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# DEVELOPMENT AND VALIDATION OF AN ANALYTICAL METHOD FOR SIMULTANEOUS QUANTIFICATION OF ASCORBIC ACID AND ASCORBYL PALMITATE IN LIPOSOMES[1]

## *DESENVOLVIMENTO E VALIDAÇÃO DE UM MÉTODO ANÁLITICO PARA QUANTIFICAÇÃO SIMULTÂNEA DE ÁCIDO ASCÓRBICO E PALMITATO DE ASCORBILA EM LIPOSSOMAS*

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**ABSTRACT**

Ascorbic acid (AA) and ascorbyl palmitate (AP) are attractive antioxidants, widely used in food industry. But they have a low stability because they can be degraded by different factors. There are several studies using nanotechnology through encapsulation to protect these compounds and increase their stability in different particles, such as liposomes. After encapsulation of AA and AP in liposomes, the actives need to be quantified. The aim of this study was to develop and validate a HPLC method for simultaneous quantification of AA and AP in liposomes. The method was validated according to the International Conference on Harmonization guidelines for validation of analytical procedures and the following parameters: specificity, linearity, detection and quantification limits, precision, accuracy, and robustness. The mobile phase was composed of acetonitrile:$NaH_2PO_4$ buffer 0.02 mol.$L^{-1}$ pH 2.5:methanol (85:10:5, v/v) at a flow rate of 0.6 mL/min, isocratic elution and the column was a LiChroCART® 250-4. The chromatographic run was set to 13 minutes and AA and AP were detected at 243 nm. The method demonstrates specificity and has no interference from the excipients. The method showed linearity between 5 - 30 μg.$mL^{-1}$, and the correlation coefficient represented by $r^2 = 0.9995$ and $r^2 = 0.9996$ for AA and AP, respectively. The analysis of precision and accuracy showed a low relative standard deviation (<1.55%) and a sufficient recovery percentage of AA (95.77%) and AP (101.24%). The procedure provided specificity, linearity, precision, accuracy and robustness, indicating that the method can be applied to the quantitation of AA and AP in liposomes.

**Keywords:** vitamin C, HPLC, L-ascorbic acid.

***RESUMO***

*O ácido ascórbico (AA) e o palmitato de ascorbila (PA) são antioxidantes atraentes, amplamente utilizados na indústria de alimentos. Mas eles possuem uma baixa estabilidade porque podem ser degradados por diferentes fatores. Existem vários estudos usando nanotecnologia através do encapsulamento para proteger esses compostos e aumentar sua estabilidade em diferentes partículas, como lipossomos. Após a encapsulação do AA e PA nos lipossomas, esses ativos precisam ser quantificados. O objetivo*

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*deste estudo foi desenvolver e validar um método de CLAE para quantificação simultânea de AA e PA em lipossomas. O método foi validado de acordo com as diretrizes da Conferência Internacional sobre Harmonização para validação de procedimentos analíticos e seguindo os parâmetros: especificidade, linearidade, limites de detecção e quantificação, precisão, exatidão e robustez. A fase móvel foi composta de acetonitrila: tampão 0,02 M NaH2PO4 pH 2,5:metanol (85:10:5, v/v) a um fluxo de 0,6 mL.min$^{-1}$, eluição isocrática e a fase estacionária foi uma coluna LiChroCART® 250-4. A corrida cromatográfica foi ajustada para 13 minutos e AA e PA foram detectados a 243 nm. O método demonstra especificidade e não possui interferência dos excipientes. O método mostrou linearidade entre 5 - 30 µg.mL$^{-1}$ e coeficiente de correlação representado por $r^2$ = 0,9995 e $r^2$ = 0,9996 para AA e AP, respectivamente. A análise de precisão e exatidão demonstraram um baixo desvio padrão relativo (< 1,55%) e uma porcentagem de recuperação suficiente de AA (95,77 %) e PA (101,24 %). O método demonstrou especificidade, linearidade, precisão, exatidão e robustez, indicando que pode ser aplicado para a quantificação de AA e PA em lipossomas.*

***Palavras-chave:*** *vitamina C, CLAE, ácido L-ascórbico.*

## INTRODUCTION

Vitamin C (or ascorbic acid) is an attractive antioxidant as it has multiple functions in food. It can sequester oxygen molecules, change redox potential, act synergistically with chelators and regenerate primary antioxidants such as tocopherol (REISCHE, LILLARD, EITENMILLER, 2002). Among vitamins, however, it has a low stability, being unstable in the presence of light, heat, oxygen and neutral and basic pH, with restricted stability between pH 2.5 and 5.5 (ORDÓNEZ *et al.*, 2005; FOOD INGREDIENTS BRASIL, 2014).

