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FLUCONAZOLE LOADED NANO-EMULSION ASSAY BY VALIDATED RAPID AND SENSITIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract:
A rapid and sensitive reverse phase high performance liquid chromatography method was developed for assay of fluconazole in olive oil based nano-emulsions. Separation of fluconazole from excipients present in nano-emulsion formulation was achieved on high efficiency core shell, micro-bore reverse phase C18 column (50 x 2.1 mm, 2.6 µ) maintained at a temperature of 30◦C by column oven. Mobile phase containing 75 volumes of 10 mM potassium dihydrogen phosphate pH 3 adjusted with orthophosphoric acid and 25 volumes of methanol was introduced at a flow rate of 0.3 ml/minute. After a very short run time of 4-5 minutes, isocratic elution followed detection at 210 nm by UV- visible detector. A sharp and symmetric peak was obtained at the retention time of 2.9 minutes. The optimized method was validated for linearity, accuracy, precision, and robustness. The linear regression data for the calibration plot are indicative of a good linear relationship between peak area and concentration (r^2= 0.996) over a wide concentration range (1 µg/ ml to 50 µg/ ml). Limit of detection and limit of quantification values were determined to be 0.10 and 0.5 µg/ml, respectively. The overall accuracy of the method was 98.2% with RSD of 0.71% indicating the acceptable accuracy of the method. Precision of the method was evaluated at two levels i.e intraday precision (repeatability) and inter-day precision (intermediate precision) with overall RSD of all determinations less than 1% indicating the acceptable precision of the method. The developed and validated method was successfully applied for the quantification of fluconazole in olive oil based nano-emulsions.

Keywords: *Fluconazole, olive oil nano-emulsions, HPLC, micro-bore, core shell column.*

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INTRODUCTION:

Fluconazole is a broad spectrum antifungal agent belonging to bistriazole group hence has significantly different pharmacokinetic properties from other imidazole based antifungal agents. Due to halogenated phenyl ring it has more antifungal activity than other counterpart however, it is less lipophilic and more hydrophilic and therefore it is supposed to have less skin penetration potential as compared to other antifungal agents [1]. Olive oil and its major component oleic acid are natural non-irritating permeation enhancer having some antifungal activity as well [2-9]. Olive oil based nano-emulsion and its antifungal evaluations have been reported by the authors [10, 11]. Purpose of the study was to develop a sensitive and rapid high performance liquid chromatographic method for evaluation of fluconazole in olive oil based nano-emulsions. Literature survey reveals various common analytical methods for determination of fluconazole in formulations such as UV spectroscopy [12-14], and HPLC [15-18], however these have certain limitations such as low sensitivity and inability to avoid interference due to excipients in the formulation during analysis (UV spectroscopy) or very complex and long analysis time per sample (HPLC). Therefore, this study was undertaken to develop a simple, sensitive and rapid high performance liquid chromatographic method and validation as per ICH norm [19, 20] for separation and evaluation of fluconazole in olive oil based nano-emulsions.

EXPERIMENTAL:

Reagents and chemicals

Fluconazole was purchased from Sigma Aldrich USA. HPLC grade acetonitrile, methanol, potassium dihydrogen phosphate, orthophophoric acid were obtained from Panreac. Milli Q water was used throughout the experiment which was prepared using Millipore water purification system.

HPLC instrumentation

Chromatographic analysis was carried on a Waters Alliance e2695 separating module (Waters Co., MA, USA) using UV detector (Waters 2998) with auto sampler and column oven. The instrument was controlled by use of "Empower pro 2" software version 6.20 installed with equipment for data collection and acquisition. Chromatographic separation was achieved on a C_{18} reverse phase column SunShell (C18 - 2.6µm, 50mm X 2.1mm I.D Origin: Chromanik Inc, Osaka, Japan) maintained at 30ºC temperature.

Method development

A working standard of 10 µg/ml of fluconazole in methanol was injected over HPLC column at ambient temperature first with equal ratio of HPLC grade methanol and water as mobile phase and the responses were monitored by UV detector at 260 nm as it was reported lamda max for fluconazole. Size and shape of peak was optimized by varying lamda max for detection, mobile phase composition (introduction of buffer and changing organic phase type and ratio), and column temperature. Optimized mobile phase consisted of 75 volumes of aqueous buffer of 10 mM potassium dihydrogen phosphate pH 3 adjusted with orthophosphoric acid, and 25 volumes of HPLC grade methanol. The mobile phase was degassed by sonication and filtered through nylon membrane of 0.45-mm pore size. Ten microliter standard samples or quality control samples or test samples were injected then eluent was monitored with a UV detector at 210 nm with flow rate of 0.3 ml/min and run time of 4 minutes. Individual peaks were identified from retention time and peak area.

