

Development and Validation of HPLC Method For the Detection of Fusidic Acid Loaded in Non-ionic and Cationic Nanoemulsion-Based Gels

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Fusidic acid (FA) is a well-known pharmaceutical antibiotic used to treat dermal infections. This experiment aimed for developing a standardized HPLC protocol to determine the accurate concentration of fusidic acid in both non-ionic and cationic nano-emulsion based gels. For this purpose, a simple, precise, accurate approach was developed. A column with reversed-phase C18 (250 mm x 4.6 mm ID x 5 m) was utilized for the separation process. The main constituents of the HPLC mobile phase were composed of water: acetonitrile (1: 4); adjusted at pH 3.3. The flow rate was 1.0 mL/minute. The optimized wavelength was selected at 235 nm. This approach achieved strong linearity for alcoholic solutions of FA when loaded at a serial concentration ranging from 12.5 to 400 µg/ml. Furthermore, the approach showed good stability and achieved full recovery and an effective separation for FA from the abovementioned formulation. Besides, the protocol validation revealed good robustness at a temperature range of 23 to 27, pH 3.0 to 3.5, detection wavelength 230 to 240 nm, flow rate 0.8 and 1.2 and mobile phase contents of (78:22 to 82:18 acetonitrile/ water). The limit of Detection was obtained 1.33 µg/ml and limit of Quantification was 4.04 µg/ml for FA that uploaded through mentioned formulations. All the validation parameters were within the acceptance criteria, as per ICH , US Pharmacopeia requirements. Overall, an affordable and reproducible method could be achieved for the detection and quantification of fusidic acid within the nano-emulsion based gels formulas.

Keywords: Cationic Nanoemulsion; Fusidic Acid; Nanoemulsion; Nanoemulsion Gel.

FA is a pharmaceutical antibiotic used to treat primary and secondary bacterial skin infections caused by Staphylococcus, Streptococcus, and Corynebacterium sensitive strains. An antibiotic with steroid-like properties¹ and a molecular formula of C₃₁H₄₈O₆ (Figure 1), FA's effectiveness belongs to its ability to inhibit the translocation of peptidyl tRNA.²

The development of novel analytical quality control methods is crucial in determining the calibre, therapeutic efficacy, and safety of FA and how it meets the requirements of the pharmaceutical industry and, by extension, the medical profession. Due to the ease with which it achieves chemical separation, identification, and quantification, reversed-phase liquid chromatography (RP-LC)

constitutes the prominent contemporary analytical method of choice. Through its use, separating drug molecules from other compounds and/or excipients, in addition to their degradation products, has been rendered feasible³.

The study's major purpose is to improve and validate a proper HPLC method, which must be an accurate, sensitive, and precise method for quantifying FA without interferences between FA and other constituent elements of NE formulation. Besides presenting the method developed, this study will discuss its advantages over other published analyses.

MATERIALS

Sgonek Biological Technology Co. (China) provided the reference standard fusidic acid (FA-RS) and stearyl amine. OREC Asia represented the supplier of ethanol (Malaysia). Soap Cart Co. (Malaysia) furnished the tea tree oil, and Sigma-Aldrich the propylene glycol (USA). Tween® 80 was acquired from Euro-Chemo-Pharma, while PEG 4000 was purchased from Merck (Germany) (Malaysia). J.T. Baker provided methanol and acetonitrile (HPLC grade) (Phillipsburg, USA). Deionized Milli-Q water constituted the purified water used (Millipore, Bedford, USA). QRC represents the source of glacial acetic acid (Selangor, Malaysia). Nylon filters (Sun Sri, USA) were used to filter the mobile phase (0.45 m) and Power sonic 405 (Seoul, Korea) was used to sonicate it. Before being injected into the HPLC equipment, 0.45 m polytetrafluoroethylene (PTFE) membrane filters of syringes (Pall Corporation, USA), were used to filter sample solutions.

