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Validation of quantitative analysis method for triamcinolone in ternary complexes by UV-Vis spectrophotometry

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ABSTRACT

Triamcinolone (TRI), a drug widely used in the treatment of ocular inflammatory diseases, is practically insoluble in water, which limits its use in eye drops. Cyclodextrins (CDs) have been used to increase the solubility or dissolution rate of drugs. The purpose of the present study was to validate a UV-Vis spectrophotometric method for quantitative analysis of TRI in inclusion complexes with beta-cyclodextrin (B-CD) associated with triethanolamine (TEA) (ternary complex). The proposed analytical method was validated with respect to the parameters established by the Brazilian regulatory National Agency of Sanitary Monitoring (ANVISA). The analytical measurements of absorbance were made at 242nm, at room temperature, in a 1-cm path-length cuvette. The precision and accuracy studies were performed at five concentration levels (4, 8, 12, 18 and 20µg.mL^{-1}). The B-CD associated with TEA did not provoke any alteration in the photochemical behavior of TRI. The results for the measured analytical parameters showed the success of the method. The standard curve was linear (r2 > 0.999) in the concentration range from 2 to 24 µg.mL^{-1}. The method achieved good precision levels in the inter-day (relative standard deviation-RSD <3.4%) and reproducibility (RSD <3.8%) tests. The accuracy was about 80% and the pH changes introduced in the robustness study did not reveal any relevant interference at any of the studied concentrations. The experimental results demonstrate a simple, rapid and affordable UV-Vis spectrophotometric method that could be applied to the quantitation of TRI in this ternary complex.

Keywords: Validation. Triamcinolone. Beta-cyclodextrin. UV- Vis spectrophotometry. Ternary complexes.

INTRODUCTION

Triamcinolone (TRI) (Figure 1) is a steroidal anti-inflammatory drug that has been used with success for the treatment of ocular diseases. However, repeated intravitreal or subconjunctival applications are necessary to maintain effective concentrations of the drug in the local tissues. Moreover, repeated injections are very traumatic for the patients and may have serious adverse effects, such as hemorrhages, eye infections and damage to the lens or retina (Paganelli et al., 2004; Cardillo et al., 2005; Magalhães et al., 2006; Paganelli et al., 2009).

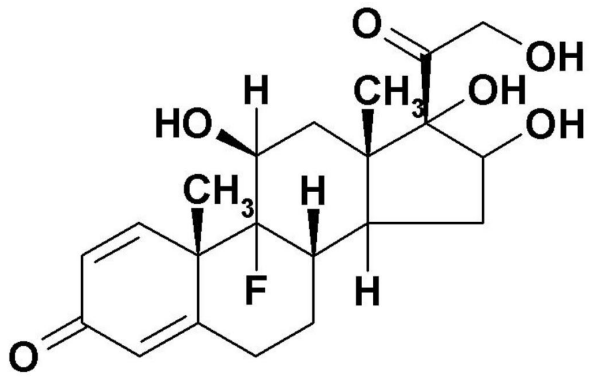

Figure 1. Two-dimensional structure of triamcinolone molecule.

The absence of eye drops containing TRI is due mainly to its low aqueous solubility, which hampers this therapeutic use. Cyclodextrins (CDs) are cyclic oligosaccharides with hydroxyl groups on the outer surface and a cavity in the center. There are three naturally-occurring

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CDs (α, β and γ) and this paper concerns the *beta* form (B-CD). Its outer surface is hydrophilic, but the cavity has a lipophilic character and can host hydrophobic compounds. The cyclodextrin inclusion complexes thus formed may alter some physicochemical properties of drugs, such as stability, solubility and consequently bioavailability (Szejtli, 1990; Giuseppe et al., 1998; Uekama et al., 1998; Hedges, 1998; Granero et al., 2008).

The use of cyclodextrin derivatives, but not specifically B-CD, as drug permeation agents, or to modify the drug release profile from delivery systems containing triamcinolone acetonide, it is described in the literature (Villar-López et al., 1999; Másson et al., 1999; Siemoneit et al., 2006; Kear et al., 2008; Tongiani et al., 2009). Shinoda and co-workers study the stability of inclusion complexes of triamcinolone and B-CD in the blood (Shinoda et al., 1999).

