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Development and validation of HPLC method for quantitative analysis of triamcinolone in biodegradable microparticles.

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ABSTRACT

A simple, rapid, selective and specific high performance liquid chromatographic (HPLC) method for quantitative analysis of the triamcinolone in polylactide-co-glycolide acid (PLGA) microparticles was developed. The chromatographic parameters were reversed-phase C18 column, 250mm x 4.6mm, with particle size 5 μm. The column oven was thermostated at 35 ºC ± 2 ºC. The mobile phase was methanol/water 45:55 (v/v) and elution was isocratic at a flow-rate of 1mL.mL^{-1}. The determinations were performed using a UV-Vis detector at 239 nm. The injected sample volume was 10 μL. The standard curve was linear (r^2 > 0.999) in the concentration range 100-2500 ng.mL^{-1}. The method showed adequate precision, with a relative standard deviation (RSD) was smaller than 3%. The accuracy was analyzed by adding a standard drug and good recovery values were obtained for all drug concentrations used. The method showed specificity and selectivity with linearity in the working range and good precision and accuracy, making it very suitable for quantitation of triamcinolone in PLGA microparticles.

Keywords: triamcinolone; HPLC analytical method; PLGA microparticles; analytical method validation.

INTRODUCTION

Triamcinolone (9-fluoro-11β , 16α ,17,21-tetrahydropregna-1,4-diene-3,20-dione) (TR), Figure 1, is a steroidal anti-inflammatory drug usually administered by the parenteral route. Recently, this anti-inflammatory compound has been administered intravitreally route for the treatment of chronic inflammation of the posterior segment of the eye (Florian et al., 2004; Cardillo et al., 2005).

Microparticles have been used as a drug delivery system to control the release of several groups of drugs including the corticosteroids (Faisant et al., 2003; Gavini et al., 2004; Martinez-Sancho et al., 2004; Silva-Júnior, 2005). One purpose of this application is to obtain a depot system for ocular application and prolong the effect of the drug (Silva-Junior, 2005).

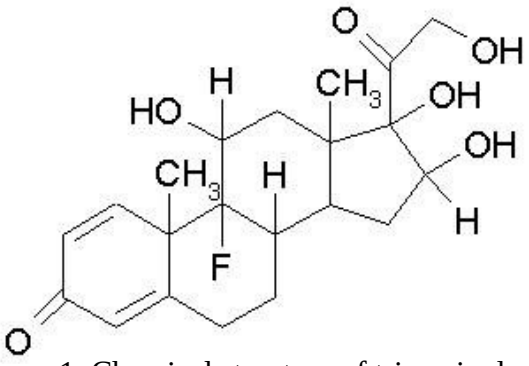

Figure 1. Chemical structure of triamcinolone

Poly (D,L-lactide-co-glycolide) (PLGA) is a copolymer of lactic and glycolic acid widely used in drug delivery systems, as it combines both biodegradability and biocompatibility (Anderson & Shive, 1997; Jain et. al., 1998; Uhrich et al., 1999; Kunou et al., 2000; Silva-Junior, 2005) (Figure 2).

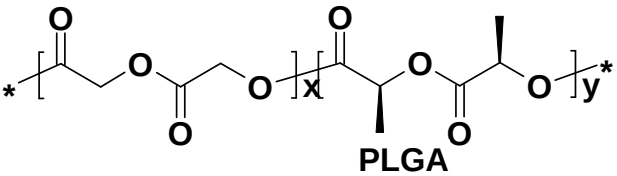

Figure 2. Schematic chemical structure of PLGA.

Analytical methodology for quantitative analysis of drugs in delivery systems is very important for quality assurance in drug administration. On the other hand the methodology used should be reliable, providing correct and reproducible results, in order to achieve the wished therapeutic effect.

