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Research Article

DEVELOPMENT AND VALIDATION OF A FAST AND SENSITIVE UHPLC-PDA METHOD FOR THE QUANTIFICATION OF URSOLIC ACID IN POLY(L-LACTIC ACID) NANOCAPSULES

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ABSTRACT

Objective: The aim of the present study is to develop and validation of a ultra-high performance liquid chromatography (UHPLC) method to determine the ursolic acid content and its encapsulation efficiency (EE) in lipid-core nanocapsules prepared from poly (L-lactic acid).

Methods: A simple UHPLC-PDA method was developed and validated for the quantitative determination of ursolic acid in poly(L-lactic acid) nanocapsules. The chromatographic conditions used were: RP-C18 column, isocratic mobile phase containing acetonitrile:water (92:8, v/v), flow rate of 0.8 ml/min, column temperature of 50°C, and detection at 203 nm. The following parameters were evaluated: Specificity, linearity, limits of detection and quantification, precision, accuracy, and robustness.

Results: The method was specific to the ursolic acid and linear (r=0.9998) in the range of 10–100 µg/ml. The limits of detection and quantification were 1.35 and 4.10 µg/ml, respectively. The precision was demonstrated by a relative standard deviation less than 2%. Adequate accuracy (98.35%±0.82) was obtained. Changes in flow rate, mobile phase, and column temperature did not significantly alter the peak area and the retention time of the ursolic acid. The mean EE was 99.89%.

Conclusion: The method proved to be fast, sensitive, and simple for quantifying ursolic acid in nanocapsules and was successfully used for determining the EE.

Keywords: Analytical development, Drug quantification, Lipid-core nanocapsules, Pentacyclic triterpenoid, Quality assurance, Quality control.

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INTRODUCTION

Pentacyclic triterpenoids are phytochemicals widely distributed in nature and ursolic acid (3β-hydroxy-urs-12-en-28-oic) (UA) is one of their main representatives [1]. It is a white crystalline solid and shows a melting point between 285 and 288°C with a molecular weight of 456.68 g/mol and molecular formula $C_{30}H_{48}O_3$ [2]. UA is usually found in human diet [3] and has a wide range of biological activities, such as the antioxidant effect [4], the anti-obesity and muscle synthesis properties [5], the hepatoprotective potential [6], the blood sugar-lowering effect [7], the neuroprotective potential [8], the anti-inflammatory activity [9], and the antitumor properties [10,11].

In spite of this pharmacotherapeutic potential, some limitations can be related to the UA use due to its low aqueous solubility and its reduced permeability through biological membranes, which decrease its absolute bioavailability to about 8% [12] and lead to a classification as a class IV drug by the biopharmaceutical classification system. For this reason, strategies to overcome these limitations have been widely studied mainly based on nanotechnology [13,14]. In brief, the aim of these approaches was to develop formulations with efficacy and safety that might guarantee at the same time improved solubility, higher bioavailability, and a controlled release pattern for this drug [15]. However, nanoparticles require extensive characterization, including the mandatory determination of the drug content and the encapsulation efficiency (EE) to achieve the expected clinical effect while assuring suitable pharmacokinetic and pharmacodynamic properties when administered *in vivo* [16,17]. Thus, a validated method that is able of quantifying the drug loading in nanoformulation is essential during the research and development process.

In this scenario, some analytical methods were previously described for the UA quantification, mainly in botanicals [18,19] and biological samples as plasma [20,21]. Concerning to the methods developed for drug delivery systems, these strategies were typically based on high-performance liquid chromatography [22-24]. Therefore, to the best of our knowledge, no previous paper was devoted to validate an analytical method for the UA determination in lipid-core nanocapsules using ultra-high-performance liquid chromatography (UHPLC). UHPLC has currently attracted great interest from the pharmaceutical industry due to its ability to achieve maximum use of chromatographic principles, to have superior features in terms of resolution, sensitivity, and efficiency, and to decrease the consumption of solvents and other reagents [25].

In this context, the present paper describes the development and the validation of a UHPLC-PDA method for determining the UA content and its EE in lipid-core nanocapsules prepared from poly(L-lactic acid) (PLA).

