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ORIGINAL ARTICLE / ÖZGÜN MAKALE

DEVELOPMENT AND VALIDATION OF AN HPLC METHOD FOR THE QUANTIFICATION OF BENZOCAINE AND FUSIDIC ACID SIMULTANEOUSLY IN MICROEMULSION FORMULATION

MİKROEMULSİYON FORMÜLASYONUNDA BENZOKAİN VE FUSİDİK ASİT MİKTAR TAYİNİ İÇİN HPLC METODU GELİŞTİRİLMESİ VE VALİDASYONU

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ABSTRACT

Objective: *The purpose of this study was to develop and validate an analytical technique for quantification in the microemulsion formulation containing fusidic acid and benzocaine developed for the treatment of mild and moderate wounds.*
Material and Method: *Acetonitrile and acetic acid (0.1%) were used as a mobile phase in the HPLC method. The flow rate and injection volume were determined. The ICH Q2 guideline was followed in the validation of the developed method. Benzocaine (2%) and fusidic acid (2%) were added to the blank microemulsion prepared using ethyl oleate, propylene glycol, ethanol, and Cremophor EL, and the selectivity of the HPLC method for both these substances was investigated.*
Result and Discussion: *The ratio of acetonitrile to acetic acid (0.1%) was determined to be 70:30 (v/v). The injection volume of 20 µl and the flow rate of 1 ml·min^{-1} were set. The developed method, which demonstrated a high correlation coefficient (0.9999) and a low variation coefficient (<2%), was simple, inexpensive, convenient, selective, and suitable for the analysis of both substances at the same wavelength (210 nm).*
Keywords: *Analytical method, benzocaine, fusidic acid, HPLC, microemulsion*

ÖZ

Amaç: *Bu çalışmanın amacı, hafif ve orta dereceli yaraların tedavisi için geliştirilen benzokain ve fusidik asit içeren mikroemülsiyon formülasyonunda miktar tayini için analitik yöntem geliştirilmesi ve valide edilmesidir.*
Gereç ve Yöntem: *HPLC metodunda mobil faz olarak asetonitril ve asetik asit (0.1%) kullanıldı. Akış hızı ve enjeksiyon hacmi belirlendi. Geliştirilen metodun doğrulamasında ICH Q2 kılavuzuna uyulmuştur. Etil oleat, propilen glikol, etanol ve Cremophor EL kullanılarak hazırlanan boş mikroemülsiyona benzokain (%2) ve fusidik asit (%2) ilave edildi ve HPLC metodunun bu iki madde için seçiciliği araştırıldı.*
Sonuç ve Tartışma: *Asetonitrilin asetik aside (%0.1) oranı 70:30 (h/h) olarak belirlendi. Enjeksiyon hacmi 20 µl ve akış hızı 1 ml·dk^{-1} olarak ayarlandı. Yüksek korelasyon katsayısı (0.9999)*

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ve düşük varyasyon katsayısı (<%2) gösteren geliştirilen yöntem basit, ucuz, seçici, kullanışlı ve her iki maddenin aynı dalga boyunda (210 nm) analizi için uygundu.
Anahtar Kelimeler: *Analitik metot, benzokain, fusidik asit, HPLC, mikroemülsiyon*

INTRODUCTION

The skin, which constitutes approximately 15-16% of body weight, is the largest organ in the body and serves as a protective barrier in opposition to external factors. However, it may be damaged due to various internal and external influences, leading to the loss of its integrity and the formation of acute or chronic wounds [1,2]. The effectiveness of active pharmaceutical agents in wound healing can be influenced by various factors such as age, gender, physiological conditions, etc. as well as dosage form and administration route. Novel drug carrier systems have the potential to improve treatment effectiveness, reduce costs, and enhance patient compliance compared to conventional dosage forms.

Dermal delivery, which provides high patient compliance and easy application, is an important alternative compared to oral administration due to the advantages that high amounts of drugs are localized in the application area, decreasing the systemic effect and, consequently, reducing systemic side effects [3,4]. Microemulsions are modern drug delivery systems with nano-sized vesicles consisting of water and oil phases. High drug loading capacity, the ability to present both oil- and water-soluble drug substances simultaneously, easy preparation, and thermodynamically stable are among the advantages of microemulsions that have made them stand out for dermal delivery in recent years. They provide high permeability with their nano size and the penetration-enhancing properties of surfactants and cosurfactants in their structure [5]. Thanks to these attractive advantages, improving pharmacological efficacy by increasing drug permeability is considered a favorable option for the delivery of different active substances in wound treatment [6-9].

