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Article

A stability-indicating HPLC-PDA method for the determination of ferulic acid in chitosan-coated poly(lactide-co-glycolide) nanoparticles

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The development and validation of a simple and efficient method for the quantification of ferulic acid in poly (D,L-lactide-co-glycolide) (PLGA) nanoparticles coated with chitosan (CS) by reverse phase high performance liquid chromatography coupled to photodiode array detection was described. For the chromatographic analysis, a reverse phase C-18 column was used, mobile phase consisting of acetonitrile and 0.5% acetic acid (37:63, v/v), isocratically eluted at a flow rate of 1 mL/min. Drug determination was performed at 320 nm. The method was validated in terms of the selectivity, linearity, precision, accuracy, robustness, limits of detection and quantification. The method was linear in the range of 10 to 100 µg/mL (r=0.999) and presented limit of detection and quantification of 102 ng/mL and 310 ng/mL, respectively. The method was precise (intra and inter-day) based on relative standard deviation values (less than 3.20%). The recovery was between 101.06 and 102.10%. Robustness was demonstrated considering change in mobile phase proportion. Specificity assay showed no interference from the components of nanoparticles or from the degradation products derived from acidic and oxidative conditions. The proposed method was suitable to be applied in determining the encapsulation efficiency of ferulic acid in PLGA-CS nanoparticles and can be employed as stability indicating one.

Uniterms: Nanoparticles. Ferulic acid/encapsulation efficiency. Ferulic acid/quantification. Stability. High performance liquid chromatography/validation. PLGA-poly(lactide-co-glycolide)/nanoparticles. Chitosan.

INTRODUCTION

Trans ferulic acid (FA) [(E) -3- (4-hydroxy-3-methoxy-phenyl) propyl-2-enoic acid] is a hydroxycinnamic acid and polyphenolic present in a variety of cereals, fruits and vegetables, such as rice, coffee, wheat, apple, peanut (Wu *et al.*, 2014), artichoke, eggplant and corn meal (Trombino *et al.*, 2013). It presents bonded to the cell wall of plants, usually linked to proteins and polysaccharides, thus, it is rarely found in its free form (Aceituno-Medina *et al.*, 2015). Studies indicate the potential use of FA as anti-inflammatory, antithrombotic, antitumor, antiviral, immunoprotective, antibacterial, protector against ultraviolet rays and especially as antioxidant (Kim *et al.*, 2013; Lima, Duarte, Esteves, 2013; Yang, Song, 2015). However, FA has some limitations that affect its therapeutic efficacy when orally administered. It presents broad hepatic metabolism (Trombino *et al.*, 2013), reduced half-life, low aqueous solubility (Zhou *et al.*, 2015), reduced ability to penetrate biological membranes (Trombino *et al.*, 2013; Yang, Song, 2015), instability against oxidation and low cellular uptake (Kim *et al.*, 2013). An alternative to overcome physicochemical, biopharmaceutical and pharmacokinetics drawbacks of drugs administered by oral route is the use of nanocarrier systems, such as the polymeric nanoparticles.

Polymeric nanoparticles improve drug absorption and bioavailability, promote prolonged and/or specific drug release, improving the drug uptake by target cells, and thus, decreasing its toxicity (De Jong, Borm, 2008). The appropriate characterization of nanoparticles is an

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important step to ensure the therapeutic efficacy. The amount of drug-loaded in nanoparticles, drug stability and drug delivery profile must be adequately determined, and thus, suitable and validated analytical methods are necessary (Das Neves *et al.*, 2010).