Preparation of the standard and quality control samples

A standard stock solution of fluconazole with a concentration of 1000 µg/ml was prepared by accurately weighting and dissolving in HPLC grade methanol. Different aliquots were taken from this stock to prepare various working standards ranging from 0.5 to 50 µg/ml. Similarly, three quality control (QC) samples at the concentration levels of 5, 20 and 40 µg/ml were prepared from the same stock solution. Individual peaks were identified from retention time and concentrations were determined from the peak area using regression equation obtained from calibration plot.

Preparation of sample of nano-emulsion

Freshly prepared olive oil nano-emulsion containing 2% w/v of fluconazole, was appropriately diluted in mobile phase. Ten microliter of the prepared sample was injected in triplicate on HPLC column for separation and evaluation of fluconazole. Individual peaks were identified from retention time and concentrations were determined from the peak area using regression equation obtained from calibration plot.

Validation of the method

System suitability

The system suitability was assessed by six replicate analyses of fluconazole working standard at a concentration of 5µg/ml. The acceptance criterion was ±2% for the percent relative standard deviation (% RSD) for the peak area and retention times of fluconazole.

Linearity

The linearity of the method was established by injecting 10 µl of series of standard solutions containing 1-50 µg/ml of fluconazole. Calibration plot was constructed by plotting the peak area

responses against their respective concentrations. Linear regression was applied and slope (a), intercept (b), correlation coefficient (r) and standard deviation (SD) were determined.

Detection and quantitation limits (sensitivity)
Limits of detection (LOD) and limit of quantitation (LOQ) were estimated through dilution method using signal-to-noise ratio (S/N) approach by injecting a 10 µl sample. LOD and LOQ were considered as the lowest concentrations level resulting in a peak height of at least three times (S/N≈3) and ten times (S/N≈10) the baseline noise respectively with precision (% RSD) and accuracy (% bias) within ±10%.

Accuracy
The accuracy of the method was determined by analyzing and calculating the % recovery of the quality control samples of fluconazole in triplicate which were prepared at three different concentration levels (5, 20 and 40 µg/ml).

Precision
Precision of the method was evaluated by analyzing the quality control samples in triplicate at three concentration levels during the same day whereas inter-day precision was evaluated by repeating the repeatability assays on second day and assessing the combined overall results of both day 1 and day 2 together.

Robustness
Robustness of the method was determined by introducing small variation in the established method parameters such as composition of mobile phase, mobile phase flow rate and column temperature followed by calculation of the responses, retention time and RSD.

RESULTS AND DISCUSSION:

Optimization of method
The method is based on separation of fluconazole from other excipients of olive oil nano-emulsions. Several parameters such as mobile phase composition, pH and flow rate along with detection wavelength were tested for their effect on location and shape of peak of the fluconazole during development phase of the method. Optimized chromatographic conditions have been mentioned in Tables 1.

System suitability
The % RSD of peak area and retention time for fluconazole were within 2% indicating the suitability of the system (Table 2).

Table 1: Chromatographic conditions for the analysis of fluconazole

PARAMETERS	OBSERVATIONS
Mobile phase	75 volumes of 10 mM potassium dihydrogen phosphate buffer pH 3 adjusted with orthophosphoric acid. 25 volumes of HPLC grade methanol.
Column used	C18 (50 X 2.1mm) 2.6 µm
Temperature	30ºC
Flow rate	0.3 ml/minute
Injection volume	10 µl
Detector	UV visible detector
Method	Isocratic elution
Wavelength	210 nm

Table 2: System suitability parameters

S N.	PEAK AREA	RETENTION TIME
1	307772	2.91
2	304658	2.92
3	301086	2.93
4	304914	2.91
5	301426	2.9
6	301549	2.93
Mean	303567.5	2.916667
SD	2664.518	0.012111
%RSD	0.877735	0.415221

Linearity, limit of detection (LOD) and limit of quantitation (LOQ)
The linear regression calibration curve was plotted by using peak area against concentration and was found linear in the range of 1 µg/ ml to 50 µg/ ml with a good linear relationship of 0.996 (Fig 2). Calibration and regression data are presented in Table 3 and Table 4 respectively. The linear regression data for the calibration plot are indicative of a good linear relationship between peak area and concentration over a wide range. LOD and LOQ values were determined to be 0.10 and 0.5 µg/ml, respectively.

