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Development and Validation of RP-HPLC Method for Quantification of Fluconazole in Pharmaceutical Formulations

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Abstract: A rapid and simple reversed phase high performance liquid chromatography method to quantify fluconazole which is a triazole antifungal drug that is available in pharmaceutical oral dosage forms (tablets and suspension) and parenteral intravenous injection. It is used mainly against Candida albicans. The chromatography was carried out using Nova-Pak C18 (3.9 x 150 mm) Column at 260 nm wave length using photodiode array detector at room temperature. Mobile phase consisted of 0.01 M phosphate buffer of pH 7 and acetonitrile in a ratio of 75:25 v/v delivered at a flow rate of 1 ml/min. Retention time was 2 min with short run time of 2.5 min. The method was validated with respect to linearity, precision, accuracy, and specificity. The linearity was established over the concentration range between 1-200 µg/ml. The percentage of drug recovered after analysis for Intra-day was between 92.64% and 108.00% while the inter-day was between 93.17% and 100.25%. The proposed method is easy to apply, it is a fast method with a retention time of only two minutes making it perfect for determining routine analysis with low fluconazole sample concentrations and it is the quickest method ever recorded for fluconazole analysis by HPLC.

Keywords: Fluconazole, HPLC, assay, validation.

التطوير والتحقق من صحة طريقة الفصل الكروماتوجرافي السائل عالي الأداء الطور العكسي لتقدير كمية الفلوكونازول في المستحضرات الصيدلانية

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الملخّص: طريقة سهلة وسريعة للفصل الكروماتوجرافي السائل عالي الأداء الطور العكسي لتقدير كمية الفلوكونازول وهو دواء مضاد للفطريات ثلاثي ازول متوفر في أشكال الدواء عن طريق الفم (أقراص ومعلق) والحقن الوريدي. يعمل بشكل أساسي ضد فطر Candida albicans. تم إجراء الفصل الكروماتوجرافي باستخدام عمود Nova-Pak C18(3.9×150مم) بطول موجة تبلغ 260 نانومتر عند الموجة بطول 360 نانومتر باستخدام كاشف صفيف الثنائي الضوئي في درجة حرارة الغرفة. تألف الطور المتحرك من 0.01 مولار محلول منظم عازل الفوسفات من الأس الهيدروجيني 7 والأسيتونيتريل بنسبة75:25 حجم لكل وحدة حجم بمعدل تدفق 1مل/ دقيقة. كان زمن

الاستبقاء دقيقتين وزمن التشغيل قصير وهو دقيقتان وثلاثون ثانية. تم التحقق من صحة الطريقة فيما يتعلق بالاستقامة الخطية، الدقة، الصحة والانتقائية. تم تعيين الاستقامة الخطية على مدى التركيز بين 1-200 ميكروغرام/ مل. كانت النسبة المئوية للدواء المسترد بعد التحليل في خلال اليوم ما بين 92.64% و 108% في حين كان ما بين الأيام 93.17% و 100.25%. الطريقة المقترحة سهلة التطبيق، وهي طريقة سريعة مع زمن استبقاء مدته دقيقتان فقط، مما يجعلها طريقة مثالية لتحديد التحليل الروتيني بتركيزات منخفضة لعينةالفلوكونازول وهي أسرع طريقة تم تسجيلها لتحليل دواء الفلوكونازول بواسطة الفصل الكروماتوجرافي السائل عالي الأداء.

الكلمات المفتاحية: فلوكونازول، فصل كروماتوجرافي سائل عالي الأداء، تحليل لتقدير كمية، تحقق من صحة.

Introduction

Fluconazole is a triazole antifungal drug that is available in pharmaceutical oral dosage forms (tablets and suspension) and parenteral intravenous injection (Zervos & Meunier, 1993). It was approved by the united states FDA (Food and Drug Administration) at 1990 (Salerno, Carlucci, & Bregni, 2010). It is used mainly against Candida albicans. Chemical structure of fluconazole is shown in Figure 1.

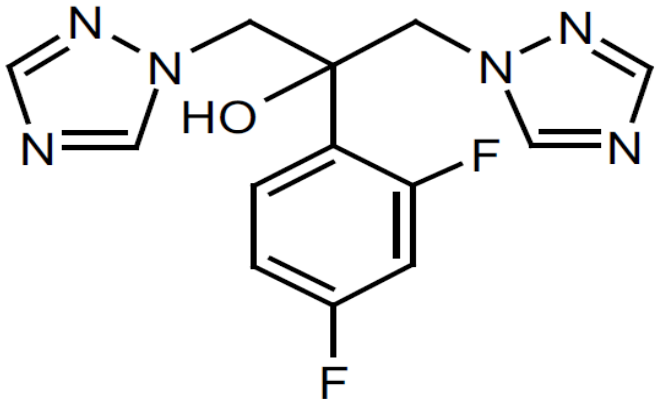

Figure (1) Fluconazole chemical structure (Peyton, Gallagher, & Hashemzadeh, 2015).