Thus, the validation of the analytical method is a necessary procedure and involves experimental studies of analytical parameters, in order to guarantee the analytical

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results (ICH, 1996; Brittain, 1998; British Pharmacopoeia, 2001; ANVISA, 2003; USP, 2004). Some important parameters such as specificity and selectivity, linearity, accuracy and precision must be verified (ICH, 1996; British Pharmacopoeia, 2001; ANVISA, 2003; USP, 2004).

The main purpose of this study was to develop a simple, rapid, specific, precise and accurate HPLC method for analytical determination of triamcinolone encapsulated in PLGA microparticles. To this end, the procedures for validation of analytical methods established by National Agency of Sanitary Monitoring (ANVISA, 2003) were followed.

MATERIAL AND METHODS

Materials

Triamcinolone was purchased from Sigma Co, Saint Louis, USA; PLGA was purchased from Birmingham polymers, USA; methanol HPLC grade from J.T.Baker, Mexico. Double distilled and deionized water were used throghout. All other chemicals and reagents used were of analytical grade.

Methods

Instrumentation and chromatographic conditions

The HPLC system included a Shimadzu LC-9A HPLC pump, Shimadzu CTO-6A column oven, UV-Vis SPD-6AV detector connected to a Shimadzu SCL-6B chromatographic control system and a Shimadzu C-R6A chromatographic integrator. The analytical column was a Merck® LiChrospher 100RP-18, 250mm x 4.6mm and particle size 5 µm. The column oven was maintained at 35ºC ± 2ºC and the mobile phase was methanol/water 45:55 (v/v). The flow-rate was isocratic at 1mL.min.$^{-1}$ and the injected sample volume was 10 µL.

Standard curve

A stock solution of 1 mg.mL^{-1} was prepared in methanol/water 45:55 (v/v). An aliquot of 300 µL was transferred to a volumetric flask and the volume completed to 5 mL with methanol, to obtain 60 µg.mL^{-1}. This solution was diluted with mobile phase, methanol/water 45:55 (v/v), in order to obtain concentrations of 100, 250, 500, 1000, 1250, 1500, 2000 and 2500 ng.mL^{-1}. To obtain the analytical curve, 10 µL of each of the solution was injected in the rheodyne valve of the HPLC system. The UV-Vis detector was set at 239 nm. The area under the curve (AUC) for each peak was plotted versus triamcinolone concentration. A straight line standard curve was obtained by linear regression of 3 independent sets of experimental data.

Triamcinolone-loaded PLGA microparticles

A suitable amount of triamcinolone and PLGA polymer were dissolved in acetone to obtain drug-polymer ratios of 1:1; 1:2; 1:3 and 1:5 (w/w). These solutions were spray dried in a mini spray dryer Büchi-191 with a 0.7 mm nozzle, using a feed rate of 4-6 mL.min^{-1}; aspirator efficiency of 50%; spray flow of 450 NL.h^{-1}; inlet of 70 ºC and outlet temperature of 44-46 ºC.

Sample preparation

The amount of microparticles equivalent to 1mg.mL^{-1} of triamcinolone was dissolved in acetone. An aliquot of 300 µL was transferred to a volumetric flask and the volume completed to 5 mL with methanol to obtain 60 µg.mL^{-1}. This solution was diluted with sufficient methanol/water 45:55 (v/v) to obtain a theoretical concentration of 1250 ng.mL^{-1} of triamcinolone. Samples of standard triamcinolone and empty PLGA microparticles were prepared by the same experimental procedure.

Specificity and selectivity

These parameters were determined by comparing the chromatograms of the triamcinolone standard, drug-loaded PLGA microparticles and empty PLGA microparticles.

Linearity

The linearity was analyzed by calculating the correlation coefficient for the (straight line) analytical curve of triamcinolone.

Precision

In this study, the intra and inter-day precision were analyzed. The intra-day precision was investigated by the relative standard deviation (RSD) of 10 analytical determinations of the middle concentration tested (1200 ng.mL^{-1}) of the triamcinolone standard solution and the sample solution. The inter-day precision was evaluated from analyses (n = 3) of samples at different concentration levels (low, medium and high) of 600 ng.mL^{-1}, 1200 ng.mL^{-1} and 2400 ng.mL^{-1} respectively, where the analyses were carried out at least two days apart.

Limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated from the mean of the standard deviation (msd) of various analytical sample responses divided by slope (s) of the standard curve (Equations 1 and 2). For the LOD, three samples at concentrations near to the smallest concentration of the standard curve (triplicate) were analyzed in order to obtain the standard deviations and to calculate the msd. For LOQ the same procedure was performed (ICH, 1996; ANVISA, 2003; USP, 2004;).

$$LOD = \frac{3.(msd)}{s} \quad (1)$$

$$LOQ = \frac{10.(msd)}{s} \quad (2)$$

Accuracy

The accuracy was investigated by the standard addition method, where samples of the microparticles without drug were added to various amounts of standard triamcinolone solution to obtain various drug concentration levels (low, medium and high). The accuracy values were calculated from the relationship:

$$\text{Accurancy} = \frac{\text{Mean experimental concentration}}{\text{Theoretical concentration}} \quad (3)$$

Statistics

Relative standard deviation was defined as (RSD = 100. (SD/ mean). All results obtained from precision and accuracy tests were initially submitted to Levene's test for homogeneity of variance and then submitted to one-way analysis of variance (ANOVA). When F values were significant, data were further analyzed by the Newman Keus test *post-hoctest* for-multiple comparison). A $p \leq 0.05$ was required for significance.

RESULTS

Standard curve

The standard curve relating the HPLC area under the curve (AUC) to the actual triamcinolone concentration (x) in the range of 100-2500 ng.mL^{-1} is shown in Figure 3. The straight line equation obtained from experimental results was found to be (Equation 4):

Y = 33.182 X - 0.602 (4)

where **y** is **10^3. AUC** and x is the concentration of the standard Triamcinolone solution in ng.mL^{-1}. The correlation coefficient (r) obtained was 0.999.

Specificity and selectivity

The specificity and selectivity of the HPLC method are show in Figure 4.

For empty PLGA microparticles, Figure 4 (B) shows an absence of the HPLC peak in the region of the retention time for triamcinolone. The retention times of 8.665 min for standard triamcinolone and 8.717 min for triamcinolone-loaded PLGA microparticles. This small difference between the retention times of the standard triamcinolone and triamcinolone-loaded PLGA microparticles may be due to the dissolution of the standard triamcinolone together with the structural polymer of the microparticles, wich could modify slightly the dielectric constant of the solvent, shifting the retention-time peak.

Linearity and working range

The correlation coefficient obtained for the triamcinolone standard curve was 0.999, wich represents a good value for the theoretical straight line in the standard curve in the range drug concentration used in this work.

Precision

The experimental results for the evaluation of intra-day precision are shown in Table 1.

