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# DEVELOPMENT, CHARACTERIZATION AND VALIDATION OF AN ANALYTICAL METHOD FOR SOLID LIPID NANOPARTICLES CONTAINING ASCORBYL PALMITATE

## *DESENVOLVIMENTO, CARACTERIZAÇÃO E VALIDAÇÃO DE UM MÉTODO ANALÍTICO PARA NANOPARTÍCULAS LIPÍDICAS SÓLIDAS CONTENDO PALMITATO DE ASCORBILA*

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**ABSTRACT**

The ascorbyl palmitate (AP) has wide application as an antioxidant additive in pharmaceutical, medical and cosmetic products. However, it is easily degraded when used as a free molecule. The nanoencapsulation has been used to overcome the degradation of several unstable molecules. In this work, solid lipid nanoparticles (SLN) containing ascorbyl palmitate were developed and characterized, and an analytical method of validation was proposed for their quantification. The results demonstrated that the SLN-containing AP had an initial particle size of 86.41 ± 0.30 nm and 89.27 ± 0.86 nm after 90 days; the values of polydispersity index were around 0.2; the initial zeta potential was -12.20 ± 0.23 mV and -18.40 ± 0.29 mV after 90 days; the pH was approximately 4.0. The method showed linearity between 9-24 µg.ml, and correlation coefficient represented by r = 0.9998. The analysis of precision and accuracy showed a low relative standard deviation (<2.98%) and a sufficient recovery percentage of AP (101.40%). The procedure provided specificity, linearity, precision, accuracy and robustness, indicating that the method can be applied to the quantitation of AP at SLN.

**Keywords:** chromatography; stability; physical and chemical parameters.

***RESUMO***

*O palmitato de ascorbila (PA) tem ampla aplicação como aditivo antioxidante em produtos farmacêuticos, médicos e cosméticos, no entanto, é facilmente degradado quando utilizado como uma molécula livre. A nanoencapsulação tem sido utilizada para melhorar a instabilidade de inúmeras moléculas. Neste trabalho, nanopartículas lipídicas sólidas (NLS) contendo palmitato de ascorbila foram desenvolvidas e caracterizadas, e um método analítico de validação foi proposto para a sua quantificação. Os resultados demonstraram que as NLS contendo PA apresentaram um tamanho de partícula inicial de 86,41 ± 0,30 nm e 89,27 ± 0,86 nm após 90 dias; os valores do índice de polidispersão foram em torno de 0,2; o potencial zeta inicial foi -12,20 ± 0,23 mV e -18,40 ± 0,29 mV após 90 dias; os valores de pH foram de aproximadamente 4,0. O método apresentou uma linearidade entre 9-24 µg/mL, e um coeficiente de correlação representado por r = 0,9998. A análise da precisão e exatidão indicou um baixo desvio padrão relativo (<2,98%) e um percentual de recuperação suficiente de PA (101,40%). O procedimento apresentou especificidade, linearidade, precisão, exatidão e robustez, indicando que o método pode ser aplicado para a quantificação de PA em NLS.*

***Palavras-chave:*** *cromatografia; estabilidade; parâmetros físico-químicos.*

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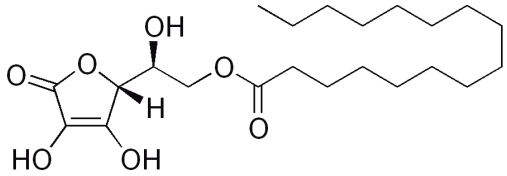
## INTRODUCTION

Ascorbyl Palmitate (AP) (or L-ascorbyl-6-palmitate) (Figure 1) is a lipophilic fatty acid ester that presents a wide application as an antioxidant additive in food, pharmaceutical, medical and cosmetic products (JAVIDIPOUR; TÜFENK; BAŞTÜRK, 2015; YOKSAN et al., 2010). This derivative has antioxidant activity similar to vitamin C, penetrates more easily into the skin and has a greater ability to protect the skin components from the action of free radicals (PANEVA et al., 2011). The compound has a neutral pH which does not irritate the skin, it also has a good efficacy in topical formulations (GUARATINI; MEDEIROS; COLEPICOLO, 2007; LEONARDI, 2004).