Benzocaine, a local anesthetic, has a fairly widespread use for pain relief in the treatment of local and temporal wounds and burns [10]. Benzocaine, when applied dermally, has a short-duration anesthetic effect because it is hydrolyzed by esterases and undergoes enzymatic biotransformation inside the skin layers rather quickly [11]. Therefore, it requires the need for frequent application for the aimed effectiveness. It is also stated that the onset of the effect of benzocaine in the topical application is between 30 min and 2 h and that its permeability is poor [12,13].

Fusidic acid, a narrow-spectrum steroid antibiotic that is predominantly active against gram-positive bacteria, usually has an effect by inhibiting the protein synthesis of bacteria [14]. This molecule, which has bactericidal and bacteriostatic activity, is used in topical dosage forms for treating mild and moderate infections such as erythrasma, burns, and painful wounds [15]. Thanks to the unique advantages of the microemulsion system, it was shown that microemulsion formulations of these active substances have the potential for clinical use by providing an extended and increased therapeutical effect [8,16]. Moreover, microemulsion formulations in which these two active substances coexist were developed by our group [6].

High performance liquid chromatography (HPLC) is a very convenient and reliable technique that is often preferred as a determination and quantification method in the pharmaceutical area [17,18]. For novel combined formulations of known active substances, new methods that determine the substances with a single method are needed to reduce time loss and cost. Although numerous HPLC methods exist for the analysis of fusidic acid and benzocaine, no single analytical technique has been developed to quantify simultaneously both active substances. This work aimed to develop and validate a novel HPLC method that allows the quantification of these active substances by a single analytical method in the microemulsion formulation, which provides high patient compliance and high pharmacological efficacy for treating mild and moderate-severe wounds and burns.

MATERIAL AND METHOD

Materials

Fusidic acid was kindly donated by Berko İlaç (Türkiye). Benzocaine was purchased from Acros Organics (USA). Acetonitrile, acetic acid, ethyl oleate, propylene glycol, Cremophor EL, and ethanol

were obtained from Sigma (USA). All chemicals used in this study are analytical grade.

Instrumentation

An HPLC method was developed to determine and quantify the amount of benzocaine and fusidic acid simultaneously. The HPLC device (1100 Series, Agilent, USA) has a UV detector, gradient pump, and thermostable column. Also, a C18 column (4.6 mm x150 mm, 5 µm, InterSustain, GL Sciences, Japan) was used in the study.

Determination of UV Spectra

Fusidic acid and benzocaine were separately dissolved in acetonitrile at a concentration of 100 µg/ml. Then, these solutions were diluted with acetonitrile, resulting in a final concentration of 10 µg/ml. UV-Vis Spectrophotometer device (UV 1800, Shimadzu, Japan) was utilized with a wavelength of 190-400 nm and 0.5 nm of sensitivity, and the measurements were made against a blind sample in quartz cuvettes. The most suitable single wavelength (λ) for the active substances was determined by evaluating the obtained spectra.

Chromatographic Conditions

Chromographic conditions (HPLC method) were created with reference to the studies of Curbete and Salgado [19] and Hirpara et al. [20]. The most suitable concentration of the mobile phase (acetonitrile and acetic acid (0.1%) was determined as 70:30 (v/v). While preparing the mobile phase, ultra-pure water was degassed in an ultrasonic bath (Super RK 255 H, Sonorex, Bandelin, Germany) for 30 min. The rate of flow was detected to be 1 $ml \cdot min^{-1}$, the injection volume was determined to be 20 µl, and the detector temperature was set to 25°C. The analyses were made at the single wavelengths obtained as a result of measurements made with the UV spectrophotometer analyses. As a result, the studies were performed at 210 nm. In the studies conducted as a gradient, the HPLC system and the column were conditioned for a minimum of one hour with the mobile phase before the analyses.

Preparation of Stock Solutions and Reference Samples

For the preparation of the stock solution of fusidic acid, 100 mg of the active substance was dissolved in 50 ml of acetonitrile. Subsequently, the volume was finalized with acetonitrile in a 100 ml flask, yielding a stock solution (1000 µg/ml). In the same way, the stock solution of benzocaine (1000 µg/ml) was obtained. The reference samples (5, 7.5, 10, 15, 20, 30, 40, 50, 60, 80, and 100 µg/ml) were separately prepared by diluting the stock solutions using acetonitrile for both active substances. All samples were filtered using membrane filters (0.2 µm, Merck Millipore, Germany).