HPLC Conditions and Apparatus

A Shimadzu pump (LC-20AT vp) (VP series, Kyoto, Japan) was employed, along with a UV-vis detector (SPD-20AD vp), a solvent delivery unit, a degasser, an auto-injector, and an oven (CTO-10S VP) (SIL20A HT vp). LabSolutions software was used to gather and process the data using that was already installed on a desktop computer system. An Agilent ZORBAX SB-C18 column (4.6 250 mm, 5 m) was used to achieve chromatographic separations (USA). The temperature of the oven was kept at 25°C. The mobile phase consisted of acetonitrile: water

with 0.001 M acetic acid (8:2, v/v). The pH was optimised at 3.3, with a constant flow rate of 1 mL/min. The isocratic elution method analysed the calibration standard solutions/test samples. The sample injection was set at 20 μ L to ensure effective detection and clearance of both drugs from the system; each injection was given a 10-minute run time. According to the results of a preliminary UV-vis spectrophotometric evaluation using a spectrophotometer, the greatest absorbance of the post-injection FA was 235 nm.

METHODS

Preparation of a calibration curve and a standard solution

Forty milligrams of FA powder were dissolved in 100 ml of mobile phase (acetonitrile: water). The solution was vortexed for 5min minutes and sonicated. The stock solution was then diluted with the mobile phase to reach the final concentrations of 400 μ g/ml to 12.5 μ g/ml. Each standard solution was injected three times into the HPLC system; then, peaks were recorded and plotted versus the concentrations to create the FA standard calibration curve. Based on error estimations, the y-intercept, slope, and correlation coefficient of the curve were computed using most minor square linear regression analysis.

Preparation of nanoemulsion and nanoemulsion gel

Preparation of FA-loaded non-ionic (NNE) and cationic (CNE) nanoemulsions

FA-loaded NE was produced with a precise ratio of oil/surfactants/water that resulted in transparent NE as indicated by a developed pseudoternary phase diagram software which gives the right ratio of mixing between the main constituents of NE. Unless otherwise stated, the non-ionic NE was prepared in the following manner using non-ionic surfactant. In the initial step, both oily phases, FA and surfactants, were mixed then, the aqueous phase was added gradually. Finally, the mixture was vortexed for 30 seconds. Finally, cationic surfactant stearyl amine (1%) was added to the mix to produce CNE.

Preparation of FA-loaded bilayered non-ionic (BNE) and cationic bilayered (CBNE) nanoemulsion gels

The FA-NNE and FA-CNE were jellified

and transformed into 2% FA-BNE and 2% FA-CBNE using Carbopol® 940 hydrogel (Mumbai, Maharashtra, India). The two formulations were rheologically suitable for topical use in this form⁴

The FA NE gel stock sample was produced by combining 100 mg of the NE gel with 10 ml of mobile phase (acetonitrile: water), which contained 200 µg mL⁻¹ of available FA. The resulting mixture was subjected to 10 minutes of sonication. Before analysis, All samples were used to filter it. With 20 microliters of the sample fed directly into the HPLC apparatus without any additional dilution. The placebo stock sample was manufactured in an identical manner to that of the other samples but without the addition of FA.

System suitability testing

The chromatographic system's repeatability and performance were confirmed by a system suitability test. The theoretical plates (N), height equivalent of a theoretical plate (HETP), and tailing factor (T) were calculated using USP (United States Pharmacopeia) guidelines and tested at 200 µg /mL FA (n = 6).

Method validation

During method validation, ICH (2005) guidelines for linearity, precision, selectivity, accuracy, robustness and detection and quantification of limits were followed.

Range and linearity

Linearity was evaluated using five calibration plots on five consecutive days with FA concentrations ranging from (12.5 to 400 µg / mL), regarding ICH guidelines Q2(R1) (November 2005). The peak area of FA was used to construct the calibration curve.