Determination of fluconazole has been carried out using many analytical methods such as high performance liquid chromatography-UV (HPLC-UV) method for quantification in human plasma (Liew, Loh, Tan, & Peh, 2012), megabore capillary gas-liquid chromatography with nitrogen-selective detection (Harris, Wallace, Foulds, & Rinaldi, 1989), automated method for determination in serum by column-switching liquid chromatography (Egle, Trittler, & Kümmerer, 2004) and ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS)(Song, Li, Wang, & Yin, 2015). Some of these methods are hard to be applicable and sophisticated and some of them are time consuming.

Reversed-phase high-performance liquid chromatography (RP-HPLC) was first applied in 1949 by Archer Martin a young chemists that joined National Institute for Medical Research in London and it was used for separation of lauric (C_{12}) to stearic (C_{18}) (Ettre, 2001). RP-HPLC works by separating molecules according to their level of hydrophobicity. Stationary phase is made of hydrophobic ligands attached to it that adsorb solute molecules from the aqueous mobile phase. Stationary phase is usually made of silica, mobile phase which is the pressurized liquid is an aqueous mixture usually made of water and methanol

or water and acetonitrile aquoas(Aguilar, 2004), sometimes buffer is added to the mobile phase since it is important to stabilize selectivity and retention by controlling pH in RP-HPLC.

RP-HPLC has many advantages: it gives high resolution under varying range of chromatographic conditions of molecules even those which have close chemical structures, easy to applicate in which mobile phase can be altered to manipulate chromatographic selectivity, high recoveries and productivities, great reproducibility that is due to stability of stationary phase over a wide range of mobile phases (Aguilar, 2004; Snyder, Dolan, & Gant, 1979). Fluconazole can be determined by using spectrophotometric method, but this method is suitable only with high fluconazole concentrations (Göğer & Aboul-Enein, 2001). The aim of this study was to establish a method to analyze fluconazole using a shorter retention time than other published studies. Accordance to that, the chromatographic separation method was optimized with respect to stationary and mobile phase compositions, detection length, flow rate and sample volume.

Experimental

Instrumentation

The HPLC method was performed on a Waters system that is equipped with Photodiode array detector (model 186002998, Waters, Singapore), an Autosampler (model 2707, Waters, Netherlands), a binary pump (model 25P, Waters, Singapore) and Breeze computer software. The chromatographic separation was carried using C_{18} Column3.9 x 150 mm (Nova-Pak, USA).

Reagents

Fluconazole was a gift from Al-Jazeera Pharmaceutical Industries (Riyadh, Saudi Arabia). HPLC-grade methanol was purchased from Fisher Chemical (Fisher Scientific UK, Bishop Meadow Road). High purity water was obtained through a Milli-Q Integral Water Purification System (Millipore, Bedford, MA, USA). Disodium hydrogen orthophosphate was purchased from Scharlau (Scharlau S.L. Gato Perez, Spain). Phosphoric acid 80% was purchased from Merck (E. Merck, D-6100 Darmstadt, F.R. Germany). HPLC-grade acetonitrile was purchased from Panerac (Panerac Quimica S.L.U, Spain).

Methods

Chromatographic conditions

Separation was achieved by a simple RP-HPLC using a mobile phase that consisted of 0.01 M phosphate buffer of pH 7 and acetonitrile in a ratio of 75:25 v/v. The pH of the buffer was adjusted using phosphoric acid 80% which was further diluted to 10% to control the change of pH. Mobile phase was freshly prepared each day and was filtered through 0.2 µm filter with the aid of a vacuum pump and then

it was degassed using a sonicator for 30 min to ensure removing of air bubbles. The flow rate was 1 ml/min. The injection volume was 10 µl. The photodiode array detector was set at a wavelength of 260 nm. The HPLC analyses were all done at ambient temperature.

Preparation of standard solutions

Fluconazole was dissolved in methanol HPLC-grade. Stock solution was prepared freshly every day of analysis by dissolving 10 mg of fluconazole in 50 ml of methanol which yield a stock solution concentration of 200 µg/ml. Working standard solution was also prepared freshly every day by taking 5 ml of stock solution via pipette and diluting it into 10 ml volumetric flask with methanol to obtain a concentration of 100 µg/ml.

Linearity

Appropriate volume of FLZ stock solution (200 µg/ml) and FLZ working standard solution (100 µg/ml) were further diluted to produce non-zero standard drug concentrations that covers a calibration range from 1 to 200 µg/ml. The calibration solutions were injected in ascending order in each run of the validation. Linear regression equation and correlation coefficient (R2) were used to evaluate statically the linearity of the results (Al-Hadiya, Khady, & Mostafa, 2010; Ali, Ghori, & Khatri, 2006).