Ascorbyl palmitate (or L-ascorbyl-6-palmitate) is a derivative of ascorbic acid used in foods containing fat, because its solubility in hydrophobic medium is better than that of ascorbic acid itself and its salts; however, it has low solubility in water and low chemical stability (YOKSAN; JIRAWUTTHIWONGCHAI; ARPO, 2010; REISCHE; LILLARD; EITENMILLER, 2002).

Ascorbic acid and ascorbyl palmitate can be beneficial to health (PRASHAR; KUMAR; YADAV, 2014), but stability only in specific situations makes these compounds have limitations of use (FOOD INGREDIENTS BRASIL, 2014; ORDÓNEZ *et al.*, 2005). Since ascorbic acid and ascorbyl palmitate are two unstable molecules, there are several studies using nanotechnology through encapsulation to protect these compounds and increase their stability, for example, to incorporate these compounds into solid nanoparticles, liposomes, nanocapsules and nanoemulsions (KRISTL *et al.*, 2003; WECHTERSBACH; ULRIH; CIGIĆ, 2012; FARHANG; KAKUDA; CORREDIG, 2012; TEERANACHAIDEEKUL; MÜLLER; JUNYAPRASERT, 2007; ZATTA, 2011). To date, there is only one coencapsulation study of ascorbic acid with vitamin E in liposomes, and there are no studies on the coencapsulation of ascorbyl palmitate and ascorbic acid in liposomes.

Liposomes are spherical vesicles that have one or more phospholipid bilayers wrapped in an aqueous nucleus (BRANNON-PEPPAS, 1993). The liposomes structure is organized according to polarity interactions (WINTERHALTER; LASIC, 1993). Structurally hydrophobic compounds are kept

in the lipid bilayer membrane, while hydrophilic compounds are found in the aqueous nucleus (BATISTA; CARVALHO; MAGALHÃES, 2007). Furthermore, liposomes are notable for being non-toxic, non-immunogenic and biodegradable, as well as being naturally amphipathic encapsulating hydrophobic, hydrophilic and amphiphilic compounds (DAUDT *et al*., 2013; BRANNON-PEPPAS, 1993).

There are several methods that can be employed to quantify ascorbic acid and ascorbyl palmitate in different matrices like nanoparticles (solid lipid nanoparticles), juices and fruits (ODRIOZOLA-SERRANO; HERNÃTEXTEXCLAMDOWNNDEZ-JOVER; MARTÍN-BELLOSO, 2007; SCHERER *et al*., 2012; DA SILVA *et al*., 2016). The physicochemical methods are more applicable because they are more accurate, faster and economical. This category includes titration, spectrophotometric, microfluorimetric and chromatographic methods (PENTEADO, 2003). The most commonly used chromatographic method for AA and PA analysis is high performance liquid chromatography (HPLC) (PENTEADO, 2003; ODRIOZOLA-SERRANO; HERNÃTEXT-EXCLAMDOWNNDEZ-JOVER; MARTÍN-BELLOSO, 2007; SCHERER *et al*., 2012; DA SILVA *et al*., 2016), because of its advantages as specificity, sensitivity, fastness and automation feasibility.

To ensure the reliability of the results it is necessary to develop and validate a chromatographic method for quantification of these actives in liposomes, since there are no methods in literature that simultaneously certify these two actives employed the same chromatographic conditions. The aim of the present study was to develop and validate a simple and reliable HPLC method for AA and AP quantification in liposomes suspensions, according to the current official guidelines.

## MATERIALS AND METHODS

### MATERIALS AND REAGENTS

Ascorbic acid (AA), ascorbyl palmitate (AP), and cholesterol were purchased from Sigma-Aldrich®. Polysorbate 80, ethyl alcohol, and monobasic anhydrous sodium phosphate were purchased from Synth®, vitamin E from Nova Derme® and Lipoid S-100 from Lipoid®. HPLC-grade acetonitrile and methanol were acquired from J.T. Baker®, phosphoric acid P.A from Nuclear®, and Milli-Q® water. Liposomes suspensions containing ascorbic acid and ascorbyl palmitate (LIP-PAC) were prepared by reverse phase evaporation method as described by Mertins *et al.* (2005), with modifications. The formulations were prepared with (1 $mg.mL^{-1}$) of each active compound and without the active compounds (LIP-B).

