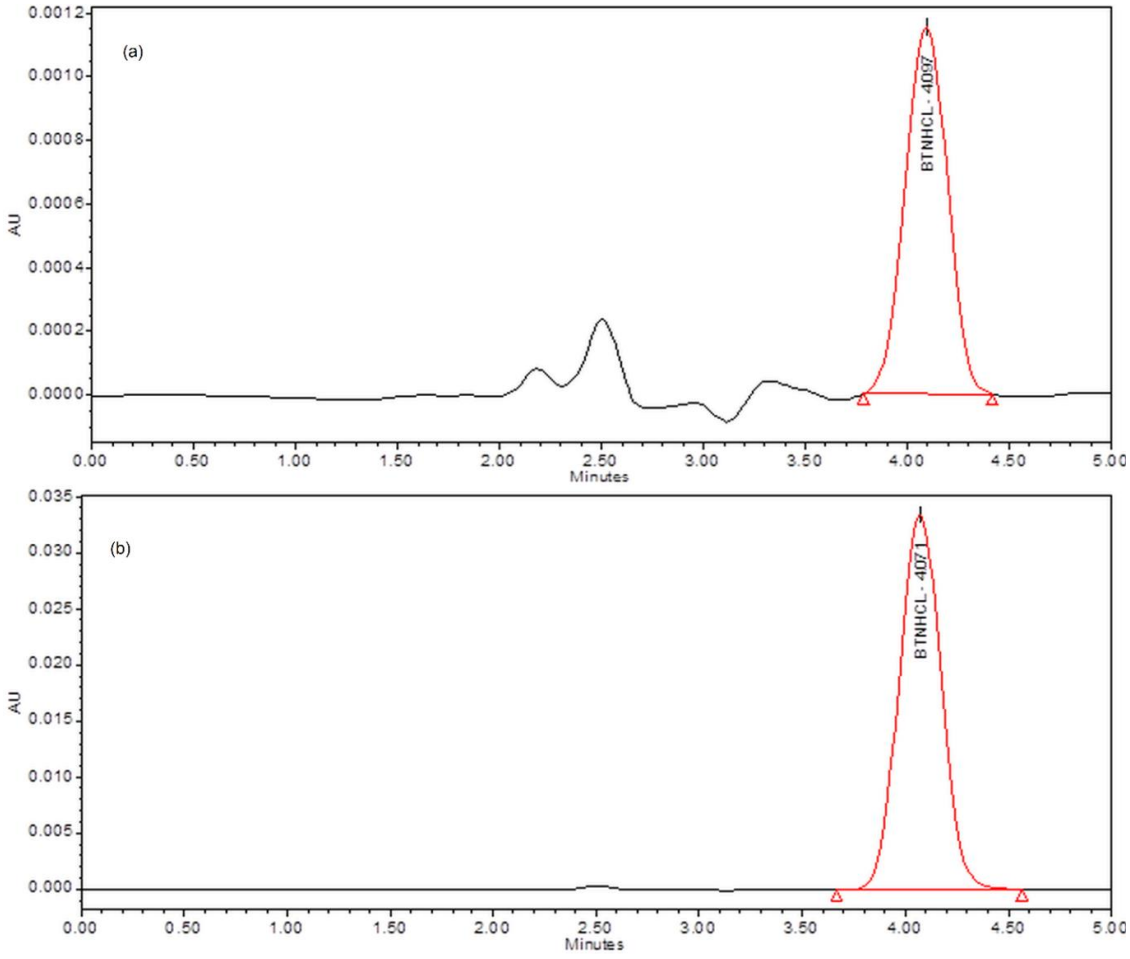

Fig. 3. The representative chromatograms of butenafine hydrochloride (a) standard solution of 1 µg/ml and (b) Nanosponge sample loaded with butenafine hydrochloride, separated on reverse phase column (C-18, 5 µm, 150 mm X 4.6 mm) set at 30ºC and detected by UV detector set at 280 nm after isocratic elution from a mobile phase containing 70:30 v/v of methanol: phosphate buffer, pH 3.0, flowing at 1 ml/min

4. CONCLUSIONS

The proposed method is rapid, simple, sensitive, accurate, and precise for isocratic elution and UV determination of butenafine hydrochloride. The low relative standard deviations of system suitability, accuracy and repeatability studies indicate that the developed method is reproducible. The method offers advantages of being simple because of its utilization of the most widely used column and mobile phase; and it is rapid due to its relatively short runtime of 5 minutes. In addition, it exhibits high sensitivity and excellent linearity and encompasses a broad range of determinations with excellent accuracy and precision.

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COMPETING INTERESTS

We declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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