**Figure 1 -** Structural formula of Ascorbyl Palmitate.




Ascorbyl palmitate presents insolubility in water and low chemical stability, which limit its broader application (PANEVA et al., 2011; YOKSAN et al., 2010). To overcome that stability problem the AP, several methods have been studied to decrease the possibility of photodegradation. Recently, several studies on nanotechnology have condunting, most of then aim to improve the selectivity and efficiency of the formulations. These studies include incorporation in microemulsions (KRISTL et al., 2003, SPICLIN; GASPERLIN; KMETEC, 2001), liposomes (KRISTL et al., 2003), solid lipid nanoparticles (SLN) (KRISTL et al., 2003; ÜNER et al., 2005) and nanostructured lipid carriers (NLC) (ÜNER et al., 2005; TEERANACHAIDEEKUL et al., 2007).

Solid lipid nanoparticles (SLN) have been developed as a new transport topic systems for pharmaceuticals and cosmetics products, and also an alternative system for encapsulating, in relation to traditional colloidal systems (such as emulsions, liposomes and polymeric nanoparticles) (ÜNER; YENER, 2007; MADAN; KHUDE; DUA, 2014). The SLN is formed by a matrix of biodegradable lipids that are physiologically well tolerated, and have great advantage as its excellent physical and chemical stability, which provides greater protection against degradation of labile drugs (SOUTO et al., 2011).

This nanoparticle model is promising as it presents unique physicochemical characteristics which include beneficial properties surpassing the traditional substances (DOAK et al., 2012), among them, the possibility of reducing the particle size, thereby increasing the contact surface area, protection from enzymatic, chemical or immunological degradation (ARORA; RAJWADE; PAKNIKAR, 2012).

There are no previous studies regarding the development and validation of SLN containing AP. In this context, we developed SLN containing AP. Since this study involves a new formulation, the first necessary and crucial step is to have a suitable analytical method to quantify the AP in the nanoparticles. However, the process for drug extraction from nanostructured system is complex and difficult to be performed. This process requires the specific combination of solvents to solubilize of the polymer and the active drug (PAESE et al., 2009). Thus, a fast and accurate method for the extraction and quantification of AP from nanostructures was also developed.

High Performance Liquid Chromatography (HPLC) is the preferential method to quantify nanoencapsulated drugs, since it presents high sensitivity and accuracy (BARRIOS et al., 2011; BIENIEK et al., 2011; VELLOSO et al., 2009). However, any method applied for drugs quantification should be validated to ensure the safety and reliability of the results. For HPLC validation, specificity, linearity, precision, accuracy, robustness and detection and quantification limit should be determined (ANVISA, 2003; ICH, 2005).

Developing and validation of methods to nanoparticles analysis have been intensively studied. Linder et al. (2013) developed and validated an analytical method using HPLC-PDA for resveratrol determination in polymeric nanoparticles. Silva-Buzanello et al. (2015) validated an analytical method based on ultraviolet-visible spectroscopy for the quantitative determination of curcumin encapsulated in poly (L-lactic acid) nanoparticles. Kumar et al. (2015) developed and validated of HPLC method to determine valsartan in nanoparticles.

Therefore, the aim of the present study was to develop and characterize SLN containing AP, and validate an analytical methodology, by HPLC, for quantification of this active incorporated in these nanoparticles.

## MATERIAL AND METHODS

### MATERIALS

Ascorbyl Palmitate and Sorbitan Monostearato were obtained from Sigma-Aldrich®, Rosehip Oil was purchased from Delaware®, Methacrylic acid copolymer - Eudragit L100 from Röhm Pharma Polymers®. Tinogard TT and Polysorbate 80 were supplied from Via Farma®. Ethylenediaminetetraacetic acid - EDTA from Nuclear®. Propyleneglycol, Shea Butter and Sorbitan Monooleate by Alpha Quimica®. Monobasic Anhydrous Potassium Phosphate was obtained from Synth®, Milli-Q® water. Ethanol HPLC grade, Methanol HPLC grade and Acetonitrile HPLC grade were supplied by J.T. Baker® and Fosforic Acid P.A by Nuclear®.

