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DEVELOPMENT AND VALIDATION OF UV SPECTROPHOTOMETRIC METHOD FOR SIMULTANEOUS ESTIMATION OF FLUCONAZOLE AND QUERCETIN FROM PHARMACEUTICAL FORMULATION

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ABSTRACT

The aim of present investigation is to establish simple, precise, economical, rapid, and accurate first order (D^1) UV-Spectrophotometric method for simultaneous estimation of Fluconazole (FCZ) and Quercetin (QCT) from developed mucoadhesive In-situ vaginal gel formulation. The method was developed in Simulated Vaginal Fluid (SVF) at two different pH, normal vaginal pH (4.31) and in Infected vaginal pH (7.00). In developed fist order UV-spectrophotometric method, FCZ was quantify at detection wavelength, 232.03 nm, and 268.03 nm in SVF, pH 4.31 and pH 7.00, respectively. While QCT can easily quantified at same detection wavelength, 400 nm in both SVF, pH 4.31 and pH 7.00. The method was also validated as per ICHQ2(R1) guidelines. The scope of developed method includes quantification of FCZ and QCT from its developed mucoadhesive In-situ vaginal gel formulation and identification of drug released profile of both analytes in both normal vaginal pH (4.31) and infected vaginal pH (7.00).

Keywords: Simultaneous estimation, Fluconazole (FCZ), Quercetin (QCT), First order (D^1) UV-Spectrophotometric method, ICH Q2(R1) Guideline

INTRODUCTION

Vulvovaginal Candidiasis (VVC) is also known as vaginal thrush which is a common gynaecological disease that occur due to excessive growth of yeast in the vagina that caused by species *Candida albicans* **[1, 2]**.

Fluconazole (FCZ) **(Figure 1)** is the commonly used first-line drug in clinical prophylaxis and treatment of mucosal and invasive *Candida* infections including Vulvovaginal Candidiasis. The extensive used of Fluconazole is due to its good bioavailability and lower toxicity **[3-5]**. The study represents that the resistance rate to *C. albicans* was between 10-20% for FCZ in patients with Vulvovaginal Candidiasis. Excessive and indiscriminate clinical use of FCZ has causes emergence of multiple-drug-resistant (MDR) strains of *C. albicans* **[6-8]**. Therefore, it is necessary to seek novel medicines to use alone or in combination with FCZ in the treatment of FCZ-resistant *C. albicans* isolated from VVC.

Quercetin (QCT) **(Figure 2)**, a dietary flavonoid that has been demonstrated to possess weak antifungal function to manage clinical *Candida albicans* biofilms and sensitize the susceptibility of FCZ-resistant *Candia albicans* isolates to FCZ **[9, 10]**.

The study also represents that after the treatments of QCT and FCZ, the fungal loading decreased, the hypha-form cells disappeared, and the inflammation of mucosal epithelial cell were greatly alleviated that represents FCZ and QCT is possible synergistic combination in treatment of resistant *Candida albicans* associated infection **[11]**.

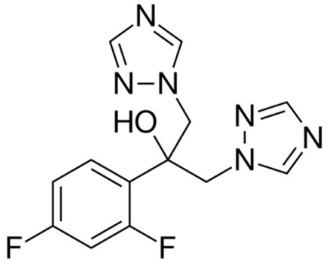

Figure 1: Chemical Structure of Fluconazole (FCZ).

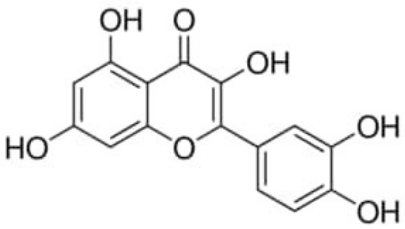

Figure 2: Chemical Structure of Quercetin (QCT)

The literature review reveals that there is no any published data related to analytical methods for combination of Fluconazole and Quercetin in their combined dosage form. Previously, an attempt was made in the laboratory for the formulation of Mucoadhesive In-situ vaginal gel formulation that contained FCZ and QCT as synergistic combination for the treatment of Vulvovaginal Candidiasis (VVC).