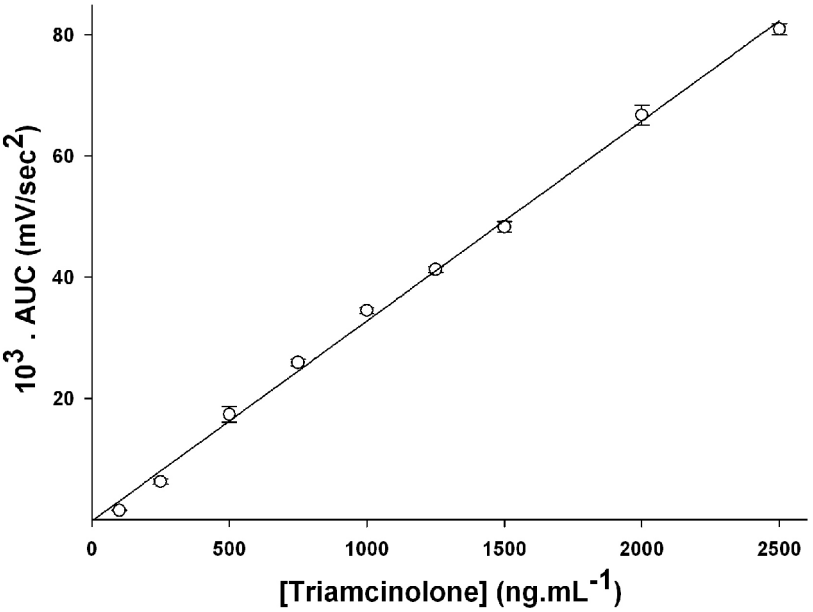

Figure 3. HPLC standard curve for triamcinolone in methanol/water 45:55 (v/v).UV-Vis detector at 239 nm. r = 0.999, mean values ± SD, n = 3.

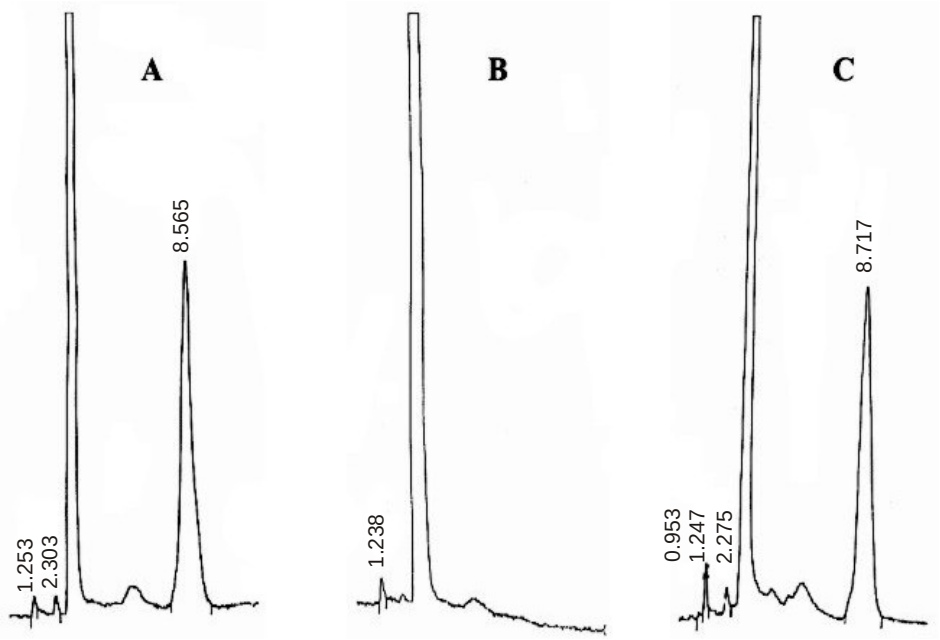

Figure 4. HLPC chromatograms for samples of triamcinolone and microparticles. UV-Vis detector at 239 nm; Merck® LiChrospher 100RP-18 column, 250mm x 4.6mm 5 µm particle size and 35ºC ± 2ºC. Key: (A) Standard triamcinolone solution 1,200 ng.mL^{-1}, (B) Empty PLGA-microparticles, (C) Triamcinolone incapsulated in PLGA-microparticles (1,200 ng.mL^{-1}).

Table 1 - Intra-day precision of experimental determinations of traimcinolone (TR).

Injection	Standard TR (%)	TR-load PLGA microparticles (%)
1	105.9	98.9
2	102.0	100.2
3	103.7	101.5
4	106.3	104.1
5	101.7	104.0
6	106.2	99.8
7	103.9	103.7
8	108.3	102.7
9	103.7	103.1
10	98.4	99.5
Mean TR (%)	104.01	101.75
SD	2.86	2.02
RSD (%)	2.76	1.98

The average values for standard triamcinoloneconcentration was not statistically different [$F(1,18) = 0.23$, $p = 0.64$] from the average value for the triamcinolone-loaded PLGA microparticles. In fact, all RSD values obtained were within the legal limits described by the Brazilian Sanitary Monitoring Agency.