METHODS

Reagents and chemicals

Ursolic acid (UA) 98% pure was purchased of Natural Field Bio-Technique (Xi'an, China). Methanol HPLC grade (Research Hexis, Phillipsburg, NJ, USA), acetone P.A. (Synth, Diadema, Brazil), acetonitrile HPLC grade (J.T. Baker, Phillipsburg, NJ, USA), L-lactic acid 90% pure (Vetec, Duque de Caxias, Brazil), and chloroform P.A. (Vetec, Duque de Caxias, Brazil) were used as received. The water was purified using a Milli-Q Plus system (Millipore, Burlington, MA, USA).

Equipment

A Shimadzu UHPLC system (Nexera X2, Kyoto, Japan) equipped with a SPD-M20A PDA detector, a DGU-20A5RA degasser, a

LC-30AD pump, a SIL-30AC autosampler, a CTO-20A oven, and a CBM-20A communicator module was used for the analytical method development. The LabSolutions software version 5.73 was used for the acquisition, the analysis and the data generation. The UHPLC analysis was performed using a C18 Shim-pack XR-ODS III reverse phase column (Kyoto, Japan) with a 2.2 µm particle size coupled to the pre-column.

Chromatographic conditions

The mobile phase consisted of a mixture of acetonitrile and water (92:8, v/v) with a flow rate of 0.80 ml/min. The elution was performed in an isocratic mode. The injection volume was 10 µl. The analyses were carried out at a temperature of 50°C, the PDA detector wavelength was set at 203 nm, and the running time was 2.1 min.

Preparation of standards and samples

The standard UA stock solution was prepared in methanol at 500 µg/ml. Dilutions were performed to obtain solutions with a concentration between 10 and 100 µg/ml. The UA samples corresponded to the supernatant obtained after the ultracentrifugation of UA-loaded nanocapsules, as described in the method applicability section. Standards and samples were suitably diluted in methanol to obtain the desired concentration. These solutions were filtered through a polytetrafluoroethylene filter (PTFE, Chromafil Xtra, 0.2 µm×13 mm, Macherey-Nagel, Düren, Germany) before injection.

Validation method

The validation of the analytical method by UHPLC-PDA was performed according to the criteria proposed by the International Conference on Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use [26]. The following characteristics were considered: Specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and robustness. Specificity was determined by analyzing the chromatograms of UA-loaded nanocapsules compared to those obtained from non-loaded formulations.

The linearity was investigated by the linear regression using the least-squares method from three authentic analytical curves obtained at 10; 30; 40; 60; 70; 80; and 100 µg/ml. The slope and other parameters of the analytical curves were calculated by the linear regression and the analysis of variance (ANOVA). The residue analysis was carried out using Statistica 8.0 (StatSoft, Tulsa, OK, USA). The LOD (Eq.1) and the LOQ (Eq.2) were determined using the following equations as recommended by ICH [26].

$$LOD = \frac{3.3 \times SD}{S} \quad (1)$$

$$LOQ = \frac{10 \times SD}{S} \quad (2)$$

where σ is the standard deviation (SD) from the response and *S* is the slope of the mean calibration curve.

Precision was assessed at two different levels: Repeatability (intraday precision) and intermediate precision (interday precision). These parameters were investigated at 60 µg/ml in sextuplicate and at 20; 50; and 90 µg/ml in triplicate. The results were expressed in terms of SD and relative SD (RSD) (DPR). Accuracy was assessed by recovery analysis in which a known amount of UA (15 µg) was added in triplicate to the solutions of 10.0; 40.0; and 80.0 µg/ml. The accuracy of the method was calculated by the percentage ratio between the experimental concentration and the theoretical concentration, and the results expressed as a percentage of the recovery. Robustness was evaluated in the samples at 60 µg/ml by intentionally varying the flow rate to 0.75 and 0.85 ml/min, and the concentration of the acetonitrile:water mobile phase to 91:9 (v/v) and 93:7 (v/v).