## PREPARATION OF SOLID LIPID NANOPARTICLES CONTAINING ASCORBYL PALMITATE

The SLN containing AP were prepared according to the method of Raffin et al. (2012). The aqueous phase containing polysorbate 80, propyleneglycol and Milli-Q water was poured into the lipid phase composed of AP, shea butter, sorbitan monooleate and Tinogard TT, under moderate magnetic stirring. To obtain the SLN, the mixture was poured into high shear mixer at 20,000 rpm for 20 min.

## PHYSICO-CHEMICAL PARAMETERS OF SOLID LIPID NANOPARTICLES

- *Particle size distribution and polydispersity index:* The particle size and polydispersity index (PDI) were measured by photon correlation spectroscopy. Samples were diluted in Milli-Q water and analyses were performed at 25 °C, using a Zetasizer® (Nanoseries, Malvern, UK). Each sample was analyzed in triplicate.

- *Zeta potential:* The zeta potential determination was performed by photon correlation spectroscopy. Samples were diluted in 10 mmol $L^{-1}$ NaCl and analyses were performed at 25°C, using a Zetasizer® (Nanoseries, Malvern, UK). Each sample was analyzed in triplicate.

- *Determination of pH:* The pH values of the SLN were determined by direct immersion of the electrode into the samples, using a calibrated potentiometer (Digimed®), at room temperature. Each sample was analyzed in triplicate.

## EXTRACTION OF ASCORBYL PALMITATE FROM SOLID LIQUID NANOPARTICLES

To release the AP from SLN, samples were treated with the combination of acetonitrile, ethanol and mobile phase (30:30:40, v/v) kept in sonication for 30 minutes, and after vórtex for 10 minutes. Lastly, this solution was centrifuged and filtered through polyacrylamide membrane with porosity 0.45 μm.

## CHROMATOGRAPHIC PARAMETERS

The chromatographic conditions were optimized for the determination of AP in SLN. The HPLC equipment used was the Prominence Shimadzu Liquid Chromatograph (Tokyo, Japan) equipped with a degasser (DGU-20A 5R), an LC-20AT pump system, an SPD-M20A photodiode array detector, a CTO-20A chromatographic column oven and a SIL-20A auto sampler. The analysis was carried out using a Lichrospher® 100 RP-18 column (250 mm x 4 mm, 5 μm particle size) from Merck, that was protected by a Lichrospher® 100 RP-18 pre-column (250 mm x 4 mm, 5 μm particle

size) from Merck. The analyses of AP content were performed at $\lambda$ = 254 nm with a mobile phase of methanol:acetonitrile:potassium phosphate buffer 0.02M pH 2.5 (40:40:20, v/v) at a flow rate of 1.5 mL $min^{-1}$. The analytical column was kept at 25°C, and the injection volume was 20 µL.

## VALIDATION OF THE ANALYTICAL HPLC METHOD FOR SOLID LIPID NANOPARTICLES CONTAINING ASCORBYL PALMITATE

The analytical method was validated according to the criteria proposed by Agência Nacional de Vigilância Sanitária (ANVISA) - Resolution RE nº 899, and the International Conference on Harmonization (ICH). The parameters evaluated were linearity, specificity, limit of detection and quantification limit, repeatability, intermediate precision, and accuracy.

- *Linearity:* The linearity assay was set using three calibration curves prepared by dilution of the standard solution. Each calibration curve was prepared using six AP concentrations (9, 12, 15, 18, 21, 24 µg $mL^{-1}$) and the analyzes was performed in three different days.

- *Specificity:* The specificity of the method was determined by comparative analyzes of the SLN without AP and SLN containing the drug at a concentration of 15.0 µg $mL^{-1}$. The samples were analyzed under the same experimental conditions, verifying the absence of interference by excipients which are part of the formulation. Furthermore, we also evaluated the specificity by analyzing the purity of the peaks, according to the recommendations of the ICH (2005).

- *Precision:* The precision was assessed by repeatability, six sample solutions (SLN containing AP) were analyzed at a concentration of 15.0 µg $mL^{-1}$ in a single day; and through the intermediate precision with injection of solutions at a concentration of 15.0 µg $mL^{-1}$, on different days.

- *Accuracy:* The accuracy of the method was determined by adding a known amount of drug (10.0 µg $mL^{-1}$) to the sample solution, resulting in concentration of 12.5; 15 and 17.5 µg $mL^{-1}$.