Precision and accuracy

Repeatability (daily) and intermediate(inter-day) evaluated precision. Precision by repeatability (n=9) covered the method's linear range, i.e., three concentrations obtained from the calibration curve: the lowest concentration was (12.5 µg/ml), the middle concentration was (50 µg/ml), and the highest concentration was (100 µg/ ml). This technique was performed three times on a single day, in one laboratory, and under consistent experimental conditions by the same analyst. On separate days, different analysts worked on the linear range of the technique (n=9) to attain intermediate precision. % RSD (the percentage relative standard deviation)

was determined as an accuracy indicator. Besides, the relative percentage relating to an error (% RE) was used to assess the accuracy of FA calculated from the previously established standard calibration curve.

Recovery of FA

A half g of blank BNE sample was solubilized in 20 ml of ultra-pure water and swirled continuously while 10 mg of FA was added to the solution, which was then sonicated using an ultrasonic bath for ten minutes. Using a 10 ml volumetric flask, 1 ml of the mentioned solution was diluted with 10 ml of the mobile phase and passed through a 0.45 µm nylon filter. In the end, the final nominal concentration was 50 µg/ml. A 20 µl FA sample was injected into the HPLC apparatus. The resulting area under the curve of the drug was coupled to the regression equation to compute the recovery of the selected sample. Three times the experiment was carried out.

Specificity

The chromatograms that resulted from the standard FA solution were compared with the chromatograms of the formulation with and without FA, the probability of interferences of FA with mobile phase and other formula's excipients during the retention time was assessed.

Limit of detection (LOD) and limit of quantification (LOQ)

The standard deviation from the calibration curve's y-intercept and the slope's average, which were obtained from Equations using 1 and 2, respectively, were used to calculate the LOD and LOQ

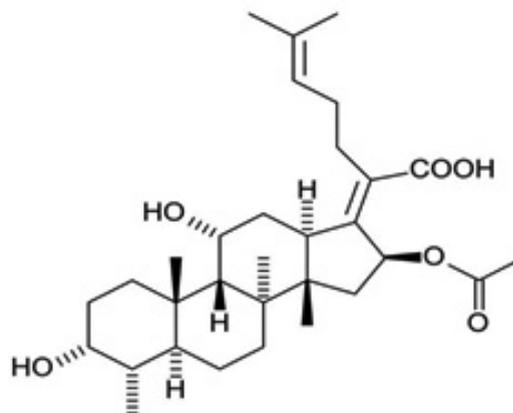


Fig. 1. Chemical Structure of Fusidic Acid

LOD = 3 (SD. a^{-1})

LOQ = 10 (SD/ a^{-1})

Where SD is the intersection's standard deviation and a is the slope's average.

Robustness

Minor modifications to the optimal chromatographic conditions were used to test the resilience of the new approach. The variables tested were the detector wavelength (230 to 240 nm), pH 3.0 to 3.5), mobile phase composition (78:22 to 82:18 acetonitrile/ water v/v), flow rate (0.8 and 1.2 ml/min), and oven temperature (23 to 27 °C). The study was carried out with a FA concentration of 200 µg mL⁻¹. The FA peak regions and percentage RSD of the test robustness were determined, and the data was statistically examined.

Stability of the stock solution

For 24 hours, the FA stock solution (20 mg/ml) was set aside at ordinary room temperature (25± 2°C). Then, FA concentrated stock solution was diluted to 200 µg/ml, which is a concentration within the standard range of the calibration curve. After 24 hours, the responses were compared to the freshly prepared sample.

Statistical analysis

All data obtained from statistical analyses were repeated three times individually. All gathered data are expressed as mean ± standard deviation (SD), with a statistical significance threshold of 0.05, using the GraphPad Prism® software.