### APPARATUS AND CHROMATOGRAPHIC CONDITIONS

HPLC analysis was performed on a Prominence Shimadzu Liquid Chromatograph (Tokyo, Japan) equipped with a degasser DGU-20A 5R degasser, a LC-20AT pump, CBM-20A system controller,

a SIL-20A HT auto sampler, SPD-M20A detector and a CTO-20AC column oven. Analytical separation was performed on a LiChroCART® 250-4 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, 100Å) from Merck®. The mobile phase was composed of acetonitrile:$NaH_2PO_4$ buffer 0.02 mol.$L^{-1}$ pH 2.5:methanol (85:10:5, v/v) which was pumped at a flow rate of 0.6 mL.$min^{-1}$, isocratic elution. The mobile phase was filtered through a 0.45 µm regenerated cellulose membrane filter and degassed before use. The analytical column was kept at 25 °C. The chromatographic run was set to 13 minutes and the volume injected was 20 µL. AA and AP were detected at 243 nm.

## SAMPLE PREPARATION

Liposomes suspensions used for the evaluation of all parameters were freshly prepared. To release the AA and AP of the LIP-PAC, 150 µL of LIP-PAC suspensions was diluted with mobile phase to a concentration of 15 µg/mL and kept in sonication for 10 minutes. Lastly, the resulting solution was filtered through a 0.45 µm regenerated cellulose membrane and injected in the HPLC system (n=3).

## STANDARD SOLUTION

Two stock solutions were prepared, the first with AA and the second with PA. First stock standard solution (1.0 mg.$mL^{-1}$) was prepared solving 50.0 mg of AA in 50.0 mL of $NaH_2PO_4$ buffer 0.02 mol.$L^{-1}$ pH 2.5. The second stock standard solution (1.0 mg.$mL^{-1}$) was prepared solving 50.0 mg of PA in 50.0 mL of methanol. Both solutions were dissolved in an ultrasound bath for 10 minutes. From these solutions, a working standard of 15.0 µg.$mL^{-1}$ was prepared using 150 µL of the first and second stock standard solution in 10.0 mL of mobile phase. And from these two solutions the calibration curve was also prepared, using 50, 100, 150, 200, 250 and 300 µL of the first and second stock standard solution in 10.0 mL of mobile phase to give six standard solutions with different concentrations of AA and AP (5.0, 10.0, 15.0, 20.0, 25.0 and 30.0 µg.$mL^{-1}$), which were used in the linearity. All solutions were filtered through a 0.45 µm regenerated cellulose membrane and injected in the HPLC system (n=3).

## METHOD VALIDATION

The method was validated according to the International Conference on Harmonization guidelines for validation of analytical procedures and following the parameters: specificity, linearity, detection and quantification limits, precision, accuracy and robustness. The system suitability test was also carried out and the parameters measured were retention time, theorical plates, tailing factor, peak asymmetry factor and peak purity.

## Specificity

The specificity of the method was determined by comparative analyzes of the liposomes without AA and AP with liposomes containing the actives at a concentration of 15.0 μg.mL$^{-1}$. The samples were analyzed under the same conditions, and it was verified the presence of interference or overlaps of the excipients which are part of the formulation with AA and AP responses. Besides that, we also evaluated the specificity by analyzing the purity of the peaks.

## Linearity, detection and quantification limits

Linearity was evaluated by the injection an analysis of six concentrations of standard stock solutions in AA and AP concentrations of 5.0, 10.0, 15.0, 20.0, 25.0 and 30.0 μg.mL$^{-1}$, as described in the "Standard solution" section. Three independent calibration curves were constructed, and linearity was evaluated by the least-squares regression analysis. Detection (LOD) and quantification (LOQ) limits were calculated directly from the calibration plot. LOD and LOQ were calculated based on 3.3 σ/S and 10/σ, respectively, where σ is the standard deviation of the intercept and S is the slope of the calibration plot (ICH, 2005).

## Precision

The precision was assessed by repeatability and intermediate precision. Repeatability (intra-day precision) was evaluated by measuring, in triplicate, six sample solutions at the same concentration (15.0 μg.mL$^{-1}$) in a single day under the same experimental conditions. For the intermediate precision (inter-day precision) samples with the same concentration (15.0 μg.mL$^{-1}$) were analyzed in three different days by two different analysts. Precision (repeatability and intermediate precision) was expressed as the relative standard deviation (RSD %).