FA has been quantified by many analytical methods, such as high-performance liquid chromatography (HPLC) coupled with photodiode array wavelength detector (PDA) or UV-Vis detector (Anselmi *et al.*, 2006; Craparo *et al.*, 2009; Kareparamban *et al.*, 2013; Li *et al.*, 2007; Li *et al.*, 2004; Li, Bi, 2003; Lu *et al.*, 2005; Nadal *et al.*, 2015; Picone *et al.*, 2009; Qi *et al.*, 2007; Wang *et al.*, 2011), liquid chromatography tandem mass spectrometry (Guy *et al.*, 2009; Wang *et al.*, 2013; Zhang *et al.*, 2009), UV-Vis spectroscopy (Lima *et al.*, 2013; Merlin *et al.*, 2012), thin layer chromatography (Mabinya, Mafunga, Brand, 2006), high-performance thin layer chromatography (Hingse, Digole, Annapure, 2014; Srivastava, Singh, Singh Rawat, 2012), gas chromatography (Olthof *et al.*, 2003), chemiluminescence (Shen *et al.*, 2013), capillary electrophoresis (Lima, Duarte, Esteves, 2007), micellar electrokinetic chromatography (Guo *et al.*, 2003), electrochemical analysis (Vilian, Chen, 2014) and paper-based platforms (Tee-Ngam *et al.*, 2013). However, HPLC is considered the most reliable and popular methodology for investigating phenolic acids (Barberousse *et al.*, 2008).

Moreover, most of these studies are about quantification of FA coupled with other phenolic compounds or other components extracted from plants (Andreasen *et al.*, 2000; Guo *et al.*, 2003; Sen *et al.*, 1991; Srivastava, Singh, Singh Rawat, 2012; Vichapong *et al.*, 2010; Waldron *et al.*, 1996), or about the quantification of FA in rat plasma (Li, Bi, 2003; Qi *et al.*, 2007; Rondini *et al.*, 2004). There are few studies about the analysis of FA in products with pharmaceutical potential and among these are even rarer those with a documented validation study (Nadal *et al.*, 2015). Some studies show the determination of FA in lipid nanoparticles (Bondi *et al.*, 2009; Carbone *et al.*, 2014; Trombino *et al.*, 2013), metallic (Vilian, Chen, 2014), magnetic (Saikia *et al.*, 2013) and in poly(lactide-co-glycolide) (PLGA) nanoparticles by UV spectrophotometer (Merlin *et al.*, 2012).

Thus, aiming the adequate characterization of the FA-loaded nanoparticles and to supply the lack of suitable validated methods for quantification of FA in pharmaceutical dosages, in this work it was developed and validated a simple and fast analytical methodology by reverse phase HPLC-PDA to quantify FA in PLGA nanoparticles coated with chitosan (CS).

MATERIAL AND METHODS

Chemical and reagents

CS (medium molecular weight, 75-85% deacetylated), FA (99% purity), PLGA (65:35, 40000-75000 Da), and polyvinyl alcohol (PVA, 31KDa, 88% hydrolyzed) were purchased from Sigma Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile and ethanol were obtained from LiChrosolv-Merck (Darmstadt, HE, Germany). Acetic acid was purchased from Vetec Química Fina (Duque de Caxias, RJ, Brazil), dichloromethane from Fmaia (Belo Horizonte, MG, Brazil), hydrochloric acid from Dinâmica (Diadema, SP, Brazil) and hydrogen peroxide and sodium hidroxyde were obtained from Biotec (Pinhais, PR, Brazil). The water used was purified with a Milli-Q Gradient® (Millipore®, Darmstadt, HE, Germany), with 18.2 conductivity MΩ/cm.

Chromatographic conditions

For the development and validation of the methodology, it was used a HPLC Waters 2695 Alliance (Waters®, Milford, MA, USA) combined with a PDA Waters 2998 (Waters®, Milford, MA, USA). HPLC system was equipped with a column compartment with temperature control, on line degasser, quaternary pump, auto sampler and auto injector. The analyses were realized using a reverse phase C18 chromatograph column (Vertical®, Bangkok, Thailand) with 5 μm particle size, 4.6 mm internal diameter and 250 mm length.