Accuracy and precision

Intra-day accuracy and precision evaluations were applied on six replicates determinations of FLZ standards within one day. Also inter-day accuracy and precision were evaluated on six replicates analysis of low, medium and high concentrations on three consecutive days. The precision of both intra-day and inter-day methods was expressed as relative standard deviation and the accuracy of intra-day and inter-day methods was expressed in terms of% drug recovered (Al-Hadiya et al., 2010).

Specificity

It is the ability of a specific method to unequivocally assess the analyte in the existence of other components that might be present in the sample for example other ingredients, excipients or impurities. Specificity is used to decide if the study is suitable enough to analyze the samples of this particular study. A blank solution that has the same components of the formulation except for the drug was injected and analyzed to be compared with the standard solution that has been also injected.

Lower limit of detection (LOD) and lower limit of quantification (LOQ)

The LOD is the lowest concentration of analyte in a sample that can be detected by the developed method but not reliable in quantification of the sample. The LOQ is the lowest concentration of analyte in a sample that can be quantified reliably, with an acceptable accuracy and precision. There are three

methods for determining the LOD and LOQ; by visual evaluation, calculating signal to noise ratio (LOD should be equal to or more than three times the noise peak, while LOQ should be equal to or more than 10 times the noise peak) or by standard deviation of response and slope, for LOD this formula should be applied LOD = 3.3 σ / S and for LOQ this formula should be applied LOQ = 10 σ / S, where S = slope of calibration curve and σ = standard deviation of the response, and this can be one of three, standard deviation of blank response or residual standard deviation of regression line or standard deviation of Y-intercept of the regression line S Y/X, i.e. standard error of estimate (Shrivastava & Gupta, 2011).

Calculations

Accuracy was calculated as the percentage (%) of the drug recovered after analysis relative to the corresponding nominal concentrations; Accuracy% = (mean / actual amount) × 100. Precision% = (standard deviation (SD) / mean) × 100.

Results and discussion

Optimization of the chromatographic conditions

In order to affect proper resolution of all the components, the mixture of methanol or acetonitrile with water and different buffer ratios were assayed as the mobile phase using Nova-Pak C18 Column 3.9 x 150 mm as the stationary phase. Water with methanol produced no peaks, while water with acetonitrile produced tiny peaks with small sensitivity. Binary mixture of 0.01 M phosphate buffer and acetonitrile proved to be the best for separation because the chromatograms were well-defined. Various ratios of mobile phase were tested of buffer:acetonitrile (70:30, 75:25, 80:20 and 85:15) and was noticed that as the buffer increased, the peak becomes nearer to the solvent front peak; among these ratios, 75:25 v/v was the best. Among different pH of the buffer tried (ph 3-8) ph 7 produced the best peak shape with almost no tailing. Flow rate used was 1 mL/min, A 260 nm wavelength was selected as it gave reasonably high absorption. Under the described conditions, fluconazole was well resolved with a retention time of 2 minutes and required a runtime of maximal 2.5 minutes. This is the quickest method ever recorded for FLZ analysis by HPLC and is even more rapid than UPLC methods used for FLZ determinations in which the retention time was at 4.1 min (Yanamandra et al., 2010). A chromatogram of fluconazole is shown in Figure 2.

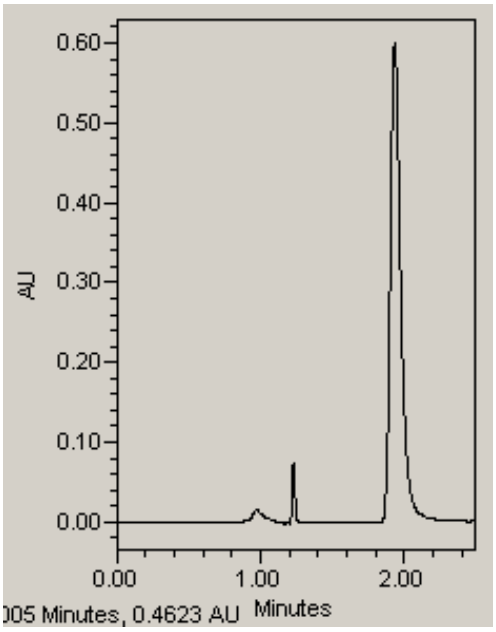

Figure (2) Fluconazole HPLC chromatogram of 100 µg/mL of fluconazole (retention time, 2 minutes).

Assay Validation

The HPLC method was validated in terms of linearity, specificity, precision and accuracy, limit of detection (LOD) and limit of quantification (LOQ) in respect with the FDA guide lines of bioanalytical method validation (FDA, 2018).