- *Detection and quantification limits:* The detection and quantification limits were calculated by dividing the standard deviation of the coefficients of calibration curves by the average linear coefficients of the angular testing these respective curves and multiplied by 3 and 10, respectively, as suggested by ICH (2005).

## EVALUATION OF THE STABILITY PARAMETERS

SLN were stored in amber vials at 5 ± 2°C, for 90 days. Samples were analyzed initially and after 90 days, in terms of the physico-chemical characteristics as particle size, PDI, zeta potential and pH values.

## RESULTS AND DISCUSSION

### PHYSICO-CHEMICAL PARAMETERS OF SOLID LIPID NANOPARTICLES

- *Particle size distribution and polydispersity index:* The particle size of SLN containing AP was measured at day of production and 90 days after. The average of particle sizes were 86.41 ± 0.30 nm at the time of preparation and 89.27 ± 0.86 nm for 90 days after. These results differ from found by Kristl et al. (2003) and Üner et al. (2005) who developed SLN containing AP and obtained the initial particle size of 150 nm increasing yo 228 nm after storage. Also, the mean particle size of SLN found by Pardike and cols was from40 to 1000 (PARDEIKE; HOMMOSS; MULLER, 2009).

The size is affected by numerous parameters such as the composition of the formulation (mixture of surfactants, incorporated drug), method of production and their established conditions such as preparation time, temperature, etc. These parameters are defined in order to improve the particle size and its polydispersity (ÜNER, 2006).

The PDI of the nanoparticles developed presented mean values of 0.204 ± 0.01, demonstrating adequate homogeneity in the distribution of particle size. The PDI indicates a narrow size distribution and values less than 0.3 are considered acceptable for colloidal suspensions (PAESE et al., 2009).

- *Zeta potential:* The values determined in our formulation were -12.20 ± 0.23 mV for initial mensure and -18.40 ± 0.29 mV 90 days after. The analysis of zeta potential allows the determination of stability and the drug-particle interaction. This parameter can be used to predict the stability, especially during storage. The results of zeta potential for SLN are in accordance with other studies that use the same surfactant, independent of the drug content. Stable resveratrol-loaded lipid-core nanoparticles presented zeta potential of −14.1 mV after preparation and values of −12.7, −13.2 and −13.2 mV for the analyses after 30, 60 and 90 days, respectively (FROZZA et al., 2010), indicating that the values obtained in the present study are satisfactory.

- *Determination of pH:* The values found for first pH determination and 90 days after were 4.34 ± 0.04 and 3.15 ± 0.13, respectively. This pH values prove the slightly acidic nature of this drug in SLN. The reduction indicates certain drug instability. Due to this limited stability, probably the pH reduction in SLN containing AP formulation is related to the molecule degradation by hydrolysis.

### VALIDATION OF THE ANALYTICAL HPLC METHOD FOR SOLID LIPID NANOPARTICLES CONTAINING ASCORBYL PALMITATE

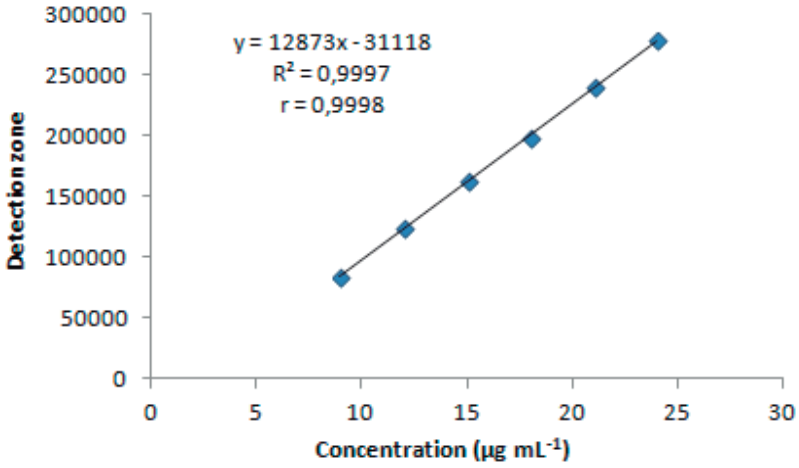
- *Linearity:* The relative standard deviation (RSD) was determined for each concentration used (Table 1). The analytical curve of SLN containing PA showed a significant linear regression ($p < 0.01$), and no significant deviation from linearity ($p > 0.01$).