To ensure optimal chromatographic conditions, variations in the proportion of components of the mobile phase, the sample dilution solvent, the flow rate and in the injection volume were performed. The most appropriate chromatographic conditions were selected from the chromatographic peak characteristics and subsequently validated. The mobile phase was composed of acetonitrile and 0.5% acetic acid (37:63, v/v), eluted at a flow rate of 1.0 mL/min in isocratic mode. The injection volume was 10 μL and the analyses were performed at 320 nm at 25.0±1.0 °C.

Preparation of standard and sample solutions

A standard stock solution of FA (1 mg/mL) was prepared in acetonitrile. After, subsequent dilutions in acetonitrile were performed in order to obtain six standard solutions (10; 30; 50; 60; 80 and 100 μg/mL). FA samples corresponded to supernatant originated after ultracentrifugation of nanoparticles containing FA, as

described further. Prior to injection, standard and sample solutions were filtered through 0.22 μm filter pore size.

System suitability

The system suitability was carried out based on the analysis of five replicates of FA standard solution (50 μg/mL). The system performance was evaluated by the following parameters: number of theoretical plates (N), tailing factor (T) and retention factor (k).

Method validation

Validation was conducted following the guide's recommendations International Conference on Harmonization (ICH) (ICH, 2005) and AOAC International Standard (AOAC) (AOAC, 2016). The parameters used for this validation were linearity, range, accuracy, precision, limit of detection and limit of quantification, specificity and robustness.

Specificity

The specificity was evaluated by comparing the chromatograms obtained for the supernatant of blank nanoparticles (without FA) and chitosan solution with chromatograms of samples containing FA and FA standard solution.

Linearity and range

Linearity of the method was evaluated from the construction of three independent calibration curves (peak area *versus* drug concentration) using six FA standard solutions (10; 30; 50; 60; 80 and 100 μg/mL). The linearity was evaluated by the linear regression and the correlation coefficient (r), and can be considered satisfactory if (r)≥0.99. The statistical analysis to evaluate the linearity and deviation from linearity was performed by analysis of variance (ANOVA).

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were obtained based upon the slope (S) of the calibration curve and least standard deviation obtained from the response (σ), according to Eq. 1 and Eq. 2 (ICH, 2005) from a specific calibration curve constructed by analysis in triplicate of five FA standard solutions with concentrations of 0.5; 2; 5; 7 and 10 μg/mL:

$$LOD = 3.3 \times \frac{\alpha}{S} \qquad \text{Equation 1}$$

$$LOQ = 10 \times \frac{\alpha}{S} \qquad \text{Equation 2}$$

Precision: repeatability and intermediate precision

The repeatability of the method was evaluated by analysis of FA sample solution (supernatant of FA-loaded nanoparticles) in concentrations of 10; 50 and 100 μg/mL, and for each concentration, five solutions were injected on the same day, in a short period of time. To the intermediate precision, samples were analyzed in the same way, however repeated on three different days. The precision was expressed as mean ± standard deviation (SD) and relative standard deviation (RSD).

Accuracy

The accuracy was verified by spiking blank nanoparticles with known concentrations of FA solution to obtain final concentrations of 10, 50 and 100 μg/mL, analyzed in quintuplicate. It was determined the RSD and the percentage of recovery (Eq. 3).

$$Recovery = 100 \times \frac{average\ measured\ concentration}{theorical\ concentration} \qquad \text{Equation 3}$$

Robustness

Robustness was determined by changes in the ratio of mobile phase (acetonitrile:0.5% acetic acid - 35:65 and 39:61 v/v) and in the flow rate (0.95 and 1.05 mL/min). There were used FA solutions with concentrations of 10, 50 and 100 μg/mL. The percentage of recovery and the RSD were determined, and to verify the presence of significant difference, analysis of variance (ANOVA) with Tukey's multiple comparisons test were performed ($p<0.05$).