Table 1. System Suitability Analysis For FA At 200 ig/ml (Mean ± SD, n=6)

Parameters	Theoretical plates (N)	Tailing factor (T)	Retention time (Rt)
Values	8296.33±30.022	1.105±0.0057	6.02±0.013
% RSD	0.361	0.522	0.771
Required limits	N > 3000	T ≤ 2	RSD ≤ 2 %

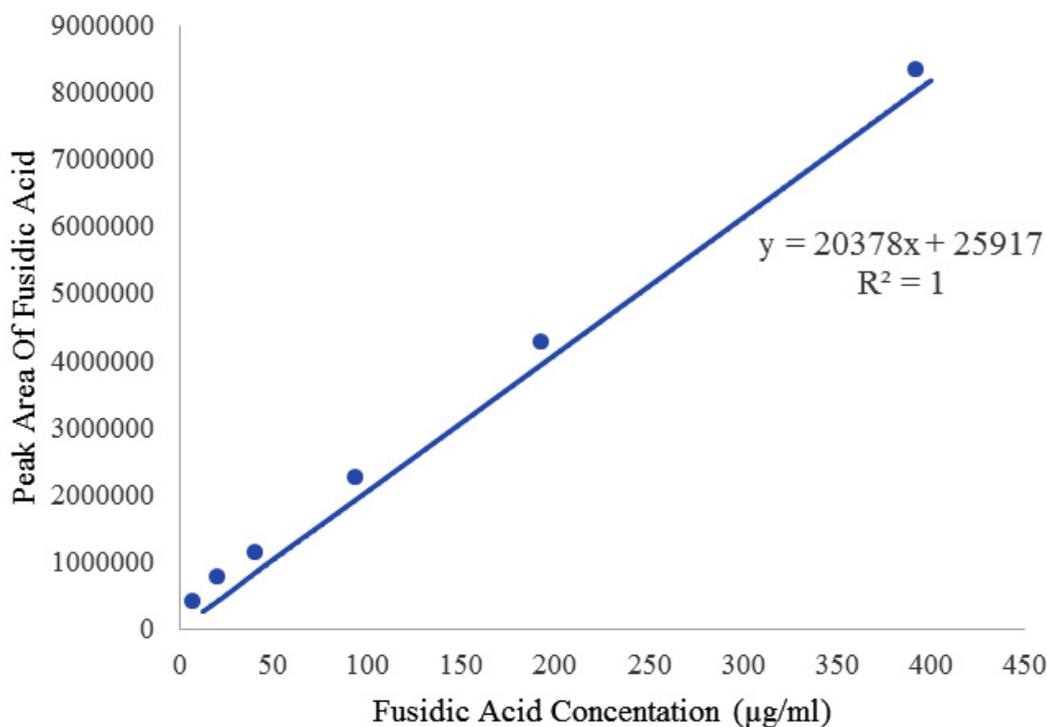


Fig. 2. Standard Calibration Curve of FA

Table 2. The Results of Intra-Day and Inter-Day Precision and Accuracy. Mean \pm SD, N = 6

Conc. ($\mu\text{g/mL}$)	Area	Intra-day		Inter-day		Accuracy (%RE)
		Mean \pm (SD)*	Precision (%RSD)	Mean \pm (SD)*	Precision (%RSD)	
100	2079731	2065036 \pm 6278	0.30	2035235	2050280 \pm 14902	-0.663
	2002018			2065036		
	2023958			2050569		
50	1049000	1047652 \pm 2042	0.19	1040689	1048691 \pm 8568	0.378
	1048655			1047652		
	1045303			1057732		
12.5	280739	277786 \pm 4316	1.55	277868	278303 \pm 1861	-0.886
	279961			276868		
	272904			280473		

(SD) = standard deviation; *RSD = relative standard deviation, RE relative error

RESULTS AND DISCUSSION

System suitability testing

Before the validation technique, a group of tests that evaluate the system's compliance (system appropriateness) must be done. This collection of compliance tests is intended to ensure that the equipment and procedure under development can generate accurate and precise results.⁵

To be declared reliable and repeatable, the system must pass at least two of the following tests, according to the FDA (1994): repeatability, resolution, plate number, asymmetry, and retention factor. Moreover, asymmetry is a significant property. Due to its inhibition of chromatographic peaks from broadening may lead to inaccuracies in detecting analytes of interest⁷.