The aim of present investigation is to establish simple, precise, economical, rapid, and accurate first order (D^1) UV-Spectrophotometric method for simultaneous estimation of Fluconazole (FCZ) and Quercetin (QCT) from developed mucoadhesive In-situ vaginal gel formulation. The developed method was validated as per ICHQ2(R1) guideline.

MATEIRALS AND METHODS

Fluconazole was purchased from Rutu Chemicals, Panoli, Gujarat and Quercetin was purchased from Sigma-Aldrich, Mumbai, Maharashtra. Methanol was purchased from Merck which was used as solvent for both drugs. For preparation of Simulated Vaginal Fluid (SVF), Sodium chloride, Potassium hydroxide, Calcium hydroxide, Bovine serum albumin, Lactic acid, Acetic acid, Glycerol, Urea, Glucose was purchased from S.D. Fine chemicals, Vadodara. Double distilled water was self-prepared in laboratory.

INSTRUMENTATION

A Shimadzu (Kyoto, Japan) model UV-1800 double beam UV-Visible spectrophotometer attached with computer operated by software with UV probe 2.35. Spectral width of 2 nm, wavelength accuracy of 0.5 nm and pair of 1 cm matched quartz cells was used to measure absorbance of the resulting solutions. The absorbance spectrum was recorded at two pH, normal vaginal pH (4.31) (Δλ: 4 nm for FCZ, and 20 nm for QCT) and in Infected vaginal pH (7.00) (Δλ: 8 nm for FCZ, and 10 nm for QCT). Scaling factor 10 was kept for whole study. Simulated vaginal fluid, pH 4.31 and pH 7.00 was used for baseline correction.

Analytical balance (Shimadzu AUW220 balance, Japan) was used for weighing all chemicals.

PREPARATION OF STANDARD SOLUTION

Preparation of Stock Solutions and working standard solutions of FCZ and QCT

FCZ and QCT, each of 10 mg were accurately weighed and transferred into 10 ml volumetric flask. Required quantity of methanol was added in both the flask and sonicated for 10 minutes and finally volume was made up to the mark with methanol. The resultant standard stock solutions have FCZ and QCT, each of 1000 µg/ml. 1ml of standard stock solution of FCZ and QCT were separately transferred into 10 ml volumetric flask and volume was made up to the mark with methanol. The resultant working standard solutions have FCZ and QCT, each of 100 µg/ml.

Preparations of Simulated Vaginal Fluid (SVF) buffer, pH 4.31 and 7.00

The normal vaginal pH is acidic (pH 3 to 4.5) but during vaginal infection, pregnancy, and pre-puberty or post-menopause the pH shifts towards somewhat alkaline (pH 7.0 or higher). Simulated Vaginal Fluid (SVF) was

prepared using the method described by Owen and Katz **[12].** The prepared SVF was stored at room temperature at 23°C-25°C in refrigerator. The solution required to be used within one week of preparation **[13, 14]**.

1st Order UV-Spectrophotometric Method development

Selection of Analytical Wavelength and Calibration Curve

Working standard solution of FCZ and QCT, each 100 µg/ml solution was transferred into 10 ml volumetric flask in different aliquot and final dilution of the resultant solution was made up to the mark with SVF, pH 4.31 and pH 7.00 separately. The resultant solutions have concentration of FCZ, 20-60 µg/ml and QCT, 5-15 µg/ml. Zero order spectra of FCZ (20-60µg/ml) and QCT (5-15µg/ml) were scanned in UV-Spectrophotometer between 200-400 nm against SVF, pH 4.31 and pH 7.00 as blank. The obtained zero order spectra were converted into first order derivative spectra (D^1) [Δλ 4 nm for FCZ; Δλ 20 nm for QCT and scaling factor 10 was set up in SVF, pH 4.31 while, Δλ 8 nm for FCZ; Δλ 10 nm for QCT and scaling factor 10 was set up in SVF, pH 7.00]. The detection wavelengths of individual analytes were selected on the basis of ZCP (Zero Crossing Point) of one analyte where other analytes have linear absorbance without interference of each other.