Validation of the HPLC Method

Linearity, precision, accuracy, stability, robustness, specificity/selectivity, limit of detection (LOD), and limit of quantification (LOD) analyses were conducted within the scope of the validation procedure following the guidelines of the International Council for Harmonization [21]. Furthermore, the system suitability was assessed by calculating parameters, including the tailing factor, capacity factor, resolution, height equivalent to a theoretical plate (HETP), etc.

Linearity

Linearity test for an analytical procedure is performed to reveal that different concentrations of the substance are proportional in certain intervals [22]. The linearity is confirmed by the graph plotted using concentration values and areas obtained from the HPLC chromatograms. The correlation coefficient (R^2) and equation of the curve were determined for both active substances.

Accuracy and Recovery

The accuracy of a method is presented as a percentage of recovery [23]. In this test, it is determined whether the substance in the sample is within the confidence intervals. Three different samples were analyzed for both substances (5, 40, and 100 µg/ml for benzocaine; 5, 40, and 100 µg/ml for fusidic acid), and the recovery was calculated by Equation 1.

$$\text{Recovery (\%)} = (C_p / C_t) \times 100 \quad \text{(Eq. 1)}$$

C_p: Practical (measurement) concentration
C_t: Theoretical (nominal) concentration

Precision

Precision is expressed as the proximity of repeated measurements of a sample of the same concentration to each other under certain conditions [24]. Precision evaluation is carried out with repeatability, intermediate precision, and reproducibility tests [21]. The coefficient of variation being below 2% in the conducted studies indicates the high precision of the method [22]. For the precision study, the samples (30 µg/ml) were analyzed ten times, and the mean, standard deviation, and variation coefficient were calculated.

Intermediate precision denotes differences within laboratories, encompassing different days, analysts, equipment, and similar factors. Within the scope of the intermediate precision study, measurements were obtained by preparing six samples from the stock solution at the same concentration (5 µg/ml) by two analysts. The mean, standard deviation, and variation coefficient of the obtained results were calculated. In these analyses, the same concentrations were studied for both active substances. Furthermore, the sample prepared from the stock solution (30 µg/ml) was analyzed five times on two days (interday) under the same conditions.

Stability of the Solution

The solution of both active substances at a concentration of 30 µg/ml was analyzed by HPLC at 15, 30, 60, 120 min, 24, and 48 h. It was assessed whether there was a change in the concentration values corresponding to the obtained area values [21].

Robustness

The method's robustness was tested separately by changing the flow rate, the column temperature, and the ratio of the organic phase [21]. The samples of 100 µg/ml were analyzed for both substances. The flow rate of 0.9 and 1.1 ml, the column temperature of 30°C and 35°C, and the mobile phase ratio of 75:25 (v/v) and 65:35 (v/v) were adjusted. These studies were made by changing each condition separately. The retention time and the area of peak obtained as a result of the analyses were compared with the results of the optimal conditions.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The lowest concentration value at which a substance can be determined is expressed as the limit of detection (LOD). It is also expressed as the limit of detectability. To determine the ratio of signal to noise, the standard deviation of the area values obtained by analyzing the samples at the lowest known concentrations was determined. LOD was calculated using the standard deviation by Equation 2. The limit of quantification (LOQ) refers to the lowest concentration at which the active substance is calculated. It is also expressed as the limit of computability. The sensitivity of the detector is determined thanks to this analysis. LOQ was calculated using Equation 3 [21,25]. In Equation 2 and Equation 3, "σ" refers to the standard deviation of the received response, and "S" refers to the slope of the linearity curve.

$$\text{LOD} = (3.3 \times \sigma) / S \quad \text{(Eq. 2)}$$

$$\text{LOQ} = (10 \times \sigma) / S \quad \text{(Eq. 3)}$$

LOD and LOQ of the active substances were calculated using the above formulas by analyzing the samples at the lowest concentration (5 µg/ml).

Specificity/Selectivity

Selectivity is achieved by separating the peak of the active substance from other peaks [26]. This was confirmed through a comparative analysis of the HPLC chromatograms of the blank and the loaded microemulsion.

Preparation of Microemulsion and Content Uniformity Analysis

As in our previous study, the microemulsion was prepared by titration method using ethyl oleate, propylene glycol, ethanol, Cremophor EL, and distilled water, and then, it was loaded with benzocaine (2%) and fusidic acid (2%) [6]. To quantify benzocaine and fusidic acid in the microemulsion, a certain amount of the formulation (500 mg) was weighed into a beaker, and 100 ml of acetonitrile was added. It was stirred for one hour. Then, one ml sample was taken and filtered. In the study conducted in three repetitions, the samples were analyzed at 210 nm by HPLC.