Recently, we have been studying the use of B-CDs as solubilizing agents and ways to enhance their effect, and interesting results were obtained by associating B-CD with triethanolamine (TEA). The increase in the aqueous solubility of TRI was about 19-fold with B-CD alone and 38-fold when it was associated with TEA (Aquino et al., 2010). However, the drug interaction with CDs and multicomponents may cause some interference in the photochemical behavior of the drug and consequently impair the quantitative analysis of the drug by UV-Vis spectrophotometry, making it necessary to use more complex techniques (Thomas et al., 1998; Ragno et al., 2003; Orgoványi et al., 2005; De León-Rodríguez & Basuil-Tobias, 2005; Granero & Longhi, 2010).

Methods that enable the quantitative analysis of drugs in delivery systems or inclusion complexes are very important for the real evaluation of drug interaction with components and crucial to obtaining valid results in stability or solubility studies (Silva-Júnior et al., 2006). Moreover, such techniques should be reliable, providing correct and reproducible results, in order to achieve the experimental goal. Thus, the validation of the analytical method is a necessary procedure, involving the experimental determination of analytical parameters, in order to guarantee the analytical results (ICH, 1995a; 1995b; Brasil, 2003). The main purpose of this study was to validate a simple, rapid, specific, precise and accurate UV-Vis spectrophotometric method, which may be used for the analytical determination of TRI in an inclusion complex with B-CD and TEA (ternary complex). The study follows the procedures for validation of analytical methods established by the Brazilian regulatory National Agency of Sanitary Monitoring (Brasil, 2003).

MATERIAL AND METHODS

Chemicals

Triamcinolone (TRI) was purchased from Sigma Co, Saint Louis, USA; beta-cyclodextrin (B-CD) from Cyclodex®, USA, and triethanolamine (TEA) from Synth®, Brazil. Double-distilled and deionized water were used throughout. All other chemicals and reagents were of analytical grade.

Methods

Equipment and instrumental conditions

The equipment used consisted of two UV-visible spectrophotometers: Libra S32 from Biochrom® (Cambridge, United Kingdom) and UV-1650PC from Shimadzu® (Tokyo, Japan). All absorbance measurements were taken in a 1-cm path-length cuvette at room temperature, at wavelengths between 200 and 400nm.

Ternary complex preparation

Equimolar amounts of TRI and B-CD were dissolved in an ethanol:water mixture (1:1v/v) and TEA (10% w/w in relation to amount of drug + B-CD) was added. This solution was evaporated in a Büchi B-191 mini Spray Dryer equipped with a 0.7mm nozzle, at a spray feed-rate about 1-3 $ml.min^{-1}$, inlet air temperature of 120ºC, outlet air temperature of 80 ºC. The solid ternary complex powder was collected and stored under vacuum at room temperature for 48 hours.

Specificity

This parameter was determined by comparing the analytical plots of absorbance, scanned in the range 200-350nm, of a matrix solution (0.125% TEA+0.001mM B-CD) and the TRI standard solution ($12\mu g.mL^{-1}$).

Standard curve

A TRI stock solution of $50\mu gmL^{-1}$ was prepared in water (with 4% ethanol as co-solvent). Aliquots of this solution were transferred to volumetric flasks and the volume completed with water, to prepare solutions with different concentrations ($2\text{-}24\mu g.mL^{-1}$). A $12\mu g.mL^{-1}$ solution was scanned from 200 to 400nm, to find the best wavelength for TRI quantifications.

Linearity and Range

The linearity was determined from the correlation coefficient for the (straight line) standard curve fitted to the TRI analytical data in the chosen concentration range ($2\text{-}24\mu g.mL^{-1}$).

Inter-day precision

The inter-day precision was estimated by calculating the relative standard deviations (RSDs) of the analyses of TRI solutions at five different concentrations (4, 8, 12, 18 and $20\mu g.mL^{-1}$), carried out on 5 different days, at intervals of at least two days, with the same spectrophotometer equipment.