Method Validation

Developed 1st Order UV-Spectrophotometric Method was validated as per ICHQ2(R1) guideline with respect to Linearity and Range, Precision study (Repeatability, Intraday and Interday precision study), Limit of Detection (LOD) and Limit of Quantitation (LOQ) and accuracy study **[15]**.

Analysis of Mucoadhesive In-situ Vaginal Gel Formulation

The drug content of FCZ and QCT was analyzed from developed mucoadhesive In-situ vaginal gel by following procedure. 2 g of newly developed mucoadhesive gel formulation was withdrawn (FCZ 10 mg & QCT 2.5 mg), transferred in to 10 ml volumetric flask and volume was made up to the mark with methanol. The resultant solution was shaken well and sonicated it for 10 minutes which gave FCZ, 1000 µg/ml and QCT, 250 µg/ml. From the resultant solution 0.28, 0.36, 0.44 ml aliquot were withdrawn and transferred into 10 ml volumetric flask and volume was made up to the mark with respective pH of Simulated Vaginal Fluid. The prepared sample was analyzed using developed UV-spectrophotometric method. The sample analysis study was performed in triplicate in both SVF, pH 4.31 and pH 7.00.

RESULTS AND DISCUSSIONS

The UV-spectrum data at pH 4.31 represented that at ZCP of QCT (232.03 nm), FCZ have linear absorbance, therefore,

FCZ can be detected at 232.03 nm, while at detection wavelength 400 nm, QCT can be detected without interference of FCZ **(Figure 3)**.

The UV-Spectrum data at pH 7.00 represented that at ZCP of QCT (268.03 nm) FCZ have linear absorbance, therefore, FCZ can be detected at 268.03 nm, while at detection wavelength 400 nm, QCT can be detected without interference of FCZ **(Figure 4)**.

Method Validation

Linearity and Range

The linearity was assessed by linear regression analysis. The calibration curve was constructed for FCZ (20-60µg/ml) and QCT (5-15µg/ml) in both SVF, pH 4.31 **(Figure 5 and 6, Table 1)** and pH 7.00 **(Figure 7 and 8, Table 1)** and quantification of each analyte were done at respective wavelength of determination as mentioned in method development. The prepared SVF, pH 4.31 and pH 7.00 was used as blank for respective determination of analytes. The calibration curve plotted was found to be linear over the concentration range for both FCZ and QCT within specified concentration range. The correlation coefficient was taken by considering five replicates and linear regression parameters were reported **(Table 2)**.

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

As per ICH guideline, the sensitivity of method was measured in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ). LOD and LOQ of the developed method were calculated from the standard deviation of the response and slope of the calibration curve of each drug using the formulae, limit of detection = 3.3 x σ/S and limit of quantitation = 10 x σ/S, where σ is standard deviation of response and S is the slope of calibration curve. The LOD and LOQ data of FCZ and QCT at SVF, pH 4.31 and pH 7.00 were represented in Table 2.

Precision

Precision of developed first order UV-Spectrophotometric method was determined at two levels, which included repeatability, Intraday and Interday precision.

The % RSD values for repeatability, intraday precision and inter day precision of FCZ and QCT were found to be less than 2 in both SVF, pH 4.31 and pH 7.00 **(Table 3)** indicating that the proposed method has excellent repeatability and reproducibility.

Accuracy Study

The accuracy study of developed 1st order UV-Spectrophotometric method was done using standard addition method in both SVF, pH 4.31 and pH 7.00 using newly developed mucoadhesive gel formulation that comprised of FCZ and QCT in combination. The accuracy study of FCZ and QCT were performed at 50%, 100% and 150% level and mean % recovery for FCZ and QCT

were represented (Table 4). The data represented satisfactory recovery of FCZ and QCT at both, pH 4.31 and pH 7.00 indicated the method is accurate, and reliable.

Analysis of Mucoadhesive In-situ Vaginal Gel Formulation

The developed method was applied to quantify FCZ and QCT from developed mucoadhesive In-situ vaginal gel. The sample analysis study was performed in triplicate in both SVF, pH 4.31 and pH 7.00 **(Table 5)**.