Forced Degradation Studies

For evaluation of drug stability and selectivity with respect to degradation products, FA standard solutions (50 μg/mL) were subjected to forced degradation. Solutions were exposed during 24 h to basic hydrolysis (1 mol/L NaOH – pH: 13.50), acid hydrolysis (1 mol/L HCl – pH 0.39), oxidative reaction (3% H_2O_2), visible light, and temperature of -20 °C.

Method applicability

Nanoparticles containing FA were obtained by the single-emulsion solvent evaporation method. Briefly, an organic solution was prepared by the dissolution of PLGA and FA in ethanol (200 μL) and dichloromethane (1.8 mL). The aqueous phase consisted of 10 mL of chitosan solution (0.16% w/v) and polyvinyl alcohol (PVA) (1%, w/v), both

dissolved in 2% acetic acid (v/v). The organic phase was added to the aqueous phase and sonicated for 5 min using a probe sonicator (QR1000, Eco-Sonic®, Indaiatuba, SP, Brazil) to produce an oil-in-water emulsion. Next, the organic solvent was evaporated under vacum for 15 min at 37 °C by a rotary evaporator (TE 120 - Tecnal®, Piracicaba, SP, Brazil). The nanoparticles were recovered and isolated of the free drug non-encapusulated by centrifugation (19000 rpm, 20 °C, 45 min) (Z36HK Centrifuge, Hermle® Wehingen, BH, Germany). The precipitate was freeze-dried (dispersed in cryoprotectant sucrose 15%, w/v) and stored for posterior use.

Mean diameter and polydispersity index (PDI) were analyzed by photon correlation spectroscopy using a Dynamic Light Scattering Brookhaven 90 Plus (Brookhaven®, Blue Point Road Holtsville, NY, United States), at 25 °C, in 90° scattering angle and wavelength of 659 nm. The determination of the amount of FA encapsulated into nanoparticles was performed indirectly. The supernatant obtained after ultracentrifugation of the nanoparticle dispersion, which contained the free drug, was diluted in acetonitrile (1:100 v/v), filtered on membrane pore 0.22 µm and analyzed by HPLC using methodology previously validated. The encapsulation efficiency (EE%) was obtained from Eq. 4 and expressed as mean EE% and SD. Analyses were performed in triplicate.

$$EE\% = 100 \times \frac{(Initial\ FA - Free\ FA)}{Initial\ FA} \qquad \text{Equation 4}$$

where Initial FA represents the amount of FA added to the formulation of nanoparticles and free FA represents the amount of free drug not incorporated into the nanoparticles, quantified by HPLC in the supernatant.

RESULTS AND DISCUSSION

Method development

British Pharmacopoeia provides a method for quantifying FA by HPLC, but its elution is by gradient, using phosphoric acid and acetonitrile (British Pharmacopoeia Commission, 2011). An isocratic elution methodology presents greater simplicity of execution, low cost and reduced time. Literature describes solvents, such as acetonitrile, methanol, acetic acid, orthophosphoric acid, acetate buffer solution and ultrapure water for FA determination in plants, plasma and some lipid particles (Carbone *et al.*, 2014; Li *et al.*, 2008; Li, *et al.*, 2004; Li, Bi, 2003; Lu *et al.*, 2005; Seo *et al.*, 2011; Trombino *et al.*, 2013; Wang *et al.*, 2015). Based on literature, initially it was tested several proportions of acetonitrile and 0.5% glacial acetic acid as mobile phase using a flow rate of 0.8 mL/min. With low proportions of acetonitrile (less than 20%), irregular and tailing peaks were obtained, therefore, the proportion of the mobile phase was changed to increase the amount of acetonitrile. The best peak, in relation to its symmetry and width was found using a flow rate of 1.0 mL/min and a mobile phase composed of acetonitrile: 0.5% acetic acid (37:63 v/v). In these conditions, FA was detected in round to 4.5 min (Figure 1), a time that allows a large number of analyzes in a short time and with low cost with reagents.