RESULT AND DISCUSSION

One of the most used devices for determining the number of active substances in a drug delivery system is HPLC. The determination and quantification analysis is performed in all processes of the drug synthesis, preformulation, formulation, and stability. Validation of the method developed for analysis against various factors such as device, instrument, and personnel is also an important requirement for the reliability of the method [21]. According to the International Organization for Standardization (ISO), "verification, where the specified requirements are appropriate for an intended use" [27]. The development of a successful analytical method depends on the precision and validation [28]. To this end, the HPLC method is validated in terms of parameters such as accuracy, precision, and selectivity [21].

The appropriate wavelength for the active substances was achieved through the execution of absorption measurements using a UV spectrophotometer. It was studied at 210 nm [19], 235 nm [29] wavelengths for fusidic acid, 285 nm [30], 254 nm [31], and 322 nm [20] wavelengths for benzocaine in the quantification analyses conducted by HPLC-UV device. The spectra of the substances are shown in Figure 1. Depending on these results, HPLC analyses were performed at a wavelength of 210 nm.

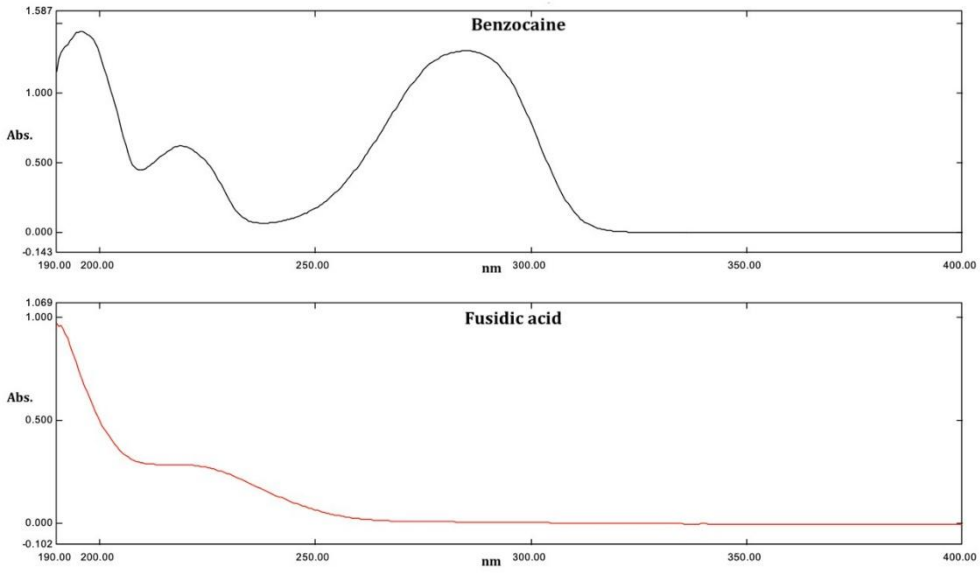

Figure 1. UV spectrum of benzocaine and fusidic acid solutions (10 µg/ml)

The samples prepared by diluting from stock solutions were analyzed using the HPLC device. A linearity graph showing the concentration against the peak areas on the chromatograms was drawn, the

equation of the curve was created, and the correlation coefficient (R^2) was calculated. The R^2 values for benzocaine and fusidic acid were determined as 0.9999 and 0.9999, respectively (Figure 2). Additionally, the system suitability data is shown in Table 1.

Table 1. System suitability results of the developed HPLC method

Active substances	Resolution	Capacity factor	Tailing factor	Assimetry factor	HETP	Theoretical plates
Benzocaine	13.33	0.93	1.03	1.05	0.01	11209
Fusidic acid	20.30	3.29	1.05	1.09	0.01	19663