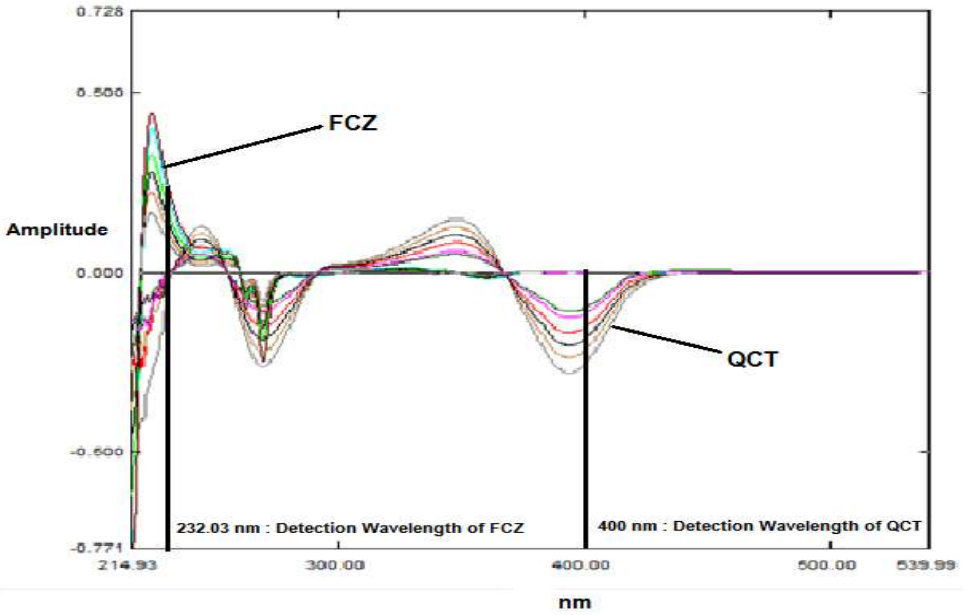

Figure 3: First order Derivative (D^1) overlay Spectra of FCZ (20-60µg/ml) and QCT (5-15µg/ml) (pH 4.31)

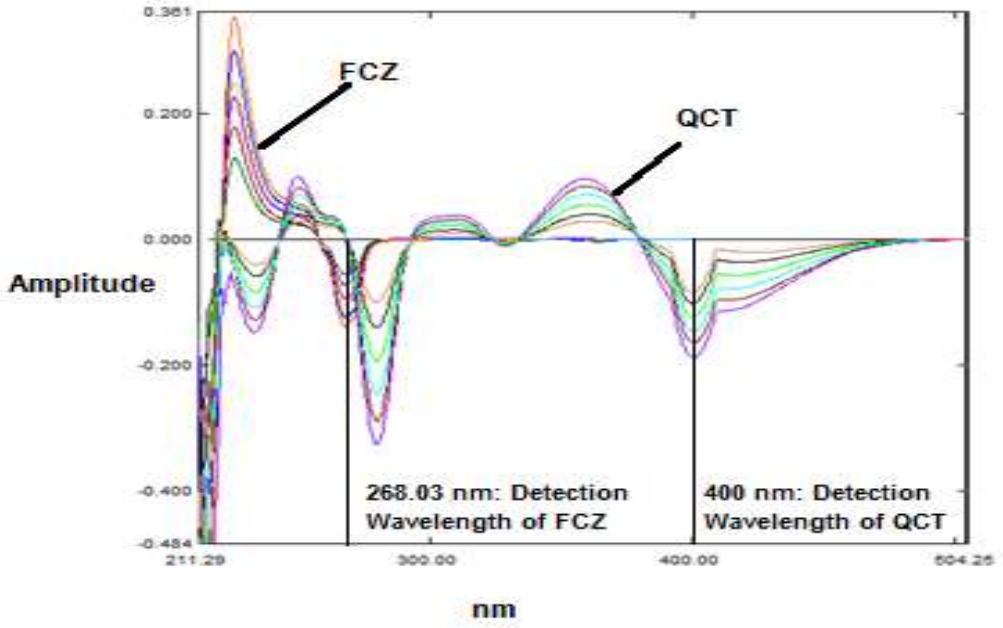

Figure 4: First order Derivative (D^1) overlay Spectra of FCZ (20-60µg/ml) and QCT (5-15µg/ml) (pH 7.00)