## Accuracy

The accuracy was evaluated assaying, in triplicate, samples of known concentrations of LIP-PAC (15.0 μg.mL$^{-1}$) spiked with three different concentrations of AA and AP standard solution at three different levels (lower - 25 %, medium - 50 %, and upper concentration - 75 %), giving sample solutions with concentrations of 18.75, 22.5, and 26.25 μg.mL$^{-1}$. Recovery (%) was calculated from differences between the concentration obtained for spiked and unspiked solutions and expressed as the relative standard deviation (RSD %) of the triplicate.

### Robustness

Robustness was evaluated by the deliberate variation of the sample sonication time (5 and 15 minutes), mobile phase [acetonitrile:$NaH_2PO_4$ buffer 0.02 mol.$L^{-1}$ pH 2.5:methanol (80:10:10, v/v) and acetonitrile:methanol:$NaH_2PO_4$ buffer 0.02 mol.$L^{-1}$ pH 2.5 (85:10:5, v/v)] and wavelength (238 nm and 248 nm). Sample solutions were evaluated (n = 3) for each variation of the method conditions.

## RESULTS AND DISCUSSIONS

In this study, chromatographic conditions were adjusted in order to obtained efficient routine analysis. Acetonitrile, methanol and $NaH_2PO_4$ buffer 0.02 mol.$L^{-1}$ pH 2.5 were chosen as the mobile phase based on the composition described by Da Silva *et al.* (2016) and Illanes *et al.* (2013).

After the mobile phase selection, the method was optimized to provide an acceptable theorical plates (4311 ± 21 and 10179 ± 57), tailing factor (1.35 ± 0.01 and 1.21 ± 0.00), peak asymmetry factor (1.30 ± 0.00 and 1.17 ± 0.00) and resolution (21.58 ± 0.22) for AA and AP, respectively. The retention time of AA and AP was 3.46 ± 0.01 and 10.02 ± 0.11 minutes, respectively, which allows rapid assay of the active compounds, but is long enough to ensure the separation efficiency and adequate system suitability values. Figure 1 shows the chromatograms obtained from the LIP-PAC, LIP-B and the reference standard solutions.

**Figure 1 -** Chromatograms of blank liposome (LIP-B), liposomes containing ascorbic acid and ascorbyl palmitate (LIP-PAC) and ascorbic acid and ascorbyl palmitate solubilized with mobile phase (F-PAC).

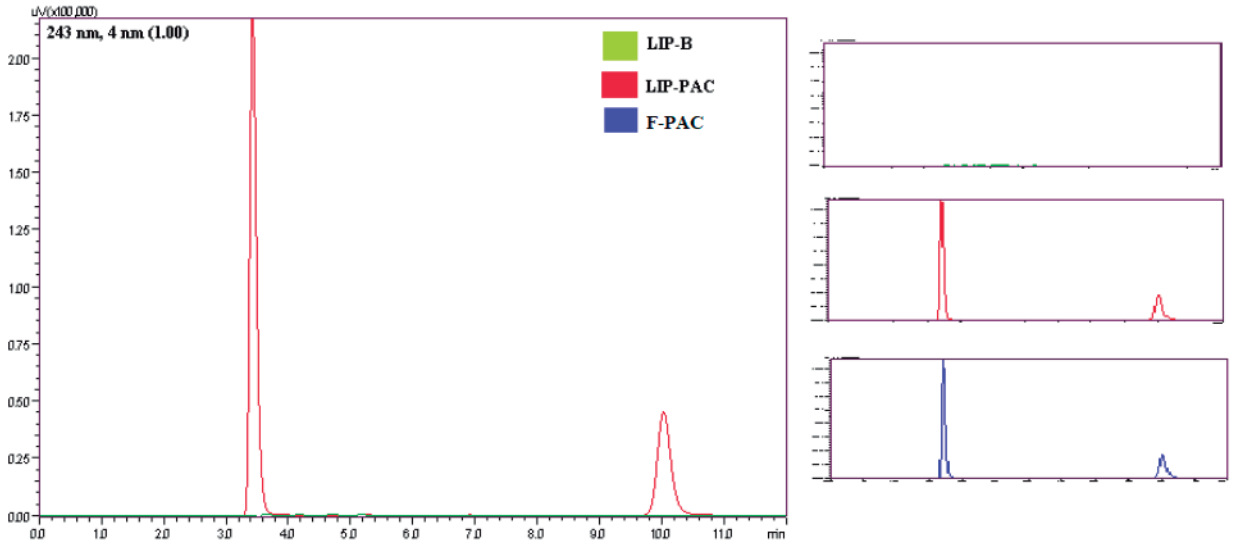


Figure 1 shows the specificity of the method, and it demonstrates that the method is specific, and no interference from the excipients was observed. In order to confirm this absence of interference,