Inter-laboratory precision (Reproducibility)

The reproducibility of the method was assessed from the RSD values obtained from analyses of TRI solutions (n=3) at five different concentrations (4, 8, 12, 18 and $20\mu g.mL^{-1}$), in two different laboratories. In this study, a Biochrom® Libra S32 UV-Vis spectrophotometer was used in 'Laboratory A' and a Shimadzu® 1650PC UV-Vis spectrophotometer in 'Laboratory B'.

Accuracy

The accuracy was investigated by the standard addition method, in which solutions containing the components of the matrix (0.001M B-CD + 0.125% TEA) were added to various amounts of standard TRI solution to obtain five different drug concentrations (4, 8, 12, 18 and 20µg.mL^{-1}). Accuracy was calculated as the mean of 5 tests at each level (n=5), from the relationship:

$$Accuracy = \left[\frac{\text{mean experimental concentration}}{\text{theoretical concentration}} \right] \quad (1)$$

Apparent robustness

In this paper, the influence on precision of several analytical parameters, such as different days, different instruments and laboratories, has been described in previous sections. Additionally, the effect of the pH of the analytical solution on the accuracy and precision of the method was investigated. To this end, the pH of the analytical solution was measured (pH= 6.2) and adjusted to lower and higher levels (pH= 5.2 and pH= 7.2) with 50mM phosphate (KH_2PO_4) buffered solutions. The analytical determinations were performed at five different concentrations (4, 8, 12, 18 and 20µg.mL^{-1}) for each pH.

Statistics

The RSD was calculated as RSD= 100·(sd/mean). All results obtained from the robustness, precision and accuracy studies were initially subjected to one-way analysis of variance (ANOVA). When F values were significant, data were further analyzed by *post-hoc* tests (Newman-Keuls) for comparisons in the accuracy and precision studies and (Dunnett´s test) for the robustness test.

RESULTS

In order to observe the maximum absorbance wavelength for TRI in aqueous solution and to estimate the interference of the matrix components with the UV-Vis spectrophotometric readings for TRI, a specificity study was carried out. Figure 2 shows the UV-Vis absorbance scans, from 200 to 350nm, for solutions of TRI and the matrix components.

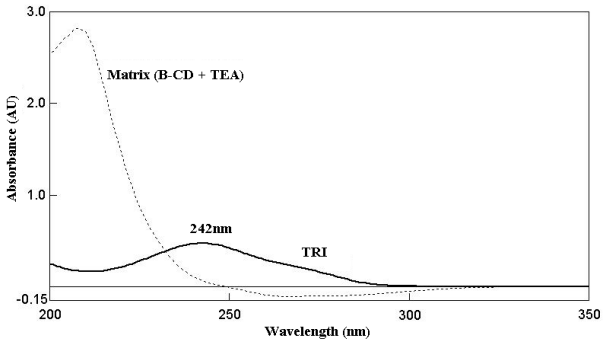

Figure 2. UV-Vis absorbance profiles of TRI solution (12µg.mL^{-1}) and matrix solution containing β-CD (0.001M) + TEA (0.125%).

A standard curve of TRI solutions at various concentrations (2-24µg.mL^{-1}) was obtained for quantitative analysis of TRI (Figure 3).

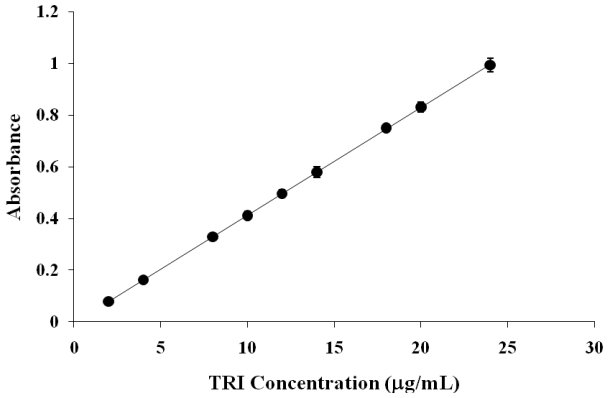

Figure 3. Standard curve of TRI solutions obtained by UV-Vis spectrophotometry at 242nm (n=5).