Table 3: Calibration data for fluconazole

Concentration (µg/ml)	Mean peak area ± SD[a] (n=3)	% RSD[b]
1	67114 ± 1540	2.30
5	305198 ± 3443	1.13
10	519023 ± 6415	1.24
15	912358 ± 4649	0.51
20	1137504 ± 2774	0.24
25	1433733 ± 6030	0.42
30	1708077 ± 5992	0.35
40	2108595 ± 8176	0.39
50	2706159 ± 5663	0.21

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

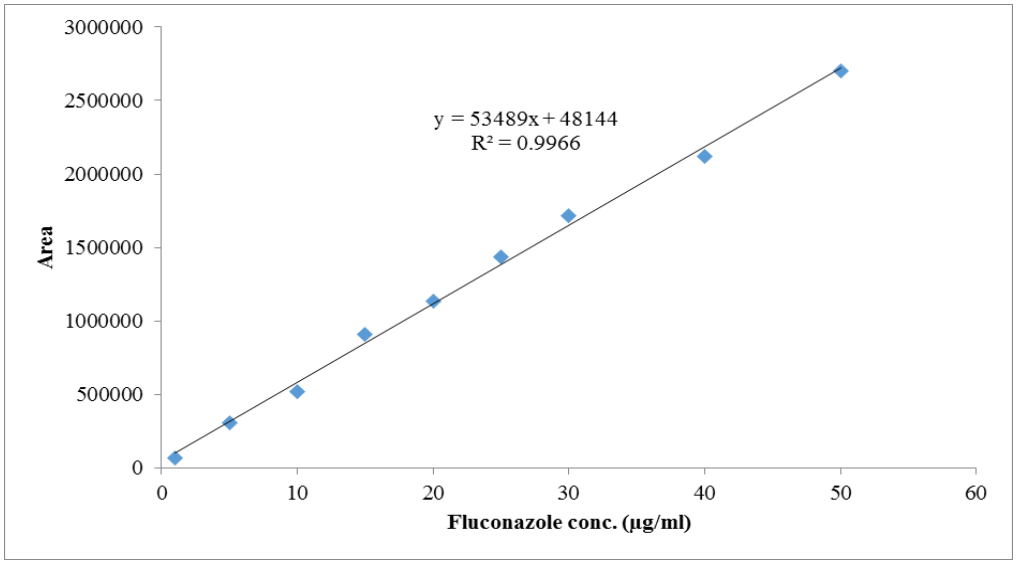

Fig. 1: Calibration plot of fluconazole

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

Parameters	**Observations**
Linearity range	1-50 µg/ml
Regression equation	$y^a = 53489x^b + 48144$
Correlation coefficient	0.998±0.0001
Slope ± SD	53439±65.6
Intercept ± SD	47064 ± 1315

[a]Peak area; [b]Concentration of standard (µg/ml)

Table 5: Accuracy of the method (n=3)

Conc. added	Concentration calculated			Accuracy %				% RSD
	N1	N2	N3	N1	N2	N3	Mean	
5	4.88	4.82	4.75	97.6	95.1	95.1	95.9	1.5
20	20.37	20.46	20.38	101.8	102.3	101.9	102	0.25
40	38.75	38.47	38.51	96.9	96.2	96.3	96.4	0.39
	Overall accuracy						98.2	0.71

Table 5: Precision of the method

SN.	AUP data Intra-day precision (repeatability-day 1)			AUP data Intra-day precision (repeatability-day 1)		
	LQC	MQC	HQC	LQC	MQC	HQC
N1	307772	1135411	2118036	307634	1136758	2102876
N2	304655	1140651	2102917	305678	1142761	2112879
N3	301088	1136441	2105005	301088	1137658	2103987
Mean	304505	1137501	2108653	304800	1139059	2106581
SD	3344	2776	8192	3360	3237	5482
% RSD	1.09	0.24	0.39	1.10	0.28	0.26
Overall RSD = 0.58 %				Overall RSD = 0.55 %		
% RSD of Inter-day precision / Intermediate precision = 0.56 %						

AUP: Area under peak, LQC: Lower level quality control sample, MQC: Middle level quality control sample, HQC: Higher level quality control sample, SD: Standard deviation of three replicate determinations, RSD: Relative standard deviation.

Accuracy

The accuracy of the method was determined by back calculation of % recovery of fluconazole quality control samples at three different concentration levels (5, 20 and 40 µg/ml) in triplicate. The results presented in Table 5 show that the % recovery ranges between 95.1-102.3% with RSD range of 0.39-1.5%. The overall recovery of all determinations was 98.2% with RSD of 0.71% indicating the acceptable accuracy of the method.

