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A spectrophotometric method for the quantification of clotrimazole from polymeric nanoparticles to *Candida albicans* vaginal infections treatment

Método espectrofotométrico para la cuantificación de clotrimazol a partir de nanopartículas poliméricas para el tratamiento de infecciones vaginales originadas por *Candida albicans*

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Abstract

In this study, a spectrophotometric method was developed in order to quantify CLT from polymeric nanoparticles of poly(lactic-glycolic acid) (PLGA) modified on the surface with chitosan (CTS) for vaginal administration in the treatment of vaginitis. The parameters of specificity, linearity, repeatability, quantification and detection limits were evaluated. The proposed dissolution medium was Simulated Vaginal Fluid solution pH= 4.2 with Sodium Lauryl Sulfate 0.5% (p/v). The wavelength used for CLT quantification was 265 nm. The results obtained meet the acceptance criteria specified in the Analytical Method Validation Guide (García et al., 2002). In addition, the spectrophotometric method developed allowed us to determine that the percentage of CLT encapsulated in the nanoparticles was 85.64% (w/w). Finally, it is concluded that the analytical method developed is reliable, low cost and easy to perform to quantify CLT from polymeric nanoparticles of PLGA and CTS.

Analytic validation, polymeric nanoparticles, clotrimazole

Resumen

En el presente estudio se desarrolló un método espectrofotométrico para cuantificar clotrimazol (CLT) a partir de nanopartículas poliméricas de ácido poli(láctico-glicólico) (PLGA) modificadas en la superficie con quitosán (CTS) para el tratamiento de vaginitis candidiásica. Se evaluaron los parámetros de especificidad, linealidad, repetibilidad, límites de cuantificación y detección. El medio de disolución propuesto fue Fluido Vaginal Simulado pH= 4.2 con Lauril Sulfato de Sodio 0.5% (p/v). La longitud de onda utilizada para la cuantificación de CLT fue de 265 nm. Los resultados obtenidos cumplen con los criterios de aceptación especificados en la Guía de Validación de Métodos Analíticos (García y col., 2002). Además, el método espectrofotométrico desarrollado nos permitió determinar que el porcentaje de CLT encapsulado en las nanopartículas fue de 85.64 % (p/p). Finalmente, se concluye que el método analítico desarrollado es confiable, de bajo costo y fácil ejecución para cuantificar CLT a partir de nanopartículas poliméricas de PLGA y CTS.

Validación analítica, nanopartículas poliméricas, clotrimazol

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Introduction

Vaginal candidiasis is one of the most frequently diagnosed vaginal infections (Roby, 2019). In Mexico, infections originating from *Candida spp*. account for 15-19% of reported vaginal infections. The species identified in cases of candidiasis vaginitis are: *C. albicans* (39.0%), C. glabrata (35.9%) and *C. tropicalis* (16.2%) (Rivera-Sánchez *et al*., 2006). Recurrent vaginitis caused by *C. albicans* is characterized by occurrence at least 4 times a year.

The most common conventional treatment for vaginal candidiasis involves the use of drugs of the azole family (clotrimazole, fluconazole, butoconazole, miconazole, ticonazole, itraconazole, ketoconazole), as well as nystatin and amphotericin B (Carbone, 2019). However, many of the pharmaceutical forms of vaginal administration can cause leakage and generate discomfort during use. This can lead to early discontinuation of treatment and failure to achieve therapeutic efficacy, in addition to promoting resistance of microorganisms.

Clotrimazole shows 85% efficacy against C. albicans with doses higher than 100 mg per day (Cararach Tur et al., 2013). However, its use for long periods of time can cause vaginal irritation and/or pain (Young and Jewell, 2001). The mechanism of action of clotrimazole is to inhibit the microsomal cytochrome P450 (CYP450)-dependent demethylation of 14-α-lanosterol; this causes an alteration of membrane permeability and fluidity, resulting in a decrease in the activity of membrane-bound enzymes and cell wall synthesis and, finally, in the leakage of cellular contents (Hitchcock et al., 1990).