The equation from fitted plot of the experimental results in Figure 3 was *y= 0.04175x – 0.00473*. The data used to build the standard curve were subjected to statistical analysis (Table 1).

Table 1. Analysis of variance (ANOVA) for linearity of the method.

Parameter	Degree of freedom	Squared sum	Mean squares	F	P
Regression	1	0.750	0.750	18652.030	<0.001
Residual	7	0.0000281	0.00000402		
Total	8	0.750	0.0937		

The experimental results from the inter-day precision test are shown in Table 2. Inter-day precision was determined by calculating the RSD, at each concentration (4, 8, 12, 18 and 20µg.mL^{-1}), of replicate assays performed on 5 different days, separated by intervals of at least two days, the acceptable value of the RSD is < 5% (Brasil, 2003).

Table 2. Experimental results from the inter-day precision test (n=5).

Concentration (µg.mL^{-1})	Analytical Concentration (µg.mL^{-1})					Mean + SD	RSD (%)*
	1stday	2stday	3stday	4stday	5stday		
4	3.97	3.85	4.11	3.99	4.07	4.00 + 0.10	2.52
8	7.90	7.59	8.21	8.23	8.02	7.99 + 0.26	3.31
12	11.97	11.32	12.21	12.28	12.13	11.98 + 0.39	3.23
18	18.01	17.60	18.24	18.36	18.26	18.10 + 0.31	1.70
20	19.92	19.27	20.42	20.28	20.21	20.02 + 0.46	2.28

* RSD (Relative standard deviation) = 100. (SD/Mean)

Besides the inter-day precision, an inter-laboratory precision (reproducibility) test was performed. The experimental results of this test are shown in Table 3.

Accuracy can be determined from at least nine analytical determinations at three different concentrations

within the linear range of the standard curve. For this purpose, at least one low, one medium and one high concentration may be used in triplicate analyses. In fact, the accuracy study was performed on five replicates at five analytical concentrations of TRI in the presence of the matrix components (B-CD + TEA). The average recoveries identified in the accuracy test are shown in Table 4.

Table 3. Experimental results from the reproducibility test (n=3).

Concentration (μg.mL^{-1})	Analytical concentration (μg.mL^{-1})		Mean + SD	RSD (%)
	Laboratory A	Laboratory B		
4	4.00 + 0.01	4.00 + 0.01	4.00 + 0.00	0.00
8	8.00 + 0.01	7.60 + 0.02	7.80 + 0.29	3.62
12	12.00 + 0.02	11.41 + 0.02	11.71 + 0.42	3.56
18	18.10 + 0.01	17.17 + 0.03*	17.63 + 0.66	3.73
20	20.02 + 0.02	19.13 + 0.03*	19.58 + 0.63	3.21

* p<0.05 vs Laboratory A

Table 4. Experimental results obtained from the accuracy test (n=5).

Theoretical concentration (μg.mL^{-1})	Analytical concentration + SD (μg.mL^{-1})	Accuracy + SD (%)
4	3.15 + 0.00	78.88 ± 0.00
8	6.29 + 0.12	79.16 ± 1.78
12	9.44 + 0.01	78.66 ± 0.11
18	14.43 + 0.05	80.34 ± 0.23
20	15.83 + 0.08	79.41 ± 0.35

The robustness consists of the capacity of the method to resist being affected by alteration of some analytical parameter, such as pH, ionic strength or temperature. In the present study, the influence of pH (pH= 5.2, 6.2 and 7.2) on the quantitative analysis of TRI was investigated at five concentrations, by assessing the accuracy and precision at these pH values. Table 5 shows the experimental results of the robustness study.

