Development and validation of an RP-HPLC/UV Method for Determination of Cholecalciferol in Polymeric Nanoparticles Suspensions

Desenvolvimento e validação de um método RP-HPLC/UV para determinação do Cholecalciferol em nanopartículas poliméricas Suspensões

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ABSTRACT
A simple and effective RP-HPLC/UV method was developed to determine cholecalciferol (COL) in suspensions of polymeric nanoparticles. Chromatographic conditions used in the methodology were a C18 RP column using an isocratic mobile phase of methanol and water (98:2 v/v) at a flow rate of 1.2 mL/min, UV detection at 265 nm. The validation parameters evaluated were: specificity, linearity, limit of quantification, limit of detection, precision, precision and robustness. The retention time was about 7.5 min. The method was linear, specific (r=0.9992) interval 10.0 to 80.0 µg/mL, the limit of quantification (LOQ) was 0.52 µg/mL and the limit of detection was 0.15 µg/mL. Intermediate precision was evaluated, expressed as inter-day variation (RSD = 1.13) and intra-day (RSD = 1.16). The accuracy obtained was the recovery of 102.87 ± 9.84, considering the exact method proposed. To evaluate the robustness, the results showed little variation, concluding that a robust method. The method was fast, robust and suitable for quantification of suspensions of COL nanoparticles and can be used to evaluate the efficiency of COL encapsulation.

Keywords: Cholecalciferol, nanoparticles, Vitamin D3

RESUMO
Foi desenvolvido um método RP-HPLC/UV simples e eficaz para determinar o colecalciferol (COL) em suspensões de nanopartículas poliméricas. As condições cromatográficas utilizadas na metodologia foram uma coluna C18 RP utilizando uma fase móvel isocrática de metanol e água (98:2 v/v) a um caudal de 1,2 mL/min, detecção UV a 265 nm. Os parâmetros de validação avaliados foram: especificidade, linearidade, limite de quantificação, limite de detecção, precisão, precisão e robustez. O tempo de retenção foi de cerca de 7,5 min. O método foi linear, específico (r=0,9992) intervalo 10,0 a 80,0 µg/mL, o limite de quantificação (LOQ) foi de 0,52 µg/mL e o limite de detecção foi de 0,15 µg/mL. A precisão intermédia foi avaliada, expressa como variação entre dias (RSD = 1,13) e intra-dia (RSD = 1,16). A precisão obtida foi a recuperação de 102,87 ± 9,84, considerando o método exacto proposto. Para avaliar a robustez, os resultados mostraram pouca variação, concluindo que se tratava de um método robusto. O método foi rápido, robusto e adequado para quantificar as suspensões de nanopartículas COL e pode ser utilizado para avaliar a eficiência do encapsulamento COL.

Palavras-chave: Cholecalciferol, nanopartículas, Vitamina D3

1 INTRODUCTION

Vitamin D comes in two forms: vitamin D2 or ergocalciferol, found in some vegetables and yeasts and vitamin D3 or cholecalciferol, which is synthesized in the skin after exposure to sunlight (HOLICK, 1981). Vitamin D obtained by diet, drugs or cutaneous is biologically inert (REICHRATH, 2007).

Currently, the area of scientific research is aimed at better understanding the mechanisms of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) to avoid the most serious cases and to avoid reactivations in the population. Some articles

suggest an involvement of vitamin D level in the severity of the SARS-CoV-2 immune process (ZWART, SMITH 2020).

The COL has molecular mass 384 g/mol and the melting point in 84.5 °C, is soluble in ethanol, acetone, fats, oils, benzene, chloroform and ether, its solubility in water is estimated to be around $1.3x10^5$ mg/dL at 25°C (MEDEIROS, 2013), when pure, in the form of small, yellowish-white, odorless crystals (BALL, 1998). It is a steroid hormone, which requires multiple metabolic pathways to become active in the body, being considered very important for calcium homeostasis, as well as presenting pleiotropic effects in a variety of non-skeletal disorders (BOUILLON et al., 2008).

Numerous factors affect the production of vitamin D by the body. The changes in lifestyle occurred in the last decades (ZITTERMANN et al., 2013), exposure to sunlight at times where the incidence of sunlight is low (HOLICK, 2004), use of sunscreen (MESQUITA et al., 2013), Individuals with more pigmented skin (ARABI et al., 2010; HOLICK et al., 2011), elderly, among others. Statistical data estimates that one billion people worldwide will be affected (HOLICK, 2007).