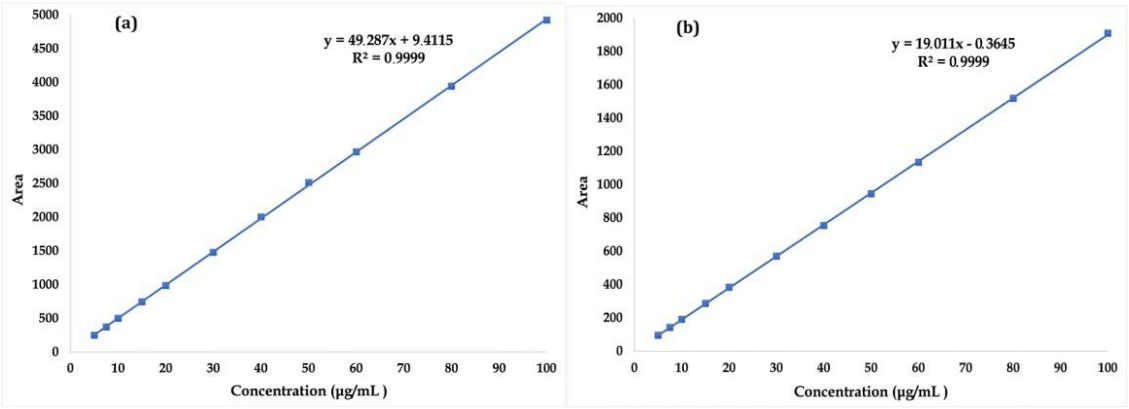

Figure 2. Linearity curves created by HPLC data. (a) Benzocaine. (b) Fusidic acid

Table 2. Accuracy and recovery study results of benzocaine and fusidic acid samples

Benzocaine				Fusidic acid			
C_t	Area	C_p	Recovery (%)	C_t	Area	C_p	Recovery (%)
5	252	4.92	98.44	5	96	5.07	101.38
	251	4.90	98.03		94	4.98	99.59
	250	4.88	97.63		96	5.05	100.96
	256	5.00	100.06		95	5.03	100.64
	256	5.00	100.06		97	5.12	102.43
		Mean	98.84			**Mean**	101.00
		SD	1.15			**SD**	1.04
		RSD (%)	1.16			**RSD (%)**	1.03
40	2009	40.59	101.45	40	761	40.05	100.12
	2008	40.54	101.36		767	40.36	100.91
	1989	40.16	100.40		765	40.26	100.65
	1996	40.31	100.78		757	39.84	99.60
	2006	40.51	101.27		766	40.31	100.78
		Mean	101.05			**Mean**	100.41
		SD	0.45			**SD**	0.55
		RSD (%)	0.44			**RSD (%)**	0.54
100	4952	100.28	100.28	100	1909	100.44	100.43
	4934	99.92	99.92		1890	99.44	99.44

Table 2 *(continue).* Accuracy and recovery study results of benzocaine and fusidic acid samples

Benzocaine				Fusidic acid			
C_t	**Area**	**C_p**	**Recovery (%)**	**C_t**	**Area**	**C_p**	**Recovery (%)**
100	4996	101.18	101.17	100	1920	101.01	101.01
	4953	100.30	100.30		1911	100.54	100.54
	4876	98.74	98.74		1905	100.22	100.22
		Mean	100.08			**Mean**	100.33
		SD	0.88			**SD**	0.58
		RSD (%)	0.88			**RSD (%)**	0.58

C_p: Practical (measurement) concentration. C_t: Theoretical (Nominal) concentration. SD: Standard deviation. RSD: Relative standard deviation (variation coefficient)

The precision results are shown in Table 3. The mean, standard deviation, and variation coefficient (%) of the obtained data were calculated. It was found that the variation coefficients for both substances were lower than 2%, and the standard deviations were low. These data showed that the analytical technique was accurate and reproducible.

Table 3. Precision study results of benzocaine and fusidic acid solutions

Benzocaine				Fusidic acid			
No	**C_t (µg/ml)**	**Area**	**C_p (µg/ml)**	**No**	**C_t (µg/ml)**	**Area**	**C_p (µg/ml)**
1	30	1498	30.20	1	30	567	29.84
2		1481	29.86	2		562	29.58
3		1481	29.86	3		568	29.90
4		1477	29.80	4		562	29.58
5		1478	29.80	5		570	30.00
6		1476	29.76	6		563	29.63
7		1477	29.78	7		572	30.11
8		1478	29.80	8		564	29.69
9		1478	29.80	9		570	30.00
10		1477	29.78	10		563	29.63
		Mean	29.84			**Mean**	29.80
		SD	0.12			**SD**	0.19
		RSD (%)	0.42			**RSD (%)**	0.63

C_p: Practical (measurement) concentration. C_t: Theoretical (Nominal) concentration. SD: Standard deviation. RSD: Relative standard deviation (variation coefficient)

Table 4. Intermediate precision results performed interday of benzocaine and fusidic acid samples