The system suitability of this method was evaluated based on the number of theoretical plates, peak symmetry (described by the tailing factor) and retention factor during the run of FA standard solution over five repetitions. The results presented in Table I and chromatographic parameters are in accordance with the criteria established by the US FDA (1994).

TABLE I - System suitability of the HPLC method (n=5)

Chromatographic parameter	Result	Acceptance criteria
Number of theorical plates (N)	3211.07 ± 29.88	N > 2000
Tailing factor (T)	1.09 ± 0.02	T ≤ 2
Retention factor (k)	2.15 ± 0.00	k > 2

Method validation

Linearity and range

Linearity indicates the ability of a method to demonstrate the results obtained are directly proportional to the concentration of analyte existing in the sample (ICH, 2005). In the present study, linearity was analyzed based on the construction of a calibration curve with six different concentrations between 10 and 100 µg/mL and by calculating the regression and correlation coefficient equation (r) by the method of least squares.

An (r) of 0.999 was obtained, evidencing the linearity of the method in this range. Additionally, the calculated line equation, y = 54629.2405 (±932.5326) x + 48094.7369 (±24960.6400), was used in subsequent assays for quantification of the standard and sample solutions. The validity of the test was confirmed by ANOVA, which showed the significance of the regression and that the deviation from linearity was not significant ($p < 0.0001$).

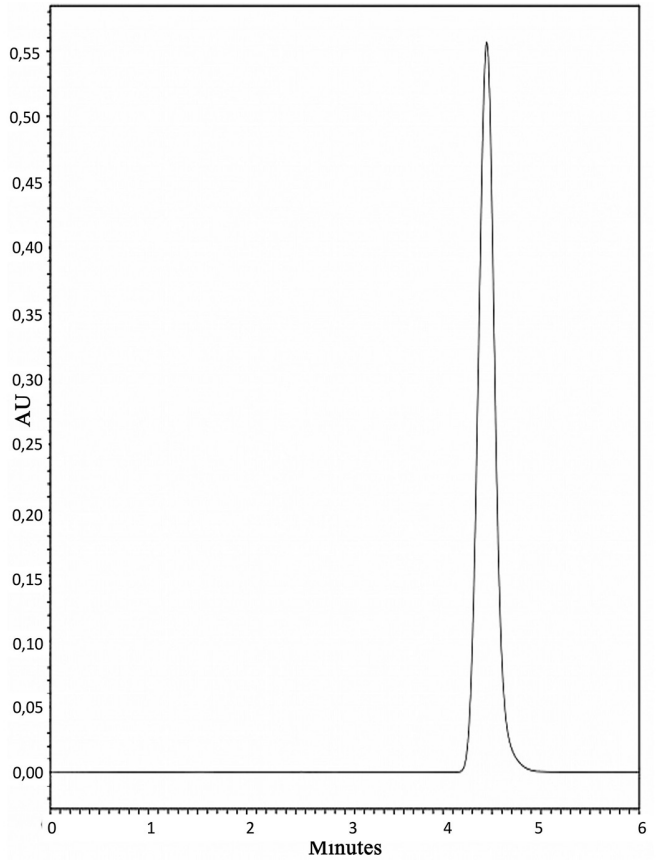

FIGURE 1 - Representative chromatogram of 100 µg/mL FA standard solution. Mobile phase: acetonitrile:0.5% acetic acid (37:63 v/v), flow rate: 1.0 mL/min, λ: 320 nm.

LOD and LOQ

LOD is the lowest amount of analyte, which can be detected in a sample, but not necessarily quantified. LOQ demonstrates the lowest amount of analyte determined with acceptable accuracy and precision (ICH, 2005). For these analyses, a specific calibration curve with concentrations below the expected range was constructed and analyzed by linear regression. From the SD of the intercept with the y-axis and the curve slope, LOD and LOQ were calculated.

The (r) found was 0.99917, thus meeting recommendations (r of at least 0.99) and confirming the linearity of the method ($p<0.0001$ - ANOVA). From line equation, y = 55890.4530 (±796.0461) x - 7244.7629 (±1730.6330), it was possible to calculate the LOD (102.18 ng/mL) and LOQ (309.65 ng/mL).