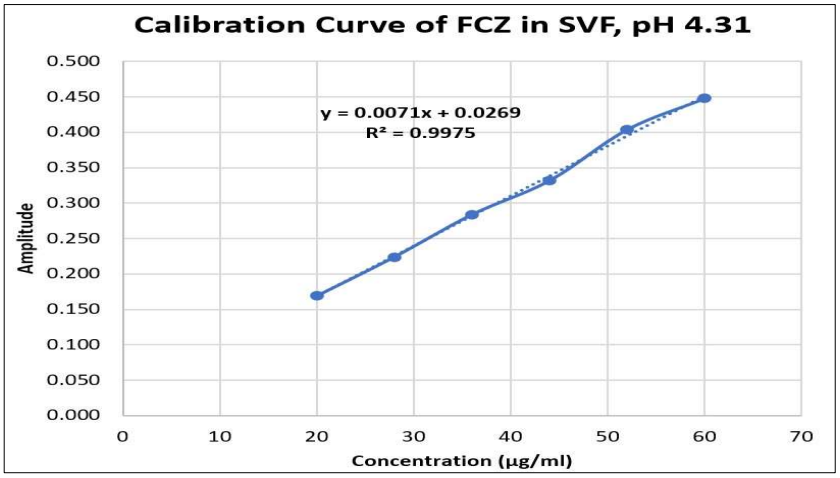

Figure 5: Calibration Curve of FCZ (20-60 µg/ml) in SVF, pH 4.31

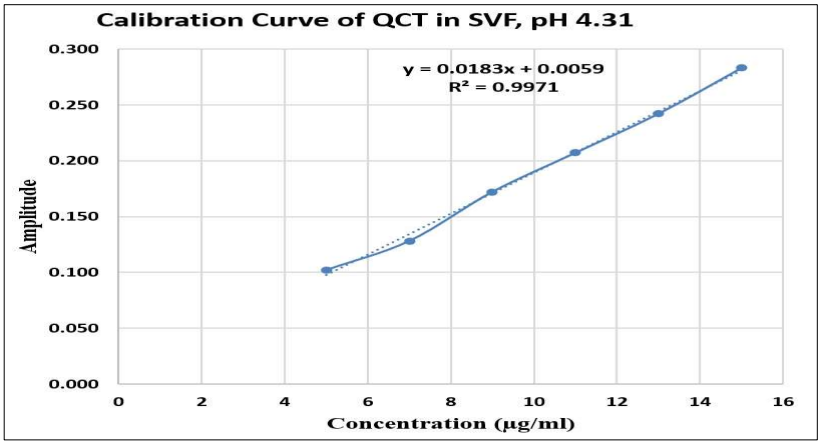

Figure 6: Calibration Curve of QCT (5-15 µg/ml) in SVF, pH 4.31

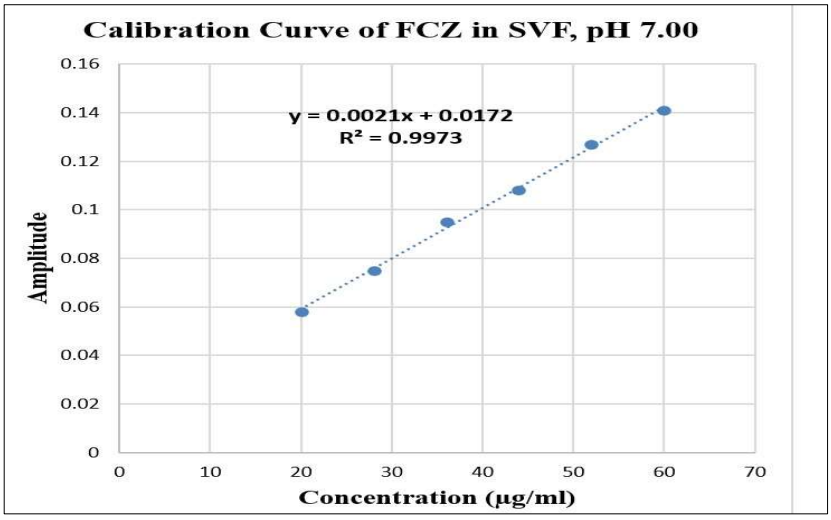

Figure 7: Calibration Curve of FCZ (20-60 µg/ml) in SVF, pH 7.00

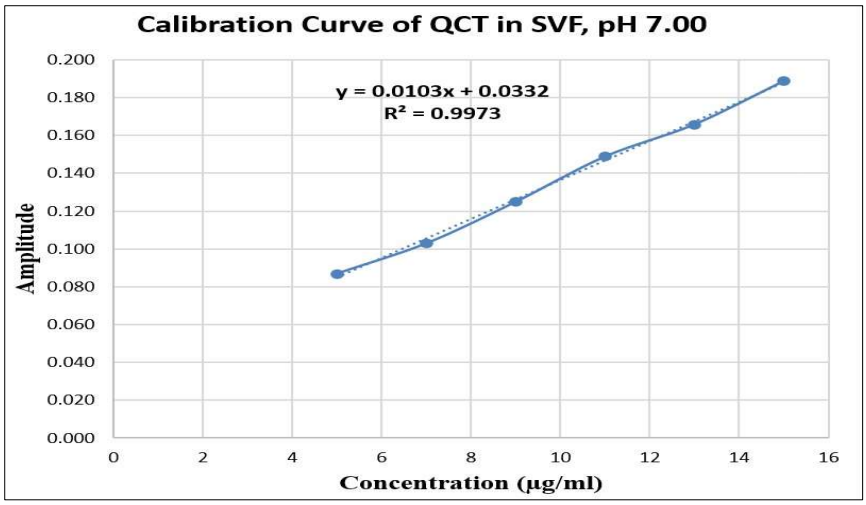

Figure 8: Calibration Curve of QCT (5-15 µg/ml) in SVF, pH 7.00

Table 1: Linearity data for FCZ and QCT in SVF, pH 4.31 and 7.00

FCZ						
Concentration (µg/ml)	SVF, pH 4.31			SVF, pH7.00		
	Average Amplitude*	S.D	%RSD	Average Amplitude*	S.D	%RSD
20	0.169	0.0019	1.1395	0.058	0.0008	1.4376
28	0.224	0.0021	0.9241	0.075	0.0010	1.3333
36	0.284	0.0019	0.6778	0.095	0.0011	1.2053
44	0.332	0.0021	0.6238	0.108	0.0011	1.0596
52	0.404	0.0023	0.5704	0.127	0.0011	0.9006
60	0.448	0.0021	0.4633	0.141	0.0011	0.8063
QCT						
Concentration (µg/ml)	SVF, pH 4.31			SVF, pH 7.00		
	Average Amplitude*	S.D	%RSD	Average Amplitude*	S.D	%RSD