The administration of topically nanoencapsulated cholecalciferol is an alternative for the replacement of this hormone, since it offers greater comfort to the patient, improving adherence to the treatment. In addition, nanoencapsulation tends to protect the drug against hydrolysis, oxidation, photolysis and moisture, while maintaining the product stable (LUO; TANG; WANG, 2012).

Nanotechnology is considered an excellent tool for transporting lipophilic drugs (LINDNER; KHALIL; MAINARDES, 2013). Nanomaterials used to transport drugs are liposomes, nanoemulsions, nanotubes, dendrimers, polymer nanoparticles, among others (JAIN; HIRST; O'SULLIVAN, 2012). The appropriate choice of the type of nanosystem and the polymer that will be used to produce the controlled drug release systems should be judicious, considering the physico-chemical characteristics of the polymers, drugs, excipients, route of administration and target tissue, we can provide profiles of constant drug concentration, significant reduction of adverse reactions, decrease of drug dosages and increase of drug treatments (SADRIEH et al., 2010).

The physico-chemical characterizations of the nanoparticles must be performed extensively and the determination of the drug content charged in the polymer nanoparticles must be verified in order to determine the amount of the encapsulated drug in order to achieve the therapeutic objective. Therefore, it is necessary to develop and validate a quantification method that is suitable for determining the amount of

encapsulated cholecalciferol. HPLC is the method of choice for this quantification, since it is considered more sensitive for dosages of small amount of analyte (SILVA et al., 2010).

The objective is to develop and validate of a fast, simple and low cost HPLC method to quantify COL in polymer nanoparticles.

2 MATERIAL AND METHODS

2.1 CHEMICAL AND REAGENTS

Cholecalciferol (97% purity) (Galena, São Paulo, Brazil), poly (ε-caprolactone) (PCL, Mn 45000 g/mol) (Sigma-Aldrich, St. Louis, MO, USA). Tween 80 (polysorbate 80) (Deleware, Porto Alegre, RS, Brazil) and acetone (Veter Química Fina, Rio de Janeiro, RJ, Brazil). HPLC grade methanol was purchased (Merck KGaA, Darmstadt, Germany). Water was purified on Purifying System Milli-Q Plus water (Merck KGaA, Darmstadt, Germany).

2.2 EQUIPMENT

Chromatographic analyzes were performed on a Merck-Hitachi LaChrom chromatograph (Tokyo, Japan), this system equipped with a D-7000 interface, an L-74000 UV detector. A L-7100 quaternary pump and an integral degasser. A system manual injector (Rheodyne, Cotati, CA, USA) equipped with a 20 µL sample of ring and a 100 µL syringe (Hamilton Microliter 710, Bonaduz, Switzerland). The software used for data collection and calculations was ChromQuest 5.0 (Thermo Fisher Scientific, San Jose, CA, USA). Chromatographic separation was performed using an Inertsil® GL Sciences ODS3 (Torrance, CA, USA) analytical reverse phase column (4.6 mm x 150 mm, 5 µm) at room temperature.

2.3 CHROMATOGRAPHIC CONDITIONS

Chromatographic analysis was performed using an isocratic mobile phase of methanol and water (98:2, v:v). The analyzes were performed at room temperature, using a flow rate of 1.2 mL/min and an injection volume of 20 µL. Detection was using a 265 nm length.

2.4. PREPARATION OF STANDARD SOLUTIONS

A standard solution of 500 µg/mL was prepared daily by dissolving 25 mg cholecalciferol in a 50 mL volumetric flask using methanol. This solution was further diluted in the mobile phase to prepare five different working solutions ranging from 10.0 to 80.0 µg/mL. These solutions were filtered through 0.45 µm x 13 mm Nylon syringe filter prior to injection into the HPLC system.

2.5 METHOD DEVELOPMENT

The wavelength used for the detection of cholecalciferol was 265 nm. The chromatographic conditions were optimized for resolution of the COL peak varying both the composition and the ratio of the mobile phase. An appropriate negative control was used prior to sample analysis. The method was validated and then used to determine the COL suspension of nanoparticles.