Precision: repeatability and intermediate precision

FA sample solutions in low, medium and high concentrations (10, 50, and 100 µg/mL) were prepared in quintuplicate and analyzed on the same day (intra-day analysis) or on three different days (inter-day analysis) to demonstrate the precision at level of repeatability and intermediate precision, respectively. According to AOAC (2016), to be considered a precise method, the RSD should not exceed 5.3% for solutions of 100 µg/mL and 7.3% at concentrations of 10 and 50 µg/mL. In this assay, there were obtained RSD below the recommended limit, being the highest value of 3.15% (Table II). Therefore, the precision of the method was confirmed.

TABLE II - Precision assay: repeatability and intermediate precision (n=5)

FA sample solution (µg/mL)	Quantified concentration ± SD (µg/mL)	Recovery ± SD (%)	RSD (%)
Repeatability			
10	9.74 ± 0.31	97.40 ± 3.07	3.15
50	49.30 ± 0.88	98.60 ± 1.76	1.79
100	101.60 ± 1.34	101.609 ± 1.34	1.32
Intermediate precision			
Day 1			
10	9.74 ± 0.31	97.40 ± 3.07	3.15
50	49.30 ± 0.88	98.60 ± 1.76	1.79
100	101.60 ± 1.34	101.60 ± 1.34	1.32
Day 2			
10	10.13 ± 0.12	101.29 ± 1.25	1.23
50	50.53 ± 1.40	101.06 ± 2.79	2.77
100	102.10 ± 1.25	102.10 ± 1.25	1.23
Day 3			
10	9.89 ± 0.27	98.88 ± 2.67	2.70
50	48.00 ± 1.05	96.00 ± 2.09	2.18
100	101.59 ± 1.18	101.59 ± 1.18	1.16

FA: Ferulic acid; SD: Standard deviation; RSD: Relative standard deviation

Accuracy

Accuracy of the method was demonstrated by the percentage of recovery of three different concentrations of FA solutions (10, 50 and 100 µg/mL) spiked in blank nanoparticles. Results are expressed in Table III, indicating the accuracy of the method. According to the AOAC (2016), to be considered an accurate method, the recovery values should be between 90 and 107% for 100 µg/mL solution and between 80 and 110% for 50 and 10 µg/mL.

Specificity

Specificity of the method was evaluated by comparing chromatograms of potential formulation interferences (supernatant from blank nanoparticles and

TABLE III - Percent recovery and RSD obtained by the accuracy test (n=5)

Standard solution (µg/mL)	Quantified concentration ± SD (µg/mL)	Recovery ± SD (%)	RSD (%)
10	10.13 ± 0.12	101.29 ± 1.25	1.23
50	50.53 ± 1.40	101.06 ± 2.79	2.77
100	102.10 ± 1.25	102.10 ± 1.25	1.23

SD: Standard deviation; RSD: Relative standard deviation

0.5% CS solution) with FA standard solution (Figure 1) and FA sample (Figure 2a).

It can be observed in Figure 2, the FA retention time in round to 4.5 min (A), however, in the chromatograms obtained from the supernatant of blank nanoparticles (B) and from the CS solution (C), no peaks were found in the same retention time. The results showed there was no interference at the retention time of FA from the formulation components. In that sense, it is possible to confirm the specificity of the purposed method.