5	0.102	0.0011	1.1135	0.087	0.0011	1.3166
7	0.128	0.0013	1.0202	0.103	0.0011	1.1027
9	0.172	0.0016	0.9193	0.125	0.0013	1.0414
11	0.207	0.0016	0.7638	0.149	0.0011	0.7632
13	0.242	0.0016	0.6534	0.166	0.0011	0.6852
15	0.283	0.0016	0.5587	0.189	0.0008	0.4422

***n= 5, mean of five replicates, SD = standard deviation, %RSD = relative standard deviation**

Table 2: Linear regression parameter for FCZ and QCT in SVF, pH 4.31

Linear Regression Parameter	SVF, pH 4.31		SVF, pH 7.00	
	FCZ	QCT	FCZ	QCT
Calibration Range (µg/ml)	20-60 µg/ml	5-15 µg/ml	20-60 µg/ml	5-15 µg/ml
Regression equation	y = 0.0071x ± 0.0269	y = 0.0183x ± 0.0059	y = 0.0021x ± 0.0172	y = 0.0103x ± 0.0332
Correlation coefficient	0.9975	0.9971	0.9973	0.9973
Slope[a] ± SD	0.0071 ± 0.0001	0.0183 ± 0.0002	0.0021 ± 0.0000	0.0103 ± 0.0001
Intercept[a] ± SD	0.0272 ± 0.0021	0.0061 ± 0.0020	0.0171 ± 0.0016	0.0329 ± 0.0008
LOD (Limit of detection (µg/ml))	0.9611	0.3654	2.5353	0.2554
LOQ (Limit of quantitation (µg/ml))	2.9124	1.1075	7.6828	0.7740