Precision

Precision of the method was evaluated at two levels i.e intraday precision (repeatability) and inter-day precision (intermediate precision). All experiments were done in triplicate and observed results are reported in terms of % RSD (Tables 6). Intermediate precision is reported as average of repeatability obtained on day 1 and day 2. The overall RSD of all determinations was less than 1% indicating the acceptable precision of the method.

Robustness

Robustness of the method was determined by introducing small variations in the experimental conditions such as mobile phase composition, flow rate and column temperature followed by calculation of the responses, retention time and RSD. The results are presented in Table 6. It should be noted that retention time varied considerably with respect to all changes that were introduced in the method whereas peak area changed minimally. However, low values of the % RSD for both peak area as well as retention time indicated the robustness of the method.

Table 6: Robustness of the method

Parameters	AUP or RT	N1	N2	N3	MEAN	SD	% RSD
Buffer: Methanol 65: 35	AREA	1135411	1140651	1136441	1137501	2776	0.24
	RT	2.11	2.18	2.16	2.15	0.036	1.68
Buffer: Methanol 70: 30	AREA	1236800	1235468	1234562	1235610	1125	0.09
	RT	2.37	2.38	2.4	2.383333	0.015	0.64
Buffer: Methanol 75: 25	AREA	1277356	1274312	1274256	1275308	1773	0.14
	RT	2.91	2.92	2.91	2.913333	0.005	0.20
Flow rate 0.2 mL/min	AREA	1963245	1962645	1964521	1963470	958	0.05
	RT	4.12	4.13	4.1	4.116667	0.015	0.37
Flow rate 0.25 mL/min	AREA	1236800	1235468	1234562	1235610	1125	0.09
	RT	3.44	3.46	3.47	3.456667	0.015	0.44
Flow rate 0.3 mL/min	AREA	1277356	1274312	1274256	1275308	1773	0.14
	RT	2.91	2.92	2.91	2.913333	0.005	0.20
Colum temp 25 ◦C	AREA	1297364	1298421	1297365	1297717	609	0.05
	RT	3.44	3.46	3.47	3.456667	0.015	0.44
Colum temp 30 ◦C	AREA	1277356	1274312	1274256	1275308	1773	0.14
	RT	2.91	2.93	2.91	2.916667	0.011547	0.40
Colum temp 35 ◦C	AREA	1316325	1315426	1324562	1318771	5035.257	0.38
	RT	2.29	2.25	2.35	2.296667	0.050332	2.19

AUP: Area under peak, SD: Standard deviation of three replicate determinations, RSD: Relative standard deviation.

Evaluation of fluconazole in olive oil based nano-emulsion sample

Freshly prepared olive oil nano-emulsion containing 2% w/v of fluconazole, was appropriately diluted in mobile phase. Ten microliter of the prepared sample was injected in triplicate on HPLC column for separation and evaluation of fluconazole. Individual peaks were identified from retention time and concentrations were determined from the peak area using regression equation obtained from calibration plot. A typical chromatogram of fluconazole standard solution and fluconazole loaded nano-emulsion is given in Fig.2 and 3 respectively.