The experimental results for the evaluation of inter-day precision are shown in Table 2.

The average value of the recovery from 600 and 1200ng.mL^{-1} was statistically bigger than value obtained from 2400 ng.mL^{-1} [$F(2,6) = 8.80$, $p < 0.05$]. However, all RSD values obtained from concentrations tested were smaller than 4%.

Table 2 - Evaluation of inter-day precision of experimental determinations of traimcinolone (TR).

Days	Recovery (%)		
	600 ng.mL^{-1}	*1200 ng.mL^{-1}*	*2400 ng.mL^{-1}*
1	107.76	107.3	98.31
2	106.34	103.07	98.63
3	104.96	99.5	97.54
Mean area	*106.35*	*103.29*	*98.16**
SD	*1.40*	*3.9*	*0.56*
RSD	*1.32*	*3.78*	*0.57*

* $p < 0.05$ vs 600 ng.mL^{-1} and 1200 ng.mL^{-1}

Table 3 - Experimental results used for limit of detection and quantifitation.

Sample	AUC from HPLC peak ($\mu V/s^2$)		
	100 ng.mL^{-1}	*200 ng.mL^{-1}*	*300 ng.mL^{-1}*
1	1203	4399	6870
2	1116	4146	4243
3	843	4280	6447
Mean area	*1054*	*4275*	*6853.33*
SD	*187.84*	*126.57*	*398.26*

Table 4 - Experimental results obtained from accuracy test.

Theoretical drug Concentration (N*g.mL^{-1}*)	Analytical drug concentration ± SD (*Ng.mL^{-1}*)	Recovery (% ± SD)	Precision RSD (%)
600 ng.mL^{-1}	646.5 ± 8.3	106.35 ± 1.39	1.31
830 ng.mL^{-1}	881.1 ± 18.8	106.16 ± 2.27	2.13
1200 ng.mL^{-1}	1239.5 ± 46.9	103.24 ± 3.91	3.78
2400 ng.mL^{-1}	2355.9 ± 13.4	98.16 ± 0.56*	0.57

* $p < 0.05$ vs 600 ng.mL^{-1}, 830 ng.mL^{-1} and 1200 ng.mL^{-1}.

Detection limit and quantitation limit

The experimental results used to calculate the LOD and LOQ are show in Table 3. The calculated values were 21.5 ng.mL^{-1} for limit of detection and 71.6 ng.mL^{-1} for limit of quantitation.

These values are much lower than those considered necessary for the quantitative analysis of triamcinolone, which lie within the concentration range of the standard curve (Figure 3).

Accuracy

The experimental results obtained from accuracy tests are showed in Table 4 and Figure 5.

Table 4 relates the average values of recovery obatined for several theoretical concentration tested and the inter-day precision achieved in each case. The average values of recovery from 2400 ng.mL^{-1} was statistically less than those obtained from the other concentrations tested [$F(3.8) = 7.73$, $p < 0.05$]. This effect can be observed in Figure 5.

The Figure 5 shows the linear fit relating the analytical concentrations determined for triamcinolone and the recovery rates calculated for the samples at the different concentrations.

DISCUSSION

The specificity and selectivity describe the capacity of the analytical method to measure the drug in the presence