Table 5. Experimental results from the robustness study (n=3)

Theoretical [ ] μg.mL^{-1}	Analytical concentration (μg.mL^{-1}+ SD)			RSD (%)
	pH= 5.2	pH= 6.2	pH= 7.2	
4	4.41 + 0.10*	4.06 + 0.06	4.30 + 0.05*	4.22
8	8.12 + 0.09	8.15 + 0.06	8.08 + 0.07	0.46
12	12.00 + 0.11	12.21 + 0.07	12.05 + 0.23	0.93
18	17.69 + 0.04*	18.30 + 0.06	17.80 + 0.03*	1.80
20	19.57 + 0.08*	20.30 + 0.11	19.50 + 0.10*	2.24

* p<0.05 vs pH= 6.2

DISCUSSION

The specificity of an analytical method describes its capacity to measure the drug in the presence of impurities, excipients, degradation products or matrix components (ICH, 1995a; 1995b; Brasil, 2003). Figure 2 shows the selectivity and specificity study. The plot of absorbance against wavelength for the matrix solution (0.001M β-CD + 0.125% TEA) reveals some interference in the TRI analysis in the range from 200 to 250nm. In order to reduce this interference, the matrix solution was used as a blank in the accuracy study.

A linear correlation was obtained between absorbance and TRI concentration in the range of 2 to 24μg.mL^{-1}. The linearity of the method may be demonstrated by the very high correlation coefficient (r=0.99998) obtained for the fit of the triamcinolone standard curve to the analytical data over the range of drug concentration used in this study. The data used to generate the standard curve were subjected to statistical analysis (Table 1) and passed in the normality test (p>0.05) and the variance test for the intercept (p=0.012), confirming the linearity of the method within the concentration range tested.

Thus, the linearity of the analytical method was tested over a specified range of drug concentrations. This range demarcates the upper and lower limits for analytical determinations that show precision, accuracy and adequate linearity (ICH, 1995a; 1995b; Brasil, 2003). Figure 3 shows the fitted curve and the standard deviations for 5 independent sets of data. The correlation coefficient (r=0.99989) indicates good linearity in the working range (2-24μg.mL^{-1}) chosen.

These values are within the limits established by ANVISA, the Brazilian regulatory National Sanitary Monitoring Agency (r^2>0.99) (Brasil, 2003). Thus, the UV-Vis spectrophotometry method can be considered suitably linear, in the concentration range (2-24 μg.mL^{-1}), for quantitative analysis of TRI under the described experimental conditions.

Precision is an important analytical parameter that represents the variability in the results in a repeated serial analysis of the sample under identical experimental conditions. The experimental results obtained from the inter-day precision study (Table 2) showed very similar RSD values, the mean RSD being 2.608%, which was not statistically different (p>0.05) from any of the values of RSD calculated for the different concentrations tested. For TRI content, the highest precision (RSD= 1.71%) was identified for the concentration of 18mg.mL^{-1}. Therefore, the UV-Vis spectrophotometric method can be considered precise for the quantitative analysis of TRI in ternary complexes, since the RSD calculated for the inter-day precision tests was less than 3.4% for all concentration levels tested.

The inter-laboratory precision is a measure of the effects of changes in the operating conditions, such as the use of different equipment, and their interference with the availability of the method can easily be identified. As expected, this study confirmed the results obtained in the inter-day precision study. The results revealed that changing the spectrophotometer and laboratory were incapable of provoking any relevant difference. Five different concentration levels were tested. The experimental measurements obtained from two different instruments and laboratories (Laboratory A and Laboratory B) revealed a statistical difference (p<0.05) only at the two highest concentrations (18 and 20μg.mL^{-1}). However, the RSD values for all the concentrations studied (RSD<3.8%) were lower than the limits (RSD<5%) established by ANVISA (Brasil, 2003), which shows that the statistical difference found at the two highest concentrations tested may be disregarded. These results confirm the precision of the proposed analytical method.

The accuracy of a method of analysis can be defined as the percent recovery of a known amount of drug added to a sample. This sample may be a pharmaceutical dosage form or only the placebo (excipient mixture). Moreover, the accuracy describes the degree of veracity of the quantitative analysis (ICH, 1995a; 1995b; Brasil, 2003). For pharmaceutical products, the established limits for reasonable recovery are 80-120%.