2.6 METHOD VALIDATION

The validation of the analytical method evaluated the parameters of linearity, specificity, detection limit, quantification limit, precision (intra-day and inter-day), precision and robustness, considering the guidelines of International Conference (ICH) on Harmonization of Technical Requirements for Pharmaceuticals for Human Use and Brazilian regulation (RE 899/2003) of the National Health Surveillance Agency.

The specificity was determined by analyzing the chromatograms of the suspensions of unloaded nanoparticles (negative control) compared to those obtained for the formulations containing COL in order to confirm that the polymer (PCL) used did not interfere in the quantification of the drug.

Linearity was used to verify that the sample solutions in a predetermined concentration range (10.0 - 80.0 µg/mL) had a linear response proportional to the drug concentration. Linearity was determined from three analytical curves obtained from HPLC experiments performed using standard solutions of COL. The experimental results were graphically plotted to obtain a mean analytical curve. Linear regression and analysis of variance (ANOVA) were performed to obtain slope and other statistics.

The limit of detection (LOD) and the limit of quantification (LOQ) were determined in three curves, averages of the linear coefficients of the y-axis intercept and the equations of the straight lines obtained (SD) and the mean angular coefficients of the

equations of the straight lines obtained (S) of the calibration curves, by means of the Equations 1 and 2.

$$LD = 3 \times DP/S \qquad \text{Equation (1)}$$

$$LQ = 10 \times DP/S \qquad \text{Equation (2)}$$

The accuracy of the method was determined according to the ICH requirements. Precision was evaluated at two levels: repeatability and intermediate precision. The relative standard deviation (RSD) of six injections at 100% assay concentration (40 µg/mL) was evaluated and analyzed intra-day and inter-day and with different analyst.

Accuracy was performed using six determinations with a concentration of 70 µg/mL in triplicate. The value of the mean concentrations was compared with the theoretical value (100%).

Robustness was determined after variations in flow rate (1.195 and 1.205 mL/min), mobile phase composition (97:3 and 99:1, methanol:water). The Tukey ANOVA test was performed to evaluate whether the variations altered the results of HPLC analysis.

2.7 METHOD APPLICABILITY

2.7.1 evaluation of encapsulation efficiency

Nanoparticle-encapsulated COL quantification was performed by the indirect method, the nanoparticles (1000 µL) were centrifuged by ultrafiltration/centrifugal ultracentrifugation (Hitachi, HIMAC CR21GII) at 22,000 rpm for 30 minutes at room temperature. With centrifugation, separation of the aqueous phase (containing the free drug) from the colloidal suspension (encapsulated drug) occurred. The aqueous portion resulting from the centrifugation was used to determine the concentration of free drug that was not encapsulated to the polymer system after centrifugation. The encapsulation efficiency (EE) was calculated according to equation 3.

$$E.E\ \% = (\text{Initial drug} - \text{Free drug}) / \text{Initial drug} \times 100 \qquad \text{Equation (3)}$$

Where the inicial drug corresponds to the amount of cholecalciferol initially incorporated into the formulation and free drug corresponds to the amount of drug not

incorporated into the nanoparticles. Samples were filtered on the 0.45 µm x 13 mm Nylon syringe filter and analyzed by HPLC in triplicate.

2.8 PREPARATION OF POLYMERIC NANOPARTICLES

The suspensions were prepared by the interfacial deposition method of the preformed polymer, developed and described by Fessi et al. (1989). Briefly, an organic phase prepared by dissolving the polymer PCL (ε-caprolactone), cholecalciferol and acetone (27 ml) was slowly poured into 45°C in an aqueous phase containing Tween® 80 under moderate magnetic stirring at room temperature. The nanoparticles were formed immediately after diffusion of the organic solvent. The system was kept under magnetic stirring for 10 minutes after dripping of heat and kept at rest for 60 minutes. After this period, the organic solvent and part of the aqueous phase were removed using a vacuum evaporator, adjusting the final volume to 10 mL with a concentration of 1.0 mg/mL. The suspensions were prepared in triplicate and for comparison purposes a suspension was prepared without addition of the drug as a negative control. To perform the HPLC analysis, suspensions of the polymer nanoparticles were subjected to ultrafiltration/centrifugation procedure (Hitachi, model HIMAC CR21GII, Tokyo, Japan) at 22,000 rpm for 30 min. Prior to injection, each sample was filtered through a 0.45 µm x 13 mm Nylon syringe filter.