Many parameters, including analytical conditions, sample or solute volume, column length, can influence the number of plates used. The retention factor is utilized to confirm that the suggested technique produces a good chromatographic utilization. This metric is inversely related to the analyte's retention time. When kept within acceptable limits, low retention factors result in reduced expense, faster analysis, and, as a result, fewer waste output⁸.

According to the results of system suitability tests, the chromatographic parameters utilized to construct this approach can deliver precise and decisive results because the computed means value for asymmetry was lower than 2.0. The average plate number and Tailing factor exceeded 3000 and 2.0, respectively, as required by FDA guidelines (1994)⁶. Furthermore, with an RSD of less than 2%, the results of FA retention time were found to be near 10 min, and chromatographic peak regions were sufficiently accurate for conformance testing (see Table 1)⁷.

Validation of Method

Linearity and range

Peak area and FA concentrations within the range of 12.5- 400 $\mu\text{g/mL}$ were shown to have a linear relationship (Figure 2). Furthermore, the linearity of the evaluated calibration curves was confirmed by the value of the regression correlation coefficients (R).

Precision and accuracy

The intra-day and inter-day precision and accuracy data are shown in Table 2. The intra-day

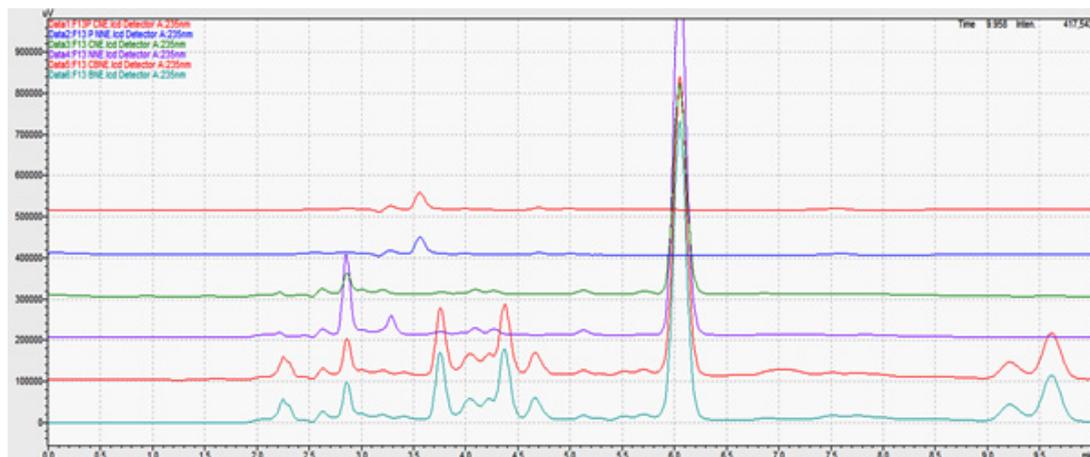


Fig. 3. Chromatograms of The Pure FA, Placebo and The Different Nanoemulsion Formulations. 1: Placebo CNE, 2: Placebo NNE, 3: FA-CNE, 4: FA-NNE, 5: FA-CBNE, 6: FA-BNE. Retention Time of FA = 6.08 Min

Table 3. Robustness at Concentration of 200 µg/ml (Mean ± SD, N=3)