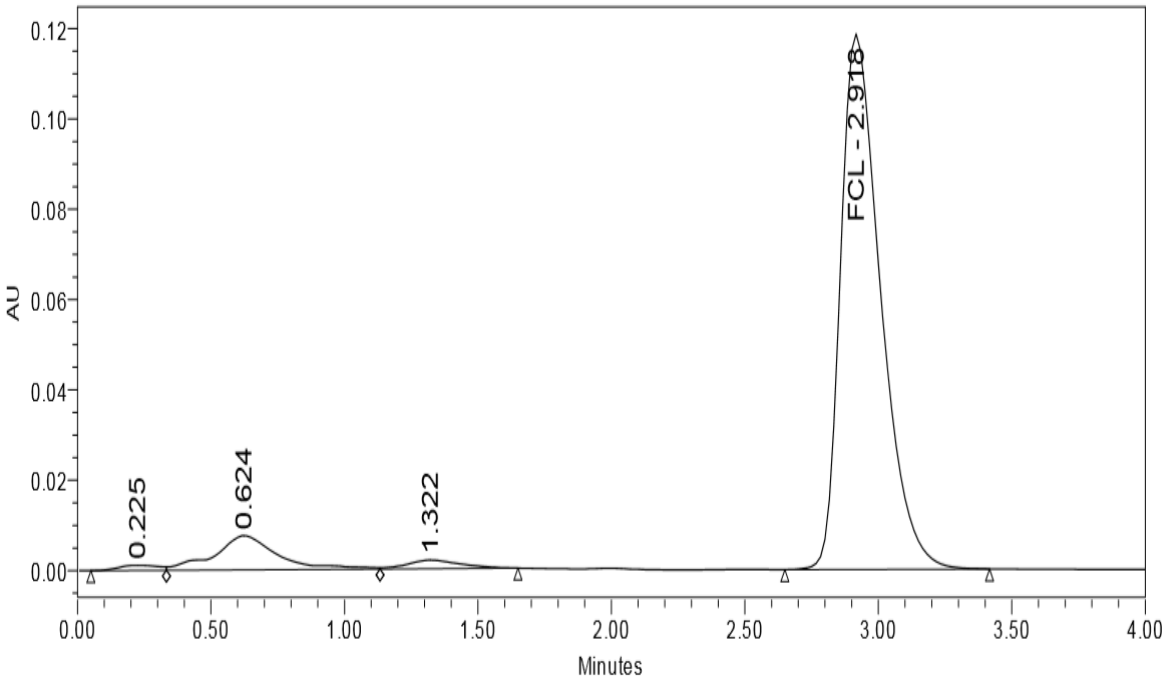

Fig. 2: A typical HPLC chromatogram of standard Fluconazole (20 µg/ml), eluted by potassium dihydrogen phosphate: Methanol (75:25) at 210 nm wavelength.

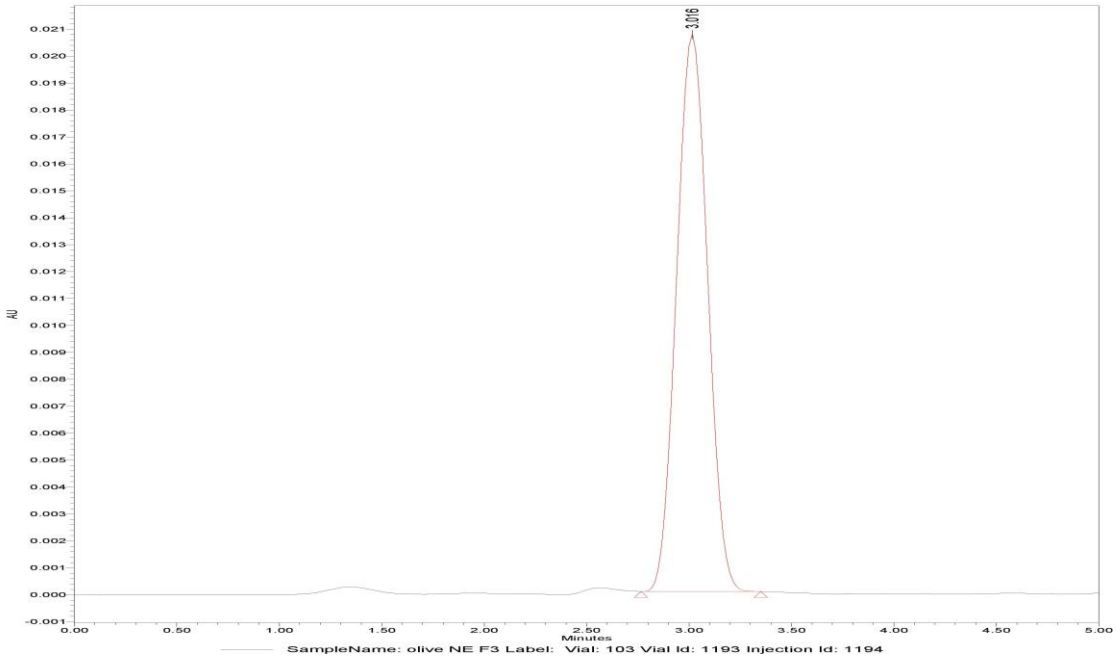

Fig. 3: A typical HPLC chromatogram of Fluconazole loaded olive oil nano-emulsion eluted by potassium dihydrogen phosphate: Methanol (75:25) at 210 nm wavelength.

CONCLUSION:

Developed reverse phase high performance liquid chromatography method for assay of fluconazole in olive oil based nano-emulsions was simple, rapid and very sensitive. A sharp and symmetric peak was obtained at the retention time of 2.9 minutes after a very short run time of 4-5 minutes following a very simple isocratic elution and detection at 210 nm by UV- visible detector. The optimized method was validated for linearity, accuracy, precision, and robustness. The overall accuracy and precision of the method was very promising as indicated by RSD less than 1%.

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