Unconventional formulations with antimicrobial agents have gained interest as an alternative to overcome drug resistance developed by microorganisms, such as *C. albicans*. In particular, nanocarriers have demonstrated efficacy in treating infectious diseases, including antibiotic-resistant ones (Huh and Kwon, 2011).

Nanotransporters have advantages such as 1) they can be designed to be activated by stimuli and targeted at a specific site in the body, 2) by decreasing the dose of drugs in nanotransporter formulations, the presence of side effects can be avoided, 3) drug delivery via nanotransporters can achieve controlled release by improving their pharmacokinetics, 4) better solubility of poorly soluble drugs can be achieved by also increasing their permeability (Pelgrift and Friedman, 2013). According to das Neves et al. (2015), the use of drug nanocarriers for vaginal delivery may allow adhesion to cervical mucus or achieve mucosal layer penetration and/or achieve intracellular delivery.

Given the growing interest in the development of new pharmaceutical dosage forms involving nanocarriers, the development of analytical methods for the quantification of drugs encapsulated in these nanosystems becomes a major area of interest.

The validation of analytical methods guarantees the quality of a product, because, through the standardization of the methodology it is experimentally demonstrated that a process is reliable to reproduce the result under the established conditions (Cardoso-San Jorge et al., 2022).

Nowadays, there is a low publication of research works reporting the development of spectrophotometric analytical methods for the quantification of substances included in nanocarriers. Therefore, in the present investigation, we report the development of a clotrimazole quantification method to determine the percentage of this drug encapsulated in polymeric nanoparticles of PLGA and CTS.

Materials and Methods

Materials

Poly(lactic-coglycolic acid) (PLGA) 50:50 (P.M. 30,000-60,000) and chitosan (CTS) (50,000-190,000) were purchased from Sigma Aldrich Química, S. de RL. de CV (Mexico). Polyvinyl alcohol (PVAL) (Mowiol® 40-88, P.M. 205,000, was purchased from Glomarza (Mexico). Clotrimazole (CLT) (99% purity) was donated by Globe Chemicals, S.A. de C.V. (Mexico). Ethyl acetate was purchased from Fermont® (Mexico).

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Equipment

The following were used: UV-Vis spectrophotometer (Thermo Scientific, Genesys 10uv Scanning, USA), IKA T 25 digital ULTRA-TURRAX homogenizer, Nanosizer® Coulter N4 plus (Beckman 3200, USA), Atomic Force Microscope (Bruker Instruments, USA).

Experimental Methods

Preparation of PLGA and CLT nanoparticles, surface functionalized with CTS (PLGA-CLT-CTS-NPs).

PLGA-CLT-CTS-NPs, were prepared by the emulsification-diffusion method (Martinez-Perez et al., 2018). Briefly, an organic phase was prepared by dissolving 100 mg of PLGA and 20 mg of CLT in 20 ml of ethyl acetate saturated with water. Then, under stirring at 6000 rpm, the organic phase was added to 40 ml of aqueous phase (0.5% w/v PVAL dissolved in water saturated with ethyl acetate), the system was kept under the same stirring conditions for 10 minutes. Subsequently, 160 ml of deionized water was added to the system. The organic solvent was evaporated under reduced pressure. The system was centrifuged at 20000 rpm for 20 minutes, the process was performed twice to remove excess PVAL. The nanoparticles were redispersed with 40 ml of an acetic solution of CTS with concentration 0.5 mg/ml. The system was kept under magnetic stirring for 24 hours and then centrifuged and lyophilized.

Analytical Method Development

The development of the analytical method was carried out considering the acceptance criteria of the Guide for the Validation of Analytical Methods of the National College of Pharmaceutical Chemists and Biologists of Mexico, A. C. (García et al., 2002).