a peak-purity evaluation using the photodiode array was carried out. This analysis showed that the AA and AP peaks are pure and have no impurities and/or excipients which co-elutes with the AA and AP peak. Fontana, Bastos and Beck (2010) developed a method for quantifying clobetasol in topical nanocapsules suspensions by HPLC. The authors also use the blank nanoformulation to a specificity test, and the method developed by them was specific for the quantification of the active in nanoparticles and showed no interference of the excipients of nanocapsules in the quantification of the active.

The analytical curves for AA and AP were constructed by plotting concentration versus peak area. The calibration plot for the method was linear over the concentration range of 5 - 30 $\mu g.mL^{-1}$. The linear equation obtained by the least-square method was y = 108 021 (mAU $\mu g.mL^{-1}$) x + 102 150 (mAU) showing an adequate linear regression ($r^2 = 0.9995$) for the AA, and for the AP was y = 37 485 (mAU $\mu g.mL^{-1}$) x + 56 871 (mAU) showing an adequate linear regression ($r^2 = 0.9996$). The values obtained for the LOD and LOQ were 0.41 and 1.23 $\mu g.mL^{-1}$ for AA, 1.12 and 3.36 $\mu g.mL^{-1}$ for AP, respectively, which indicates a good sensitivity of the method for AA and AP determining in liposomes. It is important to note that the LOQ values are within the linear range of the method. Repeatability (intra-day precision) and intermediate precision (inter-day precision) are showed in Table I.

**Table 1 -** Repeatability (intra-day) and intermediate (inter-day) precision of the method for quantifying ascorbic acid (AA) and ascorbyl palmitate (AP) in liposomes (sample solutions with a theoretical concentration of 15.0 $\mu g.mL^{-1}$).

| Parameter | N | Relative standard deviation (RSD %) of AA | Relative standard deviation (RSD %) of AP |
|---|---|---|---|
| **Intra-day precision** | 6 | 0.65 | 0.78 |
| **Inter-day precision** | | | |
| **Day 1** | 3 | 0.36 | 0.28 |
| **Day 2** | 3 | 0.22 | 0.27 |
| **Day 3** | 3 | 0.22 | 0.12 |
| **Day 1+2+3** | 9 | 0.27 | 0.22 |

According to Table 1, the intermediate precision and repeatability the RSD values were all less than 5%, which is the maximum value recommended by the RE nº 166/2017 (BRASIL, 2017) and international guidelines (ICH, 2005). Thus, these results indicate that the method presents has precision for AA and AP quantification by HPLC method. Da Silva, Alves and Junior (2016) developed a method for quantifying ascorbyl palmitate in solid lipid nanoparticles by HPLC. The method developed by them demonstrated an RSD of 1.97 and 1.42% for repeatability and intermediate precision, respectively. These values are higher than those found in the present work for repeatability and intermediate precision for AA and AP, but they are still within the legislation and international guidelines (BRASIL, 2007; ICH, 2005).

Tab. 2 shows the accuracy of the method for quantifying ascorbic acid and ascorbyl palmitate in liposomes.

**Table 2 -** Accuracy of the method for quantifying ascorbic acid and ascorbyl palmitate in liposomes.

| Active compound | Know sample (μg.mL$^{-1}$) | Added (μg.mL$^{-1}$) | Found (μg.mL$^{-1}$) | Recovery (%) | Relative standard deviation RSD (%) |
|---|---|---|---|---|---|
| AA | 15.00 | 3.75 | 16.33 | 87.08 | 1.29 |
| | 15.00 | 7.5 | 18.24 | 81.08 | 0.68 |
| | 15.00 | 11.25 | 25.14 | 95.77 | 1.23 |
| AP | 15.00 | 3.75 | 18.76 | 100.04 | 0.87 |
| | 15.00 | 7.5 | 22.78 | 101.24 | 0.88 |
| | 15.00 | 11.25 | 27.86 | 106.11 | 1.55 |

According to Table 2, the results for accuracy presented a good recovery in the range of 81 to 96% for AA and 100 to 106% for AP, indicating that the values are satisfactory, and the relative standard deviation of all concentrations was below 2%. Literature studies that quantify by HPLC drugs in different nanoparticles found recovery values close to 100 % (DA SILVA, ALVES, JUNIOR, 2016; FONTANA, BASTOS, BECK, 2010).