Parameters	Variations	Peak mean curve area ± SD	%RSD	Mean retention time (min) ± SD
Mobile Phase pH	3.0	4211670 ± 12573	0.29	6.07±0.001
	3.3	4131696±46658	1.12	6.04±0.004
	3.5	4138716±67837	1.63	6.08±0.002
Wavelength (nm)	230	4288308±79133	1.84	6.03±0.001
	235	4141966±38346	0.92	6.02±0.013
	240	3343503±7088	0.21	6.03±0.005
Flow rate (ml /min)	0.8	4264048±43858	1.02	7.53±0.006
	1	4131258±17088	0.41	6.03±0.002
	1.2	3656187±9112	0.24	5.04±0.012
Mobile phase (w/v)	78:22	4060333± 26222	0.64	6.37±0.180
	80:20	4107832± 16346	0.79	6.04±0.008
	82:18	4120439± 70773	1.71	5.84±0.303
Temperature(C°)	23	4101336±6969	0.16	6.09±0.015
	25	4135932±48326	1.16	6.05±0.006
	27	4131696±46658	1.12	6.14±0.069

Table 4. Stability of FA Solution

Time (h)	Standard solution of fusidic acid (µg/mL)	Peak area	Total drug content (µg/mL)
0	200	4216700	200.9011
		4093338	
		4093338	
24	200	4093338	200.6556
		4093338	
		4093338	
		4093338	

precision for FA was 0.19 % to 1.55 %, while the accuracy was -1.101 % to 0.277 %. While the inter-day precision started from 0.66 % To 0.81 %, and the accuracy started from -0.886 % To 0.378 %. Statistics showed that the method was exact and accurate, within the 2% range indicated by the US Pharmacopeia¹⁷.

Recovery of FA

The major purpose of the experiment was to determine the accuracy of the presented method for quantifying FA in the sample proved. Therefore, the slope and intercept of the calibration curve were obtained in the concentration range of 400–12.5 5 ig /ml, which were used to determine FA recovery. The percentage of FA recovery was 100.7% in the NE gel formulation sample.

Specificity

The chromatograms of pure FA which appeared in 6.08 min are compared to the chromatograms of several types of FA-loaded NEs and placebo formulations in Figure 3. It was demonstrated that none of the formulation components, such as surfactants and oil and mobile phase components peaks which appears in the chromatograms especially from (2-5)min and (9-10) min , influenced FA retention time . As a result, this procedure is unique to FA analysis.

Determinations of Limit of detection (LOD) and limit of quantification (LOQ)

The LOD tested samples were found to occur at 1.33 ig. mL⁻¹. Meanwhile, the LOQ was 4.04 ig. mL⁻¹.

Robustness Assays

The contents of Table 3 indicate the method's robustness. According to statistical testing, the peak area and retention times were not significantly affected by modifications in the mobile phase pH or oven temperature. However, differences in the detecting wavelength and flow rate resulted in significant variants in the peak curve area and retention time. Meanwhile, changes in mobile phase components had little effect on peak area but did produce significant alterations in retention duration.

Stock solution stability

The fusidic acid standard solution sample was determined to be stable after 24 hours at ambient temperature (25±2 C). In addition, the FA peak area did not differ significantly between 0 and 24 hours (p > 0.05). Therefore, the total

drug content was 200.9011 ig /mL at 0 hours and 200.6556 ig /mL at 24 hours, as shown in Table 4.

This method of measuring FA in biological fluids is described in several articles in the relevant literature,^{9,11,12,18} and pharmaceutical products^(13,14,6,15,4,16). Some of these procedures are time demanding, necessitate a buffer solution, or necessitate long-running durations, excess in the volume of injection, flow rates, and drug concentrations in the sample. In addition, being the first approved method for FA measurement in this type of formulation, this is a more user-friendly method for FA quantification in nanoemulsion and nanoemulsion gel.

CONCLUSION

Developing a stable HPLC–UV technique for determining FA concentration in NE and NE gels proved successful. The procedure was discovered to be simple, rapid, sensitive, and specific. The intra-day and inter-day precision and accuracy were both less than 2%, indicating that the protocol was extremely accurate, and the method followed USP recommendations. Excipients, diluents, mobile phase constituents, and degradation products did not cause any interference. The method might be employed for standard FA assays in addition to *in vitro* investigations of FA release from NE gel formulations.

Conflict of Interest

There are no conflict of interest.

Funding Sources

It is self-funded study.

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