Specificity

Solutions of CLT (0.173 mg/ml), PLGA (1 mg/ml), CTS (0.5 mg/ml) and PVAL (0.5 mg/ml) were prepared in Simulated Vaginal Fluid (SVF) pH=4.2 (Owen and Katz, 1999) and sodium lauryl sulfate (LSS) at 0.5% w/v). Specificity was evaluated by comparing UV-Vis spectrograms of drug solutions and each of the chemical components of the analytical system individually and as a physical mixture.

The specific method is considered if the response obtained (absorbance) is attributed only to the analyte (CLT).

Linearity

A stock solution of concentration 0.5 mg/ml of CLT was prepared. From the above solution, 1:10, 3:10, 5:10, 7:10, and 9:10 systems were prepared in triplicate and taken to the volumetric titration with SVF in 0.5% w/v LSS. Subsequently, their absorbance was determined at a λmax of 265 nm. Linearity was evaluated by obtaining the r2 parameter of the calibration curve described above. The method is considered linear if the r2 value is ≥ 0.98, the intercept equal to 0 and coefficient of variation (%C.V.) ≤ 3 %.

Accuracy of the method

A stock solution of concentration 0.65 mg/ml of CLT was prepared. 1:10, 3:10, 5:10, 7:10 and 9:10 systems from this solution were prepared in triplicate and brought to the volumetric titer using SVF with 0.5% w/v LSS. The absorbance of each system was determined at λ=265 nm. Accuracy was determined with 6 replicates of three CLT concentration levels. It was evaluated whether the percent recovery values were in the range: 98 % $\leq x \geq$ 102 % and % C.V. ≤ 3 %.

Repeatability of the method

A stock solution of 0.65 mg/ml concentration of CLT was prepared. Systems 3:10, 4:10 and 5:10 were prepared in triplicate and taken to the volumetric titration with SVF in 0.5% w/v LSS. The procedure was performed on three days by the same analyst. It was analyzed if the % C.V. ≤ 3%.

Limits of quantification and detection of the method

From a 0.10 mg/ml stock solution of CLT, 1:10, 3:10, 5:10, 7:10, 9:10 systems were prepared in triplicate. It was analyzed if r2 is ≥ 0.98, the intercept is equal to 0 and the coefficient of variation (% C.V.) is ≤ 3 %.

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Sample preparation to determine the % CLT loaded on PLGA-CLT-CTS-NPs

Three samples of 5-10 mg of nanoparticles were hydrolyzed with 3 ml of 1.3 M HCl for 48 hours. Samples were neutralized with 2.5 M NaOH, brought to 10 ml SVF with 0.5% w/v LSS and kept on shaking for 4 hours. The systems were filtered with a 0.22 µm membrane. The absorbance of the systems was determined at a λ=265 nm. The amount of drug loaded was obtained from Formula 1.

$$\% F.C. = \frac{C.F.NPs}{C.F.I} * 100 \quad (1)$$

Where F.C., is the amount of drug loaded on the nanoparticles; C.F.NPs, is the amount of drug loaded on the nanoparticles; C.F.I., is the amount of initial drug.

Results and discussion

Preparation of PLGA and CLT nanoparticles functionalized on the surface with CTS (PLGA-CLT-CTS-NPs)

The emulsification-diffusion method, allowed the formation of PLGA-CLT-CTS-NPs with polydispersity indices of 0.07-0.17. The average particle size of the nanoparticles was 424.0 ± 4.90 nm. The average surface charge was +12.89 ± 4.09 mV. The particle size and surface charge parameters are factors that determine their transport through the cervical mucus (das Neves and Sarmento, 2015). The positive surface charge of PLGA-CLT-CTS-NPs enhances their interaction with cervical mucus mucins (negative charge at acidic pH), improving their bioadhesive properties (da Silva et al., 2016). The spherical shape of the nanoparticles is evident in the images taken by Atomic Force Microscopy (Figure 1).

Development of the analytical method.

Specificity

Specificity is defined as the capacity of an analytical method to obtain a response due only to the analyte of interest and not to other components of the sample (García et al., 2002).