Tab. 3 shows the robustness of the method for quantifying ascorbic acid (AA) and ascorbyl palmitate (AP) in liposomes.

**Table 3** - Robustness of the method for quantifying ascorbic acid (AA) and ascorbyl palmitate (AP) in liposomes (sample solutions with a theoretical concentration of 15.0 μg.mL$^{-1}$).

| Conditions | % AA | Relative standard deviation (RSD %) of AA | %AP | Relative standard deviation (RSD %) of AP |
|---|---|---|---|---|
| **Mobile phase** | | | | |
| Acetonitrile:$NaH_2PO_4$ buffer 0.02 mol.L$^{-1}$ pH 2.5:methanol (80:10:10, v/v) | 97.69 | 0.74 | 103.05 | 0.55 |
| Acetonitrile:$NaH_2PO_4$ buffer 0.02 mol.L$^{-1}$ pH 2.5:methanol (85:5:10, v/v) | 97.41 | 0.85 | 103.70 | 0.31 |
| **λ (nm)** | | | | |
| 238 | 90.30 | 0.45 | 88.86 | 0.21 |
| 248 | 101.23 | 0.58 | 98.85 | 0.24 |
| **Sonication time (min)** | | | | |
| 5 | 96.02 | 1.62 | 93.55 | 0.49 |
| 15 | 106.41 | 1.54 | 104.57 | 0.57 |

Regarding the evaluation of robustness, the deliberate variation of the method conditions (mobile phase, λ and sonication time) had no significant effect on assay data or on chromatographic performance, indicating the robustness of the method. No statistical difference was found with the original method by changing the mobile phase of the method to 85:5:10 and 80:10:10 (v/v) of acetonitrile:$NaH_2PO_4$ buffer 0.02 mol.L$^{-1}$ pH 2.5:methanol, the wavelength to 238 and 248 nm and the sonication time of the sample to 5 and 15 minutes.

After all analysis performed for the simultaneous quantification of AA and AP in liposomes, it is recommended to use a LiChroCART® 250-4 column (250 mm x 4 mm, 5 μm particle size)

protected by a Lichrospher® 100 RP-18 pre-column (250 mm x 4 mm, 5 µm, 100Å). The defined mobile phase was composed of acetonitrile:$NaH_2PO_4$ buffer 0.02 mol.$L^{-1}$ pH 2.5:methanol (85:10:5, v/v) with a flow rate of 0.6 mL.$min^{-1}$ and isocratic elution. The analytical column was kept at 25°C. The chromatographic running time required for the quantification of AA and AP was 13 minutes, the volume injected was 20 µL and both actives were detected at 243 nm.

It was possible to develop and validate a chromatographic method for AA and AP quantification in liposomes, which was simple, fast, isocratic and allows simultaneous quantification of the two actives using the same chromatographic conditions. It is important to emphasize the importance of developing and validating a chromatographic method that quantifies both liposome actives, because without this method it would be impossible to quantify both actives in the same time and ensure the quality of liposomal formulations containing AA and AP. Without this method, a separate method would be needed to quantify each active, which results in increased time, solvents and HPLC operating time.

## CONCLUSION

A simple, fast, isocratic, economic and reliable HPLC method has been developed and validated for the simultaneous quantification of ascorbic acid and ascorbyl palmitate in suspensions of liposomes. The method provides adequate system suitability, specificity because there is no interference from the excipients of the formulations and the peaks of AA and AP are pure and have no impurities and/or excipients which co-elutes with them. The linear equation obtained by the least-square method showed an adequate linear regression demonstrating that the method is linear in the concentration range of 5.0 - 30.0 µg.$mL^{-1}$. The values obtained for the LOD and LOQ indicates a good sensitivity of the method for AA and AP determining in liposomes. The precision RSD values were all less than 5% indicating that the method has precision for AA and AP quantification by HPLC method. Accuracy and robustness demonstrated satisfactory values for AA and AP recovery and the relative standard deviation of all concentrations was below 2%. Hence, this HPLC method can be used as a tool to ensure quality control of liposomal formulations containing ascorbic acid and ascorbyl palmitate.

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