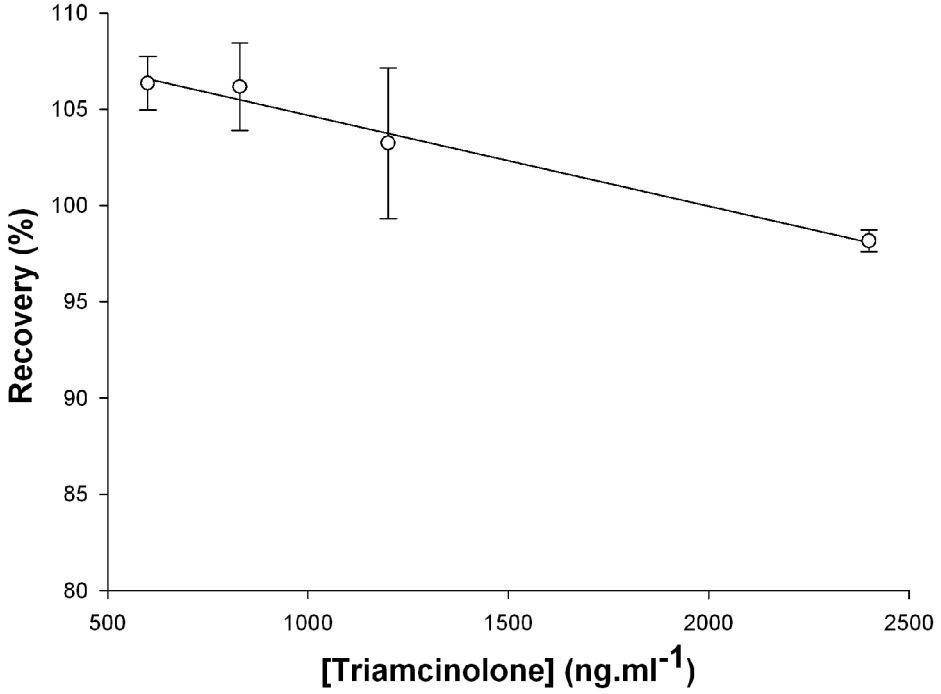

Figure 5. Recovery profile of triamcinolone from PLGA microparticles (n=3). r = 0.9926. Mean of data ± SD.

of impurities, excipients, degradation products or matrix components (ICH, 1996; ANVISA, 2003; USP, 2004). Figure 4 shows that the HPLC method used here was able to detect the drug around a retention time of 8.665-8.717 min in the standard and in the PLGA microparticles, respectively. No other peak was detected in this region of retention time, showing that quantitative analysis of triamcinolone by the method proposed is a real possibility.

The linearity of the analytical method was tested over specified range of drug concentrations. The range of concentration corresponds to the upper and lower limits for quantitation of analytical determination showing precision, accuracy and adequate linearity (ICH, 1996; ANVISA, 2003; USP, 2004).

Linear least-squares regression for the equation $y = a + bx$ resulted in a best fit (r = 0.999) for the variables $a = 0.602$ and $b = 33.182$. Figure 3 displays the fitted curve and the standard deviations for 3 independent sets of data. The correlation coefficient value obtained (0.999) lies within suitable limits established by the National Sanitary Monitoring Agency (ANVISA, 2003). Thus, the HPLC methodology used can be considered to show adequate linearity in the concentration range (100-2500 $ng.mL^{-1}$) for quantitative analysis of triamcinolone in the experimental conditions described.

Precision is an important analytical parameter representing the variability in the results in a repeated serial analysis of the sample under identical experimental conditions. In this work the intra and inter-day precision were evaluated. The intra-day precision was determined through the relative standard deviation (RSD) obtained from a given number of analyses of the homogeneous sample. The inter-day precision was determined by the investigating the RSD of the analyses of samples at different concentration levels (low, medium and high) over various days. (ICH, 1996; ANVISA, 2003; USP, 2004). The acceptable value of the RSD is < 5% (ANVISA, 2003). The experimental results from the intra and inter-day precision tests showed that the HPLC method could be considered precise for the quantitative analysis of triamcinolone in the PLGA microparticles, since the SRD obtained for the intra-day test was less than 3% (Table 1), while in the inter-day test it was less than 4% (Table 2).