Linearity

The Calibration curve that resulted from data obtained from FLZ absorption was linear over the concentration range between 1-200 µg/ml. Calibration curve was linear with a square of correlation coefficient (R2) = 0.9999.

Accuracy

Intra-day accuracy (Table 1) is between 92.64% and 108.00% while the inter-day accuracy (Table 2) is between 93.17% and 100.25%. These vales of the percent recovered reflect the accuracy of the assay method and match with the acceptance criteria for FDA guidelines (FDA, 2018).

Precision

The results of precision of intra-day are presented in Table 1 while the results of precision of inter-day are presented in Table 2. This method used in the study found to be precise since the intra-day standard deviation values (SD) of five replicate analysis at the concentration range of 1 – 200 µg/ml, varied from 0.03 to 0.14 µg/ml with the coefficient variation (CV; precision) or sometimes called relative standard deviation (RSD) ranging from 0.03% to 2.54%. While the inter-day SD values of six replicates determinations at the concentration range of 1 – 200 µg/ml in three consecutive days were between 0.07

– 1.99 µg/ml with CV of the range 0.15% – 11.35%. This is within the limit stated in the FDA guidelines (FDA, 2018).

Specificity

There was no interference between the analyte to be studied and the excipients in the formulation. A blank solution that has the same components of the formulation except for the drug was injected and analyzed to be compared with the standard solution that has been also injected. The obtained chromatogram showed no peaks near to FLZ peak, which suggest that the method is specific for fluconazole.

LOD and LOQ

LOD was determined using the signal to noise ratio method, which was 0.25 µg /ml that is 3 times the base line noise, while LOQ was 0.5 µg /ml which was higher than 10 times the noise peaks, and respecting that the LOQ must be the lowest analyte concentration of the drug that we are able to measure with acceptable accuracy and precision.

Conclusion

This HPLC analysis method is found to be reproducible, reliable and specific for FLZ drug in pure and pharmaceutical formulations. It is easy to apply, and more importantly it is a fast method that is perfect for determining routine analysis with low FLZ sample concentrations so it is a time safer. It is considered to be very economic because it consumes only a few amount of the mobile phase which also make it environment friendly and it is safer for the researcher's health than other HPLC methods in that the researcher will take less time in the process of analysis because of the short retention time so less contact with the chemicals is needed since these chemicals are volatile and harmful. This method has shown acceptable accuracy, precision and sensitivity to be used in further studies.

Table (1) Intra-day back-calculated fluconazole concentrations with accuracy and precision of FLZ samples in methanol.

Back calculated concentrations (µg/ml)									
Concentration (µg/ml)	1st	2nd	3rd	4th	5th	Mean	SD	%Precision	%Accuracy
1	1.10	1.10	1.05	1.05	1.10	1.08	0.03	2.78	108.00
5	4.62	4.69	4.60	4.63	4.64	4.63	0.03	0.65	92.60
10	10.60	10.67	10.46	10.36	10.35	10.49	0.14	1.33	104.90
50	49.60	49.80	49.70	49.60	49.70	49.68	0.08	0.16	99.36
100	96.77	96.80	96.55	96.50	96.70	96.67	0.13	0.13	96.67
200	201.60	201.57	201.70	201.56	201.64	201.64	0.06	0.03	100.82

Table (2) Inter-day back-calculated FLZ concentrations with accuracy and precision of FLZ samples in methanol.

Day of analysis	Concentrations (µg/ml)					
	1	5	10	50	100	200
Day 1	1.03	4.62	10.60	49.61	96.77	201.60
	1.02	4.69	10.67	49.53	96.80	201.57
	1.05	4.60	10.46	49.69	96.55	201.70
	1.05	4.61	10.36	49.62	96.50	201.56
	1.04	4.64	10.35	49.66	96.70	201.64
	1.02	4.70	10.31	49.79	96.80	201.66
Day 2	0.94	4.48	9.44	49.63	100.86	199.50
	0.94	4.46	9.43	49.67	100.86	199.50
	0.93	4.67	9.84	49.82	102.27	198.89
	0.97	5.05	9.49	49.71	100.41	199.82
	0.94	4.41	9.46	49.61	101.00	199.41
	0.94	4.72	9.88	49.63	101.78	199.12
Day 3	0.75	4.60	9.24	49.76	99.66	200.22
	0.67	4.52	9.84	49.64	100.10	199.99
	0.90	4.82	9.65	49.62	97.93	201.05
	0.88	4.70	9.59	49.74	98.46	200.80
	0.90	4.96	10.72	49.68	98.59	200.68
	0.80	4.90	10.18	49.76	99.53	200.22
Mean	0.93	4.68	9.97	49.68	98.98	200.50
SD	0.11	0.17	0.49	0.07	1.99	0.99
Precision	11.83	3.63	4.91	0.14	2.01	0.49
Accuracy	93.00	93.60	99.70	99.36	98.98	100.25

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