Robustness

Robustness is the ability of the method to resist on small and deliberate variations of the analytical parameters (ICH, 2005). Table IV shows the results of the quantification of FA, represented as percentage of recovery and RSD, after changes in the flow rate and in the mobile phase. There was no statistical difference in recovery obtained by the reference method and when variations in the proportion of mobile phase were applied ($p>0.05$), therefore, the method is robust for this change. However, the methodology showed to be sensible to changes in the flow rate, requiring greater caution and attention for small variations in this chromatographic parameter ($p<0.05$)

Forced degradation studies (stability indicating property)

In order to verify the specificity of the method as regards the impurities and the degradation products, as well as to promote information about the drug stability,

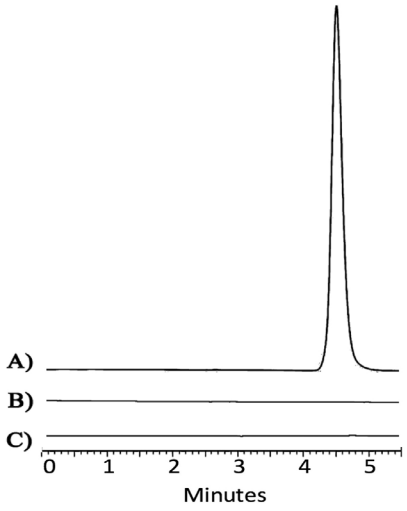

FIGURE 2 - Representative chromatograms of: A) ferulic acid sample solution, B) supernatant from blank PLGA nanoparticles, C) 0.5% chitosan solution.

a forced degradation study was performed. To evaluate this parameter, FA standard solutions (50 µg/mL) were exposed to basic hydrolysis, acid hydrolysis, oxidation, visible light and temperature of -20 °C, and subsequently quantified. The chromatograms and percentage of recovery are shown in Figure 3 and Table V, respectively.

The chromatograms of the samples exposed to acid hydrolysis, oxidation, visible light and temperature of -20 °C, did not present alteration in FA retention time, allowing its quantification. However, when the FA sample was

TABLE IV - Robustness results at different levels of flow rate and mobile phase proportion (n=3)

Variation	Recovery ± RSD (%)		
	10 µg/mL	50 µg/mL	100 µg/mL
Reference	99.32 ± 4.67[a]	98.04 ± 1.57[a]	101.02 ± 1.41[a]
Flow rate 0.95 mL/min	102.40 ± 2.93[b]	107.25 ± 1.66[b]	109.12 ± 0.49[b]
Flow rate 1.05 mL/min	91.27 ± 3.18[c]	92.59 ± 1.48[c]	95.57 ± 0.86[c]
Mobile phase acetonitrile:0.5% acetic acid; 35:65 v/v	100.29 ± 1.89[a]	102.21 ± 1.05[a]	104.67 ± 0.99[a]
Mobile phase acetonitrile:0.5% acetic acid; 39:61 v/v	99.98 ± 1.64[a]	101.97 ± 1.97[a]	104.49 ± 0.06[a]

RSD: relative standard deviation; a, b, c: Means in a column with different superscripts differ significantly ($p<0.05$) by Tukey's test

exposed to alkaline pH, there was a displacement of the peak, and the retention time was 4 min. Furthermore, the possible degradation product obtained from the alkaline hydrolysis changed the FA characteristic peak shape (Figure 3b). The chromatogram of the acid degraded sample showed one additional peak with very low intensity at retention time of 2.2 min (Figure 3c). The chromatogram of the FA treated with H_2O_2 showed an additional peak at 2.5 min (Figure 3d).

Percentage of recovery (Table V) was adequate for exposure to acid medium, visible light and temperature of - 20 °C (between 97.49±0.40 and 101.52±0.27), indicating stability in these conditions. For the exposure to oxidation, the recovery was slightly lower, although still in accordance with the AOAC (2016) recommended for the concentration of 50 µg/mL (80 to 110%). However, after the exposure to basic medium, due to the displacement and change of the peak shape by possible degradation products, quantification was not possible to be performed.