In this study, the specificity of the method to quantify CLT was determined by comparing the absorption spectra of CLT, PLGA and PVAL (main components of the NPs-PLGA-CLT-CTS system) in simulated vaginal fluid pH= 4.2 in a wavelength range from 200 to 400 nm.

As can be seen in Graph 1, at a wavelength of 265 nm, CLT can be quantified without interference from any of the other components of the system (PLGA, CTS and PVAL), which shows that the method is specific for CLT.

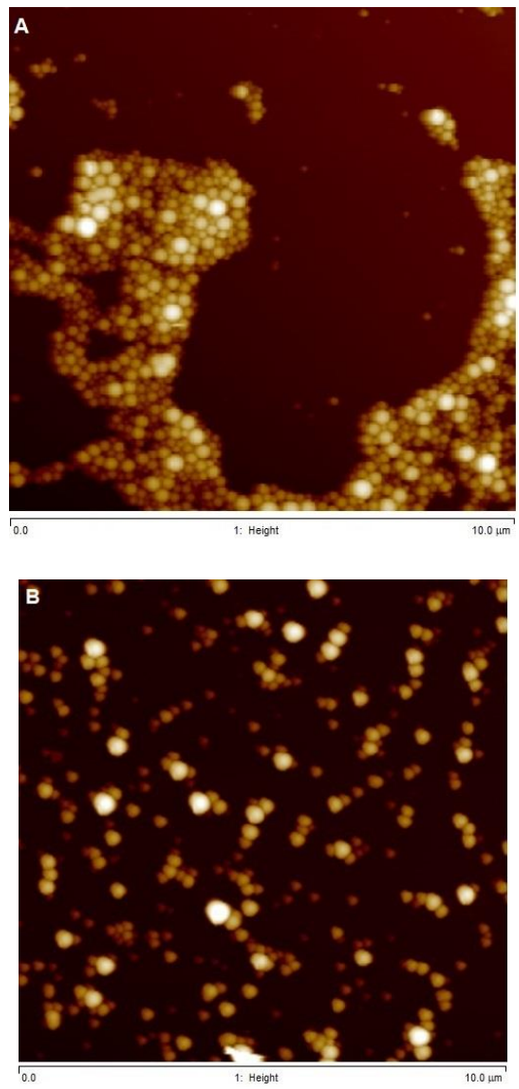

Figure 1 Atomic Force Micrographs. A) PLGA-CLT-NPs y B) PLGA-CLT-CTS-NPs

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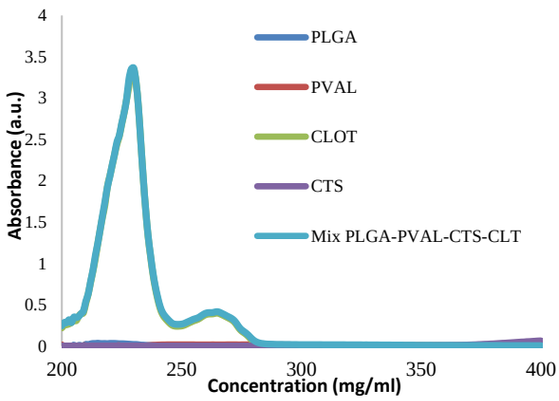
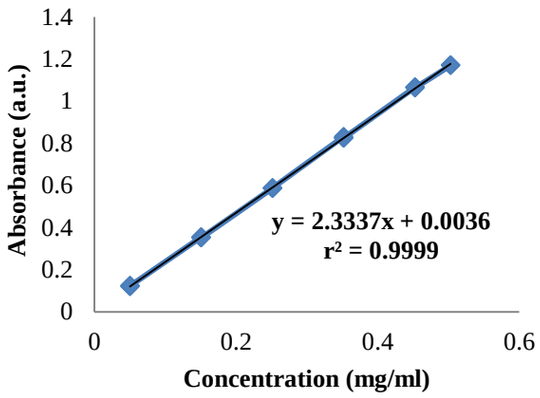


Graphic 1 CLT specificity evaluated in SVF pH=4.2 and LSS 0.5% (w/v)