**Table 1 -** Average areas of different concentrations of ascorbyl palmitate to prepare the calibration curve.

| Concentration / (µg mL$^{-1}$) | RSD / (%) |
|---|---|
| 9 | 2.71 |
| 12 | 3.38 |
| 15 | 0.84 |
| 18 | 2.61 |
| 21 | 1.25 |
| 24 | 2.42 |

As shown in table 1 the RSD values for each concentration tested were lower than 5%, maximum acceptable value recommended by the RE nº. 899/2003 (ANVISA, 2003). The straight line equation was obtained by linear regression studies between the concentration of AP and their respective areas, obtaining a correlation coefficient (r) of 0.9998, as observed in figure 2.

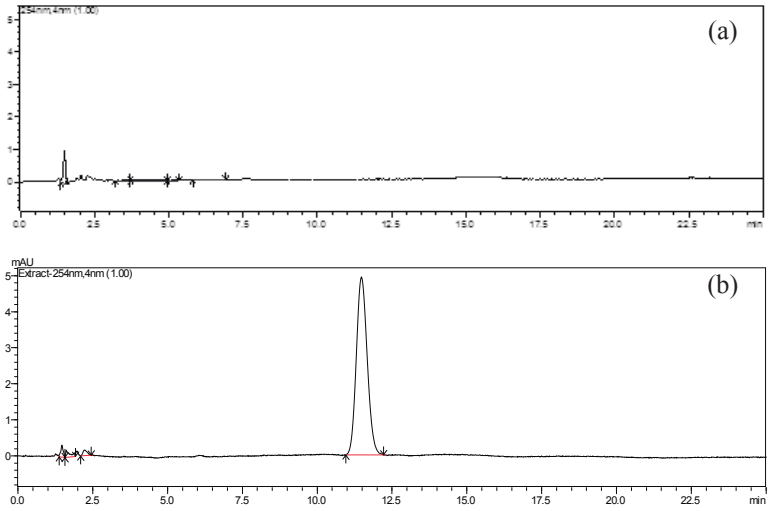
**Figure 2 -** Graphical representation of the average of the three standard curves, the concentration range from 9.0 to 24.0 µg mL$^{-1}$, quantified by HPLC.




Taken together, these data indicate that the calibration curve is suitable to be used for experimental quantification of AP. The quality of the analytical curve was demonstrated by the correlation coefficient that was higher than 0.99.

- *Specificity:* The method validated was specific for this type of analysis and is in agreement with the official specifications (ANVISA, 2003). Figure 3a shows the chromatogram obtained for SLN without AP and figure 3b shows the chromatogram obtained of SLN containing the active molecule at the concentration of 15.0 µg mL$^{-1}$.

**Figure 3** - Chromatograms of solid lipid nanoparticles without Ascorbyl Palmitate (a) and the solid lipid nanoparticles containing ascorbyl palmitate (b) at a concentration of 15.0 µg $mL^{-1}$, the chromatographic conditions developed.




As expected, no peak was observed in chromatogram from the SLN without the active molecule (Figure 3a). By other hand, a peak in the retention time of 11.47 min was detected in SLN with AP (Figure 3b). It was also confirmed by analysis of the photodiode detector arrays, a peak purity of 100%, indicating that the other components of the formulation did not affect the analysis. These data indicate that the method applied for extraction was efficient and present a good sensitivity to detection of the drug using HPLC and also this methodology has good specificity.

- *Precision:* The repeatability of the method was evaluated by the intraday RSD, and the intermediate precision was determined from the RSD interdays values. These RSD values obtained for AP extracted from SLN are listed in tables 2 and 3, which define the recovery values obtained for test repeatability and intermediate precision.