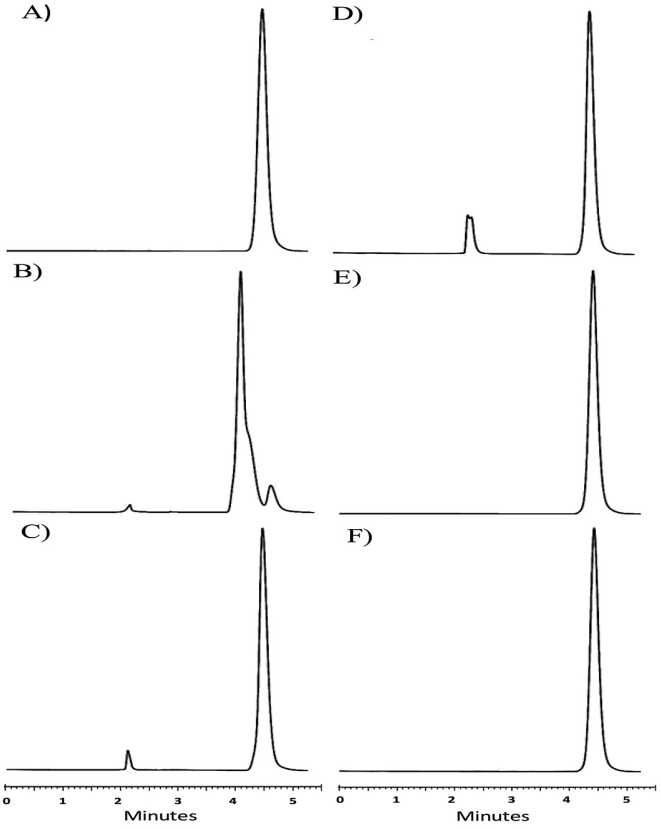

FIGURE 3 - Chromatograms obtained after forced degradation test for 24 h. A) Ferulic acid standard solution - Reference (50 µg/mL) B) After basic hydrolysis (NaOH 1 mol/L) C) After acid hydrolysis (HCl 1 mol/L) D) After oxidation (H_20_2 3%) E) After exposure to visible light F) After storage at -20 °C.

TABLE V - Results of quantification of FA after forced degradation test for 24 h (n=3)

Stress condition	Quantified concentration ± SD (µg/mL)	Recovery ± SD (%)
Reference	50.53 ± 1.40	NA
Basic hydrolysis	NQ	NQ
Acid hydrolysis	49.26 ± 0.20	97.49 ± 0.40
Oxidation	46.28 ± 1.30	91.59 ± 2.58
Visible light	51.30 ± 0.14	101.52 ± 0.27
-20 °C	50.94 ± 0.82	100.81 ± 1.63

SD: Standard deviation; NA: Not applicable; NQ: Not quantified

Method applicability

PLGA nanoparticles containing FA coated with CS were properly obtained and presented mean size of 234±15 nm and polydispersity index of 0.195±0.018, indicating homogenous size distribution. EE% was assessed by the HPLC method and the results showed about 60±5% of FA encapsulated in nanoparticles. Literature shows a similar result for FA encapsulated in PLGA nanoparticles (76%) (Merlin *et al.*, 2012).

Due to the relative absence of studies in which quantification of FA is carried out in polymeric nanoparticles by HPLC, as well as the lack of validation and further detailing on the chromatographic methods used in other pharmaceuticals products, HPLC-PDA method here validated can be considered an alternative for the quantitative analysis of FA. The method showed to be simple, fast, reliable and it fulfill the requirements to be applied in the encapsulation efficiency of FA in nanoparticles. Also, the method can be employed as a stability indicating one.

CONCLUSIONS

A simple, efficient and reliable method to quantify FA in PLGA nanoparticles coated with CS by reversed-phase HPLC with PDA was developed and adequately validated according to ICH and AOAC. The reliability of the method has been proven by parameters of linearity, range, LOD, LOQ, precision, accuracy, specificity, robustness and a study of forced degradation. The analytical methodology presented a short retention time, allowing rapid quantification of FA with low quantities of reagents. Also, the method proved to be suitable for evaluating the FA encapsulation efficiency in PLGA-CS nanoparticles.

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