Benzocaine						Fusidic acid					
Theoretical concentration – 30 µg/ml						**Theoretical concentration – 30 µg/ml**					
1. day			**2. day**			**1. day**			**2. day**		
No	**Area**	**C_p (µg/ml)**	**No**	**Area**	**C_p (µg/ml)**	**No**	**Area**	**C_p (µg/ml)**	**No**	**Area**	**C_p (µg/ml)**
R-1-1	1476	29.76	R-2-1	1472	29.68	R-1-1	563	29.63	R-2-1	568	29.90
R-1-2	1477	29.78	R-2-2	1468	29.59	R-1-2	572	30.11	R-2-2	570	30.00
R-1-3	1478	29.80	R-2-3	1484	29.92	R-1-3	564	29.69	R-2-3	574	30.21
R-1-4	1478	29.80	R-2-4	1478	29.80	R-1-4	570	30.00	R-2-4	571	30.05
R-1-5	1477	29.78	R-2-5	1477	29.78	R-1-5	563	29.63	R-2-5	564	29.69
R-1-6	1478	29.80	R-2-6	1483	29.90	R-1-6	567	29.84	R-2-6	574	30.21

Table 4 ***(continue).*** Intermediate precision results performed interday of benzocaine and fusidic acid samples

Benzocaine						**Fusidic acid**					
Theoretical concentration – 30 µg/ml						**Theoretical concentration – 30 µg/ml**					
1. day			**2. day**			**1. day**			**2. day**		
No	**Area**	**C_p (µg/ml)**	**No**	**Area**	**C_p (µg/ml)**	**No**	**Area**	**C_p (µg/ml)**	**No**	**Area**	**C_p (µg/ml)**
R-1-7	1497	29.18	R-2-7	1478	29.80	R-1-7	562	29.58	R-2-7	568	29.90
R-1-8	1481	29.86	R-2-8	1471	29.66	R-1-8	568	29.90	R-2-8	571	30.05
R-1-9	1481	29.86	R-2-9	1484	29.92	R-1-9	562	29.58	R-2-9	572	30.11
R-1-10	1477	29.78	R-2-10	1484	29.92	R-1-10	570	30.00	R-2-10	565	29.74
	Mean	29.84		**Mean**	29.79		**Mean**	29.80		**Mean**	29.99
	SD	0.13		**SD**	0.12		**SD**	0.20		**SD**	0.18
	RSD (%)	0.44		**RSD (%)**	0.41		**RSD (%)**	0.66		**RSD (%)**	0.60

C_p: Practical (measurement) concentration. SD: Standard deviation. RSD: Relative standard deviation (variation coefficient)

The intermediate precision performed interday was determined by analyzing the samples of 30 µg/ml over two days. The data are shown in Table 4. The alterations between the practical concentration values were not statistically significant for both benzocaine and fusidic acid ($p>0.05$), and the variation coefficient was found to be lower than 2%. The data obtained from studies conducted by two analysts on the same day under the same conditions are shown in Table 5. The differences in the peak areas were not statistically significant for both active substances ($p>0.05$). The data obtained in the intermediate precision studies proved that the system was highly reproducible and that there was no difference between the data obtained in the studies conducted by different analysts.

Table 5. Intermediate precision test results performed by two analysts of benzocaine and fusidic acid samples

Benzocaine						**Fusidic acid**					
Theoretical concentration – 5 µg/ml						**Theoretical concentration – 5 µg/ml**					
1. Analyst			**2. Analyst**			**1. Analyst**			**2. Analyst**		
No	**Area**	**C_p (µg/ml)**	**No**	**Area**	**C_p (µg/ml)**	**No**	**Area**	**C_p (µg/ml)**	**No**	**Area**	**C_p (µg/ml)**
1	251	4.90	1	250	4.88	1	95.8	5.06	1	92.4	4.88
2	247	4.82	2	252	4.92	2	96.0	5.07	2	94.2	4.97
3	255	4.98	3	247	4.82	3	97.6	5.15	3	92.8	4.90
4	256	5.00	4	250	4.88	4	94.3	4.98	4	94.7	5.00
5	254	4.96	5	251	4.90	5	97.8	5.16	5	97.2	5.13
6	253	4.94	6	247	4.82	6	95.6	5.05	6	95.1	5.02
	Mean	4.94		**Mean**	4.87		**Mean**	5.08		**Mean**	4.99
	SD	0.07		**SD**	0.04		**SD**	0.06		**SD**	0.08
	RSD (%)	1.34		**RSD (%)**	0.86		**RSD (%)**	1.25		**RSD (%)**	1.67

C_p: Practical (measurement) concentration. SD: Standard deviation. RSD: Relative standard deviation (variation coefficient)

To determine the stability of benzocaine and fusidic acid solutions prepared by dissolving in acetonitrile, the samples (30 µg/ml) were analyzed by HPLC at 15, 30, 60, 120 min, 24, and 48 h. The analysis revealed that there was no significant difference in the concentrations of the active substances (Table 6). These results showed that the prepared solutions were stable throughout the validation process.