[a]n=5, SD = standard deviation

Table 3: Precision study of FCZ and QCT in SVF, pH 4.31 and 7.00

Conc. (µg/ml)	Inter day Precision of FCZ and QCT at pH 4.31	Inter day Precision of FCZ and QCT at pH 7.00	Intraday Precision of FCZ and QCT at pH 4.31	Intraday Precision of FCZ and QCT at pH 7.00	Repeatability study of FCZ and QCT at pH 4.31	Repeatability study of FCZ and QCT at pH 7.00
	%RSD*	%RSD*	%RSD*	%RSD*	%RSD#	%RSD#
	FCZ					
28	0.6680	1.3889	0.6779	1.3889	-	-
36	0.5385	1.0526	0.5410	1.0870	0.6622	1.0868
44	0.4542	0.9346	0.3003	0.9091	-	-
	QCT					
7	1.2059	1.4456	0.7634	1.4878	-	-
9	0.9449	0.8197	0.5988	0.8065	0.8500	1.1314
11	0.7741	0.6667	0.5000	0.6667	-	-

*n = three replicates, #n = six replicates, SD = standard deviation, %RSD = relative standard deviation

Table 4: Accuracy study for FCZ and QCT in SVF, 4.31 and 7.00

	FCZ				QCT			
% Spike	Mean % Recovery* ±SD	% RSD	Mean % Recovery* ±SD	% RSD	Mean % Recovery* ±SD	% RSD	Mean % Recovery* ±SD	% RSD
	SVF, pH 4.31		SVF, pH 7.00		SVF, pH 4.31		SVF, pH 7.00	
50	99.73 ± 0.7171	0.7191	101.27±1.5857	1.5674	101.11±1.1130	1.1008	99.42± 1.2945	1.3021
100	100.27±0.5379	0.5364	100.95±1.1905	1.1792	100.42±0.8347	0.8312	101.75±0.9709	0.9542
150	99.93± 0.4303	0.4306	100.76±0.9524	0.9452	100.88±0.6678	0.6619	100.82±0.7767	0.7704

*n =3 replicate, S.D = standard deviation, %RSD= relative standard deviation

Table 5: Analysis of formulation of FCZ and QCT in SVF, pH 4.31 and 7.00

Drug	Drug content (% w/w)	Conc. (µg/ml)	Mean % Recovery*	SD	% RSD	Mean % Recovery*	SD	% RSD
			SVF, pH 4.31			SVF, pH 7.00		
FCZ	0.5	28	97.97	0.7684	0.7843	99.43	1.9638	1.9750
		36	99.28	0.4518	0.4550	100.26	1.3228	1.3193
		44	98.30	0.6402	0.6513	99.71	1.6532	1.6580
QCT	0.125	7	98.80	0.9014	0.9123	98.66	0.8008	0.8116
		9	101.54	0.7011	0.6905	99.03	1.0787	1.0893
		11	100.79	0.4968	0.4929	99.85	1.3482	1.3502

*n =3 replicate, %RSD= relative standard deviation

CONCLUSION

The results of method validation demonstrated that the proposed first order derivative (D^1) UV spectrophotometric method is simple, rapid, precise, accurate and sensitive for quantification of FCZ and QCT from newly developed mucoadhesive In-situ Vaginal gel formulation.

The developed method can also be applied to check drug released profile of both FCZ and QCT in SVF, pH 4.31 (normal Vaginal pH) and pH 7.00 (infected vaginal pH).

First order derivative (D^1) UV spectrophotometric method also have its own advantage like, high signal to noise ratio, used in qualitative analysis to identify the presence of two analytes with very similar λmax values that are not resolved in the absorbance spectrum and it always

eliminates baseline shifts and improves the accuracy of quantification.

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