**Table 2** - Repeatability values for the quantification of Ascorbyl Palmitate when associated with solid lipid nanoparticles

| Repetitions | Concentrations / (µg $mL^{-1}$) | Sample content / (%) | RSD / (%) |
|---|---|---|---|
| N1 | 12.31 | 82.07 | 1.97 |
| N2 | 11.70 | 78.01 | |
| N3 | 12.10 | 80.69 | |
| N4 | 12.38 | 82.50 | |
| N5 | 12.08 | 80.55 | |
| N6 | 12.22 | 81.45 | |

As shown in table 2, the percentage of AP recovered/quantified in the repetitions N1 to N6 were between 78.01 and 82.50% and the RSD value was 1.97. The results demonstrate that there is compliance among the parameters set for the validation of chromatographic methods used.

**Table 3** - Values of intermediate precision for quantification of Ascorbyl Palmitate when associated with solid lipid nanoparticles.

| Day | Concentra-tion / (μg mL$^{-1}$) | Sample content / (%) | Average | RSD of day / (%) | RSD between average / (%) |
|---|---|---|---|---|---|
| 1st day | 11.94 | 79.61 | 79.29 | 0.31 | 1.42 |
| | 11.85 | 79.00 | | | |
| | 11.89 | 79.27 | | | |
| 2nd day | 12.21 | 81.42 | 81.99 | 0.86 | |
| | 12.23 | 81.57 | | | |
| | 12.45 | 82.97 | | | |
| 3rd day | 12.27 | 81.82 | 79.87 | 1.73 | |
| | 11.78 | 78.54 | | | |
| | 11.89 | 79.25 | | | |

At the 1st day of the analyses the percentage of sample content were between 79.00 and 79.61%, and the RSD value was 0.31%. At the 2nd day of analysis, the recovery percentage was between 81.42 and 82.97% and the RSD was 0.86%; and finaly aat the 3rd day, the values were between 78.54 and 81.82% with RSD of 1.73%. The initial values (~80%) are due to AP instability as consequence of oxidative degradation.

Considering the data observed for intermediate precision and repeatability the RSD values were all less than 5%, which is the maximum value recommended by the RE nº 899/2003 (ANVISA, 2003). Thus, these results indicate that the method presents has accuracy for AP quantification in HPLC method.

- *Accuracy:* The accuracy was determined from recovery tests, in triplicate, by addition of standard solution of known concentration in AP SLN samples, at 3 different levels: low, medium and high, corresponding to final concentrations of 12.5, 15.0 and 17.5 μg mL$^{-1}$. The percentage results obtained are shown in table 4.

**Table 4** - Experimental values of method accuracy for solid lipid nanoparticles containing ascorbyl palmitate.

| Theoretical concentration / (μg mL$^{-1}$) | Recovery concentration / (μg mL$^{-1}$) | Recovery / (%) | Average / (%) |
|---|---|---|---|
| 12.5 | 12.61 | 100.88 | 101.40 |
| 15.0 | 15.18 | 101.20 | |
| 17.5 | 17.87 | 102.11 | |

Considering that the initial percentage of AP in SLN was around 80% from the theoretical concentration, the amount of AP recovered in R1, R2 and R3 were satisfactory. The recovered percentages were between 100.88 and 102.11%. Again, it indicates that the method presents a good accuracy for the range of concentration used in the formulation.

- *Detection and quantification limits:* The values obtained for the detection and quantification limits were 2.30 and 6.96 μg $mL^{-1}$, respectively, which indicates a good sensitivity of the method for AP determining in SLN.

Thus, the results observed in tests for repeatability, intermediate precision and accuracy, are in agreement with the correlation coefficients obtained in the calibration curves. The values for the limits of detection and quantification showed that the proposed method is suitable to quantitative determination of AP loaded in SLN by HPLC.

## CONCLUSIONS

In this work, SLN prepared usign high shear rates technique, were efficient obtained. Shea butter worked as good lipid to encapsulate AP, since high drug load was achieved. Besides that, it was developed and validated an analytical method for quantification of AP, by high-performance liquid chromatography, according the ANVISA (2003) and ICH (2005) recommendations.

Thus, it can be concluded that SLN produces are in agreement with nanoscale, which was confirmed by physicochemical characterization of the nanoparticles. The validation was also satisfactory for all parameters, indicating that the validated method presents adequate sensitivity for quantification AP in SLN. The SLN loaded with AP developed in this study are ready to by evaluated as a clear agent for skin.

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