Table 6. Stability test results of benzocaine and fusidic acid samples (30 µg/ml)

Benzocaine				Fusidic acid			
Time	**Area**	**C_p (µg/ml)**	**Recovery (%)**	**Time**	**Area**	**C_p (µg/ml)**	**Recovery (%)**
0th min	1477	29.78	99.26	**0th min**	567	29.84	99.48
15th min	1480	29.84	99.46	**15th min**	566	29.79	99.31
30th min	1479	29.82	99.39	**30th min**	565	29.74	99.13
60th min	1474	29.72	99.05	**60th min**	569	29.95	99.83
120th min	1474	29.72	99.05	**120th min**	567	29.84	99.48
24th h	1475	29.74	99.12	**24th h**	569	29.95	99.83
48th h	1487	29.98	99.93	**48th h**	572	30.11	100.36
	Mean	29.80	99.32		**Mean**	29.89	99.63
	SD	0.09	0.29		**SD**	0.11	0.38

C_p: Practical (measurement) concentration. SD: Standard deviation

Table 7. The robustness results of the method

Condition	Value	Benzocaine			Fusidic acid		
		Peak area	**Retention time**	**Recovery (%)**	**Peak area**	**Retention time**	**Recovery (%)**
		Change (%)	**Change (%)**		**Change (%)**	**Change (%)**	
Flow rate (ml·min^{-1})	0.9	5.05	14.77	102.18	6.36	14.73	104.66
	1.1	-13.48	-5.58	84.12	-12.23	-5.53	86.37
Percent of organic phase (%)	65	-4.52	14.58	92.84	-4.38	35.59	94.09
	75	-4.75	-3.88	92.62	-2.74	-16.21	95.70
Column temperature (°C)	30	-3.68	1.94	93.67	-3.19	1.23	95.26
	35	-4.84	0.28	92.54	-3.65	-0.85	94.81

The investigation into robustness was conducted by analyzing the solutions of the substances, which were prepared to introduce the changes in the organic component ratio of the mobile phase, the flow rate, and the column temperature. An assessment of the change in parameters (e.g., retention time and peak area) was conducted (Table 7). In particular, it was evaluated that the change in the flow rate and the mobile phase ratio affected the retention time. In addition, it was found that fusidic acid was more sensitive to changes in conditions in terms of retention time, while benzocaine was more sensitive with respect to peak area. However, the recovery values obtained indicated the robustness of the method against minor alterations (<5%) in the conditions.

To demonstrate the specificity and selectivity of the method for the substances, the blank and the loaded microemulsion were analyzed. The retention time of benzocaine and fusidic acid was determined as 2.3-2.5 min (Figure 3a) and 5.2-5.5 min (Figure 3b), respectively. No impurities were observed in the chromatogram of either active substance (Figure 3a and 3b). While certain peaks attributable to excipients were observed in the blank microemulsion chromatogram (Figure 3c), it was detected that these peaks were not coincident with those of the active substances (Figure 3d). As stated in our previous study findings, the drug content uniformities of benzocaine and fusidic acid were also determined to be 99.40±1.62% and 99.53±0.97%, respectively [6]. While the LOD and LOQ values for benzocaine were calculated as 0.127 and 0.384 µg/ml, respectively, the LOD and LOQ values for fusidic acid were calculated as 0.209 and 0.633 µg/ml, respectively.