2.9 STATISTICAL ANALYSIS

The statistical analysis was performed by analysis of variance (ANOVA), t-student test and residue analysis, using software Statistica 8.0 (Statsoft, Inc.).

3 RESULTS AND DISCUSSION

3.1 METHOD DEVELOPMENT

The method developed and validated in this work represents an alternative for the quantification of cholecalciferol in polymer nanoparticles. To evaluate the best condition for the chromatographic run, an exploratory gradient experiment was performed, varying the concentration of 5-100% methanol in 60 minutes. Thereafter, the chromatographic conditions were optimized to obtain a better peak resolution. The best result found was methanol:water (98:2, v:v) at room temperature with a flow of 1.2 mL/min. The time of analysis and the retention time were 10 and 7.5 minutes (Figure 1), respectively, which is

considered very suitable for routine analysis, allowing the analysis of a large number of samples per time period and reduction of costs due to the small amount of solvents used.

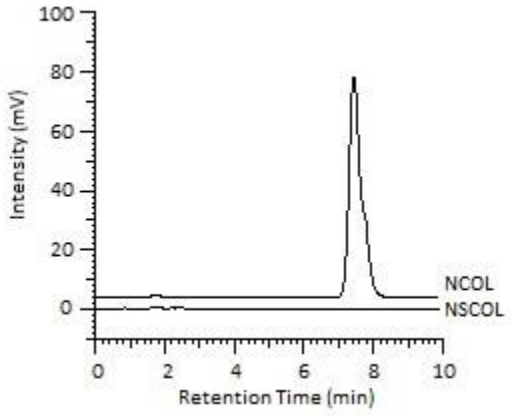
3.2 METHOD VALIDATION

The validation of the analytical method by HPLC was performed according to the criteria proposed by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceutical Products for Human Use (ICH 2005) and Resolution 899 of May 29, 2003. The parameters evaluated were: specificity, linearity, limit of detection, limit of quantification, precision, precision and robustness.

3.2.1 Specificity

Specificity is considered the first step of analytical validation, ensuring that formulation components, such as excipients, do not interfere with drug quantification. It was demonstrated by comparing the chromatograms of the cholecalciferol nanoparticles (NCOL) and negative control (NSCOL) (Figure 1). The results show that there is no interference in the quantification and retention time of COL. Thus, it is possible to confirm the specificity of the proposed method.

Figure 1. Chromatograms of the cholecalciferol nanoparticles (NCOL) and negative control (NSCOL).



3.2.2 Linearity

The linearity of the method was evaluated by six levels of concentration. There was a linear relationship between peak area and COL concentration in a concentration range of 10.0 to 80.0 µg/mL. The linear equation obtained was y = -45422.5335x - 85.4229, r^2 = 0.9999, where y is the peak area and x is the concentration of standard solution g/mL. An adequate correlation coefficient (r = 0.0092) was found, which

demonstrates that the method is linear with the value of 1 in the proposed range. The relative standard deviation (RSD) of slope was 1.42%, which is consistent with the limit proposed by ICH and ANVISA up to 5%.

According to the Analytical Methods Committee (AMC), a correlation coefficient value close to 1 is not necessarily the result of a linear relationship and therefore the non-fit test must be applied. This test evaluates the change in residual values (HADAD, EMARA, MAHMOUD, 2009). The ANOVA test for linearity is shown in Table 2. For the lack of fit, the value of F is less than the tabulated value of F for the 95% confidence level range ($\alpha = 0.05$), then linear regression did not show a lack of fit (KLEIN; LONGHINI, MELLO, 2012).

Table 2. Result of ANOVA for linearity of the method.

Cholecalciferol	**SS**	**df**	**MS**	***F***	***F*tab**
Model	7.372454 x 10^{13}	1	7,372454E+13	11278.06	4.600
Residue	9,151783 X 10^{10}	14	6,536988E+09	Linear	-
Lack of adjustment	2,402060E+10^{10}	4	6,005150E+09	0.889688	3.478
Pure error	1,079363E+10^{10}	10	1,079363E+09	There is no lack of adjustment	

SS: sum of squares; df: degrees of freedom; MS: mean squares of residues; *F: F* value of the test; *F*tab: value *F* tabulated.

3.2.3 Detection limit and quantification limit

In this study, these parameters were calculated from the specific calibration curve (15.0 - 80.0 µg/mL). The values obtained for LOD and LOQ were 0.15 and 0.52 µg/mL, respectively, indicating a good sensitivity of the method for determination of COL.