Graphic 2 Calibration curve for linearity evaluation of the spectrophotometric method UV-vis for the quantification of CLT at a wavelength of maximum absorption (λ) of 265 nm in SVF pH=4.2 with LSS at 0.5% (w/v)

Linearity

The linearity of an analytical method indicates its ability to ensure that its results are directly proportional to the concentration of the analyte (Garcia et al., 2002). In the present study, linearity was evaluated through the calibration curve with a concentration range of 50 to 500 µg CLT (Graphic 2). According to the Analytical Methods Validation Guide, a method is linear when the coefficient of determination is greater than 0.98; in this case, the value of the coefficient of determination (r^2) obtained was 0.99, a value that confirms the relationship of absorbance as a function of CLT concentration.

According to the Analysis of Variance study of the linear regression (Table 1), it is observed that, Fcal >Fcrit, for a probability of 95%; it is demonstrated that, the regression analysis does provide a good fit to the data. Table 1 shows that the confidence interval (CI) of the intercept (b0) is 0, and the confidence interval of the slope (CIb1) is not 0.

	dl	*S.S.*	*M.S.*	*F*	*Sig. F.*	
Regression	1	2.26	2.26	12.22E4	9.37E-31	
Residual	15	2.0E-4	1.85E-5			
Total	16	2.26				
	Coeff.	***S. Error.***	***t Stat***	***P-value***	***Low. 95%***	***Upp. 95%***
Intercept (b_0)	3.6E-3	2.1E-3	1.6599	0.12	-1E-3	8.1E-3
Slope X1 (b_1)	2.34	6.7E-3	349.69	9.37E-31	2.32	2.35
Note: dl degrees of freedom; S.S., Sum of Squares; M.S., Mean Squares; Sig. *F*., Significance *F; Coeff., Coefficients; S. Error, Standard Error; Low., Lower; Upp., Upper*						

Table 1 Analysis of Variance (p=0.05) to assess the linearity of the CLT quantification method

Method accuracy

The accuracy of a method indicates the degree of agreement between a value obtained using the method and the reference value (Garcia et al., 2002). Table 2 shows the experimental results obtained to evaluate the accuracy. The range of ICµ values of the experimental data is from 98.99 to 99.39 (p=0.05). The recovery percentages shown in table 3 are within the specified limits (97-103%), therefore, it can be stated that the method is accurate.

Concentration (mg/ml)	% Recovery
0.19	99.32
	99.99
	99.32
	99.77
	99.99
	98.87
0.25	98.78
	99.28
	99.12
	99.45
	99.12
	98.78
0.31	98.99
	98.73
	98.86
	98.99
	99.13
	98.99
Average	99.19
Standard Deviation	**0.39**
% C.V.	**0.34**

Table 2 Experimental values to evaluate the accuracy of the spectrophotometric method to quantify CLT in SVF pH=4.2 and LSS 0.5% (w/v)

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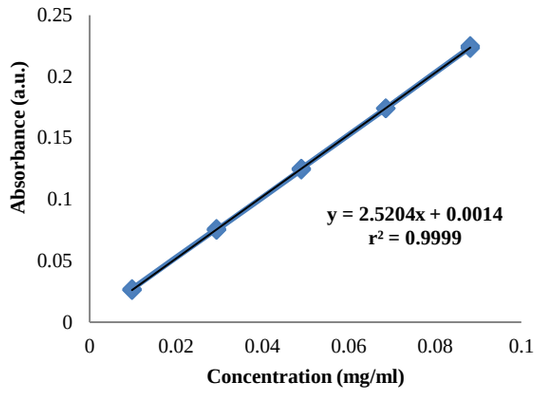
Method repeatability

The repeatability of a method demonstrates the precision of an analytical method expressed as the agreement obtained between determinations performed by a single analyst using the same method (Garcia et al., 2002). In this case, repeatability was evaluated with a single analyst with the values of % recovery evaluated at three concentration levels obtained on three different days (Table 3). The ICμ of the experimental data is from 100.15 to 101.08, therefore, it can be mentioned that the method is repeatable.