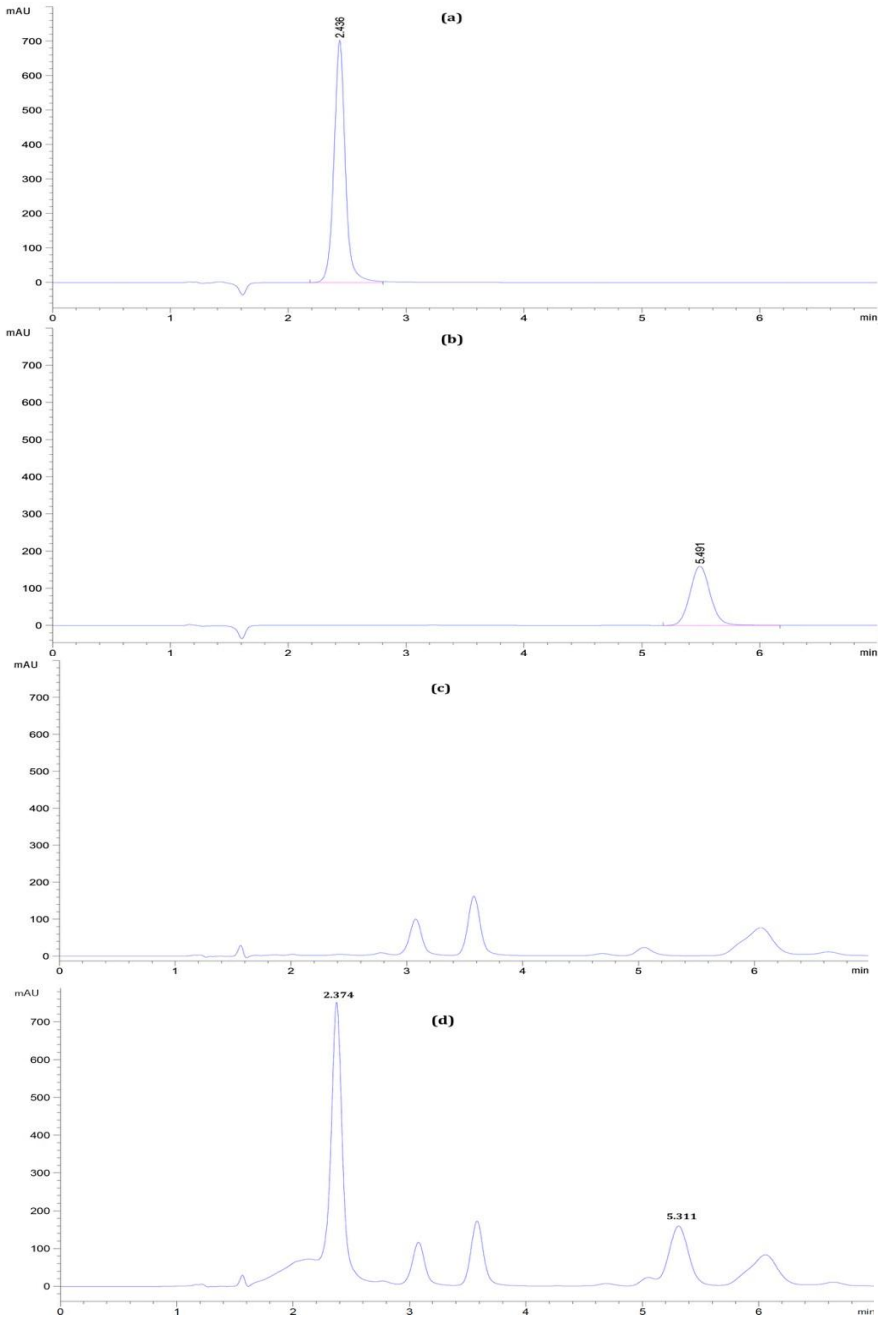

Figure 3. (a) HPLC chromatogram of benzocaine (100 µg/ml); (b) HPLC chromatogram of fusidic acid (100 µg/ml); (c) HPLC chromatogram of the blank microemulsion; (d) HPLC chromatogram of benzocaine- and fusidic acid-loaded microemulsion

To the best of our knowledge, no HPLC method currently exists that enables the simultaneous analysis of both benzocaine and fusidic acid using a single wavelength. However, separate methods have been developed for these substances contained in some marketed products. Curbete et al. [19] utilized a pre-column combined with a C18 column to quantify fusidic acid in a cream formulation, reporting a retention time of approximately 8 min. Yaseen *et al.* [33] employed a mobile phase consisting of acetonitrile and 0.001 M acetic acid (80:20, v/v), achieving a retention time of around 6 min for fusidic acid. Hirpara et al. [20] used acetonitrile and acetic acid (65:35, v/v) as the mobile phase and a detection wavelength of 243 nm in their method for the quantification of benzocaine, achieving a

retention time of approximately 4 min. On the other hand, the method developed in this study offered several advantages over existing methods, including the elimination of the need for a pre-column, the reduction of mobile phase consumption due to shorter retention times, and the capability to operate using a single wavelength.

An inexpensive, simple, and easily applicable HPLC method was developed for the determination and quantification of both active substances in fusidic acid and benzocaine-loaded microemulsions. The validation of the developed method demonstrated a very high correlation coefficient (0.9999 for both substances), high accuracy and recovery (98.84%, 101.05%, and 100.08% for benzocaine; 101.00%, 100.41%, and 100.33% for fusidic acid), and low variation coefficient for intermediate precision (<1.67). The results confirmed that this method is unique and highly reproducible for the quantification of both active substances.

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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