To standardize the methodology, a standard triamcinolone sample was run and the limits of detection and quantitation of the equipment were determined. A randomized experimental design was used. Thus, the smallest amount of drug that can be reliable detected in the sample is the limit of detection (LOD) under the experimental conditions established. The LOD could be determined as the smallest drug concentration sufficient to provoke the appearance of noise in the base-line of the system (signal: noise ratio 3:1).

For the limit of quantitation (LOQ), the lowest content of the drug, which can be measured with reasonable statistical certainty, was used. If both accuracy and precision are constant over a concentration range around the limit of detection, then the limit of quantitation is numerically equal to six times the standard deviation of the mean of blank determination. Thus, the LOQ could also be determined

within acceptable values, precision and accuracy are previously established. The LOQ must be determined by using drug concentrations that produce a signal:noyse ratio of 10:1. The limits of detection and quantitation were 21.5 $ng.mL^{-1}$ and 71.6 $ng.mL^{-1}$, respectively. These experimental results showed that the HPLC method suggested is capable of to identifying and measuring very low drug concentrations.

Accuracy is one of the most important analytical parameters of a methodology and it can be expressed as the percent recovery of the known amounts of drug added to a sample. Accuracy describes the degree of veracity of the quantitative analysis. For repeated analysis of a reference drug, the deviation of the mean from the certified value, expressed as a percentage of the certified value, shall not lie outside the limits ± 10 % (ICH, 1996; ANVISA, 2003; USP, 2004).

The accuracy can be determined from at least nine analytical determinations at three different concentrations, distributed in the linear range of the standard curve. For this purpose, low, medium and high concentration levels were used in triplicate analyses. From the experimental results for accuracy, showing in Table 4 and Figure 5 it can be seen that greater recovery is obtained at smaller drug concentrations. The data in Figure 5 also reveal that there is a linear relationship between recovery rate and triamcinolone concentration, with a straight-line fit (r = 0.9926). By analyzing the in Table 4 and Figure 5, it was also possible to verify that biggest recovery rate deviations were associated with the lowest drug concentrations. This phenomenon may be explained by the larger proportion of drug relative to the structural polymer of the microparticles.

For pharmaceutical products, the established reasonable percent recovery limits are 80-110%. The recovery values from accuracy tests are in agreement with established limits (ANVISA, 2003). The experimental results show that the HPLC method used can be considered suitable for analytical determination of triamcinolone in the PLGA microparticles, owing to high selectivity and specificity, to linearity in the concentration range used and precision and accuracy at the concentrations studied. Thus, the HPLC method suggested here can be safely employed in the quantitative analysis of triamcinolone-loaded PLGA microparticles.

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RESUMO

Desenvolvimento e validação de método de análise quantitativa para triancinolona em micropartículas biodegradáveis por CLAE.

Neste trabalho, foi desenvolvido um método de cromatografia líquida de alta eficiência (CLAE) para a determinação quantitativa da triancinolona contida em micropartículas de ácido polilático-co-glicólico (PLGA). As condições cromatográficas utilizadas foram Coluna de Fase Reversa C18, 250mm x 4,6mm com diâmetro partícula 5 μm. O forno de coluna foi termostatizado a 35 ± 2ºC, a fase móvel usada foi metanol:água 45:55 (v:v), com fluxo isocrático de 1 $mL.min^{-1}$ e volume de injeção de 10 μL. Foi utilizado detector de UV-Vis selecionado em 239nm. A curva padrão obtida apresentou linearidade (r^2 > 0,999) na faixa de concentração 100-2.500 $ng.mL^{-1}$. O método proposto apresentou precisão adequada com desvio padrão relativo < 3%. Os resultados mostraram que a exatidão do método apresentou valores de recuperação dentro dos limites recomendáveis em toda a faixa de concentração estudada. O método de CLAE mostrou especificidade e seletividade com linearidade dentro da faixa de concentração de trabalho utilizada e precisão e exatidão que permitem a quantificação da triancinolona em micropartículas de PLGA.

Palavras-chave: Triancinolona; método de CLAE; micropartículas de PLGA; validação de método analítico.

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