The values of accuracy observed in this study (Table 4) were not statistically different ($p>0.05$) between different concentrations. All values identified were about 80%. These results confirmed that there was some interference by the matrix components in the apparent TRI recovery, as already demonstrated in the specificity study (Figure 2). This phenomenon can be explained by the absorbance of the matrix components, in the range from 200-250nm, and the wavelength chosen (242nm) for the quantitative analysis of the drug. However, these components did not provoke any alteration in the photochemical behavior of the drug and this wavelength (242nm) was thus maintained as the absorption maximum for TRI in the presence of TEA and B-CD, although the recovery values identified at all concentration levels are close to the lower sanctioned limit of 80% (Brasil, 2003).

In the robustness study (Table 5), a wide range of pH was used to test this parameter, in view of the ionization that might be provoked by addition of a third component to the inclusion complex with TRI and B-CD. The pH identified for TRI aqueous solution was 6.2 and the accuracy was adequate at all concentrations investigated at the various pHs. At two concentrations (8 and 12 $\mu g.mL^{-1}$), there were no statistical differences ($p>0.05$) between assays (vs pH=6.2). However, the differences identified at the other concentrations tested were statistically different ($p<0.05$). Nevertheless, the RSD values calculated from the analyses performed at the three pH were less than 4.5%, demonstrating the precision of this test and the irrelevance of the statistical differences found in the analyses at 4, 14 and 18$\mu g.mL^{-1}$. These results show that the alteration of this parameter (pH) was incapable of changing the accuracy and precision results for the UV-Vis spectrophotometric method.

The present study demonstrates a properly conducted validation study of a UV-visible spectrophotometric method. The experimental results show that this method can be considered suitable for the quantitative analysis of TRI in ternary complexes (TRI/B-CD/TEA). The B-CD and TEA in the complex did not provoke any photochemical alteration in the TRI. Furthermore, the paper describes a simple and rapid method with good linearity, precision and accuracy over the whole range of concentration tested. All parameters stipulated by ANVISA were investigated and the data suggest a good and suitable analytical method that may be safely employed in the quantitative analysis of TRI in this multicomponent complex system.

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RESUMO

Validação de método de análise quantitativa para a triancinolona a partir de complexo ternário por espectrofotometria de UV-Vis

A triancinolona (TRI) é um fármaco amplamente utilizado no tratamento de doenças inflamatórias do globo ocular e é praticamente insolúvel em água, o que limita sua utilização na forma de colírio. As ciclodextrinas (CDs) têm sido utilizadas com sucesso para aumentar a solubilidade ou velocidade de dissolução de fármacos. O presente estudo teve como objetivo a validação de uma metodologia analítica para a TRI a partir de complexos de inclusão com beta-ciclodextrina (B-CD) associada com a trietanolamina (TEA) (complexo ternário) por espectrofotometria de UV-Vis. A validação do método proposto foi realizada de acordo com os parâmetros analíticos estabelecidos pela Agência Nacional de Vigilância Sanitária (ANVISA). As análises quantitativas foram realizadas a 242nm a temperatura ambiente, utilizando cubeta de quartzo de 1cm. Os estudos de precisão e exatidão foram realizados para cinco níveis de concentração (4, 8, 12, 18 e 20$\mu g.mL^{-1}$). A B-CD associada a TEA não alterou o comportamento fotoquímico da TRI. Os resultados da avaliação dos parâmetros analíticos demonstraram o sucesso da metodologia. A curva padrão apresentou linearidade ($r^2 > 0.999$) na faixa de concentração de 2 a 24$\mu g.mL^{-1}$. A metodologia apresentou bons níveis de precisão para o estudo inter dia (desvio padrão relativo-DPR <3.4%) e reprodutibilidade (DPR<3.8%). A exatidão ficou em torno de 80% e a variação de pH inserida no estudo de robustez não revelou uma interferência significativa em todas as concentrações estudadas. Os resultados experimentais demonstraram um simples, rápido e viável método de espectrofotometria de UV-Vis com aplicabilidade para a análise quantitativa da TRI a partir do complexo ternário.

Palavras-chave: Validação. Triancinolona. Beta-ciclodextrina. Espectrofotometria de UV- Vis. Complexo ternário.

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