On the other hand, the values of the limits of quantification and detection obtained by the method are 5.42 μg /ml and 1.79 μg/ml, respectively. The Analysis of Variance study (Table 4) of the regression analysis to determine the limit of quantification (Graph 3), indicates that, Fcal >Fcrit, with a probability of 95%, it is pointed out that the regression analysis does provide a good fit to the data. In addition, the confidence interval (CI) of the intercept (b0) has values close to 0 and the values of the confidence interval of the slope (ICb1) are different from 0.

Day	Concentration (mg/ml)	% Recoery
1	0.1887	99.32
	0.1887	99.99
	0.1887	99.77
	0.2516	98.78
	0.2516	99.12
	0.2516	99.45
	0.3145	98.73
	0.3145	98.99
	0.3145	99.13
2	0.1866	101.65
	0.1866	101.89
	0.1866	102.37
	0.2488	101.26
	0.2488	100.21
	0.2488	100.35
	0.3110	100.61
	0.3110	100.19
	0.3110	99.91
3	0.1881	102.46
	0.1881	101.99
	0.1881	101.52
	0.2508	101.72
	0.2508	101.54
	0.2508	101.54
	0.3135	101.14
	0.3135	101.14
	0.3135	101.69
	Average	**100.61**
	Standard Deviation	**1.18**
	% C.V.	**1.17**

Table 3 Experimental values to evaluate the repeatability of the spectrophotometric method to quantify CLT in SVF pH=4.2 with LSS at 0.5% (w/v)



Graphic 3 Calibration curve for evaluation of the CLT quantification limit by spectrophotometric method UV-vis, at a wavelength of maximum absorption (λ) of 265 nm in SVF pH=4.2 and LSS at 0.5% (w/v)

	dl	*S.S*	*M.S.*	*F*	*Sig.F.*	
Regression	1	7.3E-2	7.3E-2	19.29E4	1.67E-2	
Residual	13	4.93E-6	3.8E-07			
Total	14	7.3E-2				
	Coeff.	***S. Error.***	***t Stat***	***P-value***	***Low. 95%***	***Upp. 95%***
Intercept (b_0)	1.3E-3	3.0E-3	4.2305	9.82E-3	7.0E-4	2.1E-3
Slope X1 (b_1)	2.52	5.8E-3	439.23	1.67E-28	2.51	2.53
Note: dl degrees of freedom; S.S., Sum of Squares; M.S., Mean Squares; Sig. F., Significance F; Coeff., Coefficients; S. Error, Standard Error; Low., Lower; Upp., Upper						

Table 4 Analysis of Variance (p=0.05) to evaluate the CLT quantification limit

Sample preparation to determine the % of CLT loaded in PLGA-CLT-CTS-NPs

From the development of the analytical method, the amount of CLT loaded on the polymeric nanoparticles was determined. In PLGA-CLT-NPs, the percentage of loaded CLT was 72.48% and in PLGA-CLT-CTS-NPs it was 85.64%. When comparing the percentages of loaded CLT in nanoparticles with CTS and without CTS, a higher amount of CLT was observed in nanoparticles with CTS, this could be explained because the CTS adsorbed on the surface of PLGA nanoparticles, prevents the CLT from leaving the nanoparticles during the centrifugation process.

Conclusions

A method for quantification of CLT contained in PLGA polymeric nanoparticles functionalized with CTS was developed, meeting the acceptance criteria specified by the Guide for Validation of Analytical Methods proposed by the National College of Pharmaceutical Chemists and Biologists of Mexico. In addition, the development of the quantification method allowed us to reliably quantify the amount of CLT loaded in the polymeric nanoparticles (PLGA-CLT-NPs and PLGA-CLT-CTS-NPs) with percentages of 72.48% and 85.64%, respectively.

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Finally, the method being spectrophotometric is easy to reproduce and low cost compared to other analytical methods such as chromatographic methods.

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