3.2.4 Precision

The repeatability expresses the accuracy under the same operating conditions for a short period of time. The intermediate precision, expressed as intra-day and inter-day variation, was evaluated. The Tukey test assesses whether there is any difference between the different levels of a factor. Up to 5% there is no significant difference in peak area and COL retention time. The repeatability of DPR for the six concentrations of 40 µg/mL was 0.42%. The intra-day precision between the RSD value was 1.16% and 1.13% between days. The results are shown in the Table 3, confirming the accuracy of the developed method.

Table 3. Intermediate precision and repeatability data for cholecalciferol analysis.

	Theoretical concentration (µg.mL^{-1})	Concentration (µg.mL^{-1}, ± DP*)	DPR** (%)
Repeatability (n = 6)	40.0	40.07 ± 0.45	0.42
Intermediate Accuracy			
Intra-day (n = 3)	40.0	40.35 ± 0.46	1.16
Inter-day (n = 3)	40.0	40.52 ± 0.45	1.13

*DP: standard deviation; ** DPR: relative standard deviation.

3.2.5 Accuracy

The accuracy of the method by recovery assays was determined by the preparation of a sample with a known amount of colecalciferol. The mean recovery ± SD and DPR were 102.87 ± 9.84 and 4.78%, respectively. Therefore, these results indicate that the proposed method is considered accurate.

This percentage of recovery was expressed between the experimentally determined average concentration and the corresponding theoretical concentration, with acceptance criteria between 95 and 105% of recovery, the result obtained is in accordance with the ANVISA requirement.

3.2.6 Robustness

The robustness of an analytical method indicates the reliability of the method in relation to small variations of analytical parameters. To evaluate the robustness, the flow of 1,200 ml/min and mobile phase of methanol: water 98:2 (v:v) was considered. The Tukey test at the 5% level showed no significant difference in peak area and COL retention time when flow ranged from 1,200 to 1,205 mL/min (RSD = 0.13%) and 1,005 mL/min (RSD = 0.18%) and when the mobile phase concentration ranged from 98:2 (v:v) to 97:3 (v:v) (RSD = 0.11%) and 99:1 (v:v) (RSD = 0.02%). Therefore, the method proved to be robust for the analysis of the drug under the conditions evaluated.

3.3 Evaluation of encapsulation efficiency

The analytical method developed and validated (Table 4) was used to evaluate the encapsulation efficiency of colecalciferol in the suspension of polymeric nanoparticles.

Table 4. Summary of calibration curve parameters.

	Cholecalciferol
Linear Band (µg/mL)	10 - 80
Detection limit (µg/mL)	0.15
Quantification Limit (µg/mL)	0.52
Regression data *	
N	3
Inclination (a)	45422.53
Standard Slope Deviation	647.30
Relative Standard Slope Deviation (%)	1.42
Intercept (*b*)	-85422.94
Correlation Coefficient (r)	0.9992

By the indirect method it was possible to determine the encapsulation efficiency. The results of the drug concentration incorporated into the nanoparticles (µg/mL) and the encapsulation efficiency (%), are described in Table 5.

Table 5. Encapsulated cholecalciferol content and encapsulation efficiency indirect method.

Sample	Theoretical concentration (µg/mL)	COL Unencapsulated (µg/mL)	EE (%)
NPCOL	100.000,00	2.000,00 (± 5,85)	98 %

NPCOL: nanoparticles cholecalciferol; EE: Encapsulation efficiency.

Considering the content of the drug, the formulation presented concentration with values very close to the theoretical, indicating that there was practically no loss during its preparation. In addition, the formulation had an encapsulation efficiency of 98.0%, which is related to the low aqueous solubility of the drug, thus leading to an increase in the concentration of the drug incorporated into the nanoparticles. The tests were performed in triplicate.

4 CONCLUSION

In this work, a new RP-HPLC UV method was developed and validated to quantify the polymer nanoparticles colecalciferol. The method developed was simple, sensitive, specific, linear, precise, accurate and robust in the conditions evaluated. The validation of the results, following the rules of Re No. 899/2003 (BRAZIL, 2003) and considered a simple, fast and easy procedure to be applied in the analysis of laboratory routines to quantify suspensions of COL polymer nanoparticles.

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