Method applicability: Synthesis of PLA, preparation of ursolic acid-loaded lipid-core nanocapsules, and determination of EE

The preparation of PLA was carried out through the polycondensation reaction of L-lactic acid [27,28]. Briefly, 200 ml of L-lactic acid (90% w/v) were added into a round bottom flask and a distillation system was then connected. The flask was kept into an oil bath at 180°C for 8 h under magnetic stirring. A reduced pressure of –350±10 mmHg was coupled to the distillation system for removing the water. After PLA synthesis, the polymer was dissolved in acetone, precipitated twice in purified water, and dried at 30°C. This polymer had its chemical composition characterized by Fourier-transform infrared spectroscopy using a Shimadzu IR Prestige-21 spectrometer (Kyoto, Japan) from 4000 to 400 cm^{-1} in potassium bromide pellets with 32 scans and resolution of 4 cm^{-1}. The viscosity-average molecular weight was recorded by intrinsic viscosity measurement [29] using chloroform as standard.

Suspension of lipid-core nanocapsules was prepared by interfacial deposition of this preformed polymer, as described by Fessi *et al*. [30,31]. In brief, PLA (100 mg) was dissolved in 20 ml of acetone and 10 ml of ethanol in the presence of Span 60® (80 mg) and UA (100 mg). This organic phase was added to the aqueous phase containing 80 mg of Tween® 80 and 60 ml of purified water by dripping and under vigorous magnetic stirring at 40°C. The organic solvent was then evaporated under reduced pressure in a rotary evaporator to the final volume of 10 ml. For comparative purposes, a suspension of nanoparticles was prepared with no UA (NPLA-0) as a negative control. All formulations were prepared in triplicate from three independent batches.

The proposed UHPLC-PDA method was used for determining the UA amount in the loaded nanocapsules. The drug quantification was carried out by indirect analysis [17]. The supernatant containing free UA was obtained by ultracentrifugation using an Amicon® device (*Mw* cutoff=10,000 g/mol, Merck Millipore, Bedford, MA, USA), was then appropriately diluted in methanol, and analyzed by the aforementioned method. The EE was calculated by the following equation (Eq. 3) in triplicate.

$$EE = A_{initial} - \frac{A_{free}}{A_{initial}} \times 100 \quad (3)$$

where $A_{initial}$ is the amount of UA initially added to the formulation and A_{free} is the concentration of the free drug quantified in the supernatant after ultracentrifugation and suitable dilution.

Statistical analysis

All data were expressed as mean±SD. The linearity data were evaluated by simple linear regression. RSD was shown as required. Data were compared by ANOVA with Tukey's post-hoc test at a significance level of 5% (α=0.05). GraphPad Prism software version 5.03 (San Diego, CA, USA) was used for statistical analysis.

RESULTS AND DISCUSSION

Validation method

The method was validated using acetonitrile:water (92:8, v/v) as a mobile phase and a flow rate of 0.80 ml/min. The total analysis time and the retention time were 2.1 and 1.78 min, respectively. This fast response is very important for the analysis routine. These conditions also provided a lower tail size and a more symmetrical peak for the UA quantification when compared to other methods that used HPLC [22,23].

The specificity is a validation parameter used for investigating if some particular component of the formulation can interfere in the drug retention time, as well as ensuring that no spikes of other substances or impurities appear in the UA retention time [26]. For this purpose, the UA chromatogram was compared with the chromatogram of non-loaded nanocapsules and no peak was detected at the UA retention time (Fig. 1). This result proved the specificity since there was no interference in the drug retention time.

Linearity was performed in triplicate in the concentration range from 10 to 100 μg/ml. The linear equation achieved was *y*=8065.1 *x*–6096, where *y* is the peak area and *x* is the standard solution concentration in μg/ml. A correlation coefficient of r=0.9998 was recorded, which suggested that the method was linear since an r value close to 1.0 could indicate the calibration curve suitability [32].

The RSD of the slope was 0.77%, a value lower than that recommend by ICH (5%) [26]. The negative b value (–6096) was in the 95% confidence interval of the analytical calibration curve by the ANOVA test. These results are summarized in Table 1.

Although the correlation coefficient value was close to the unit, it does not certainly represent that the method showed a linear relationship. Therefore, it is mandatory to apply the test for a lack of adjustment to evaluate the residual values [33]. The F value obtained for the lack of adjustment was less than the critical F value for a 95% confidence interval (α=0.05). Hence, the linearity was confirmed due to the linear regression that did not present a lack of adjustment according to the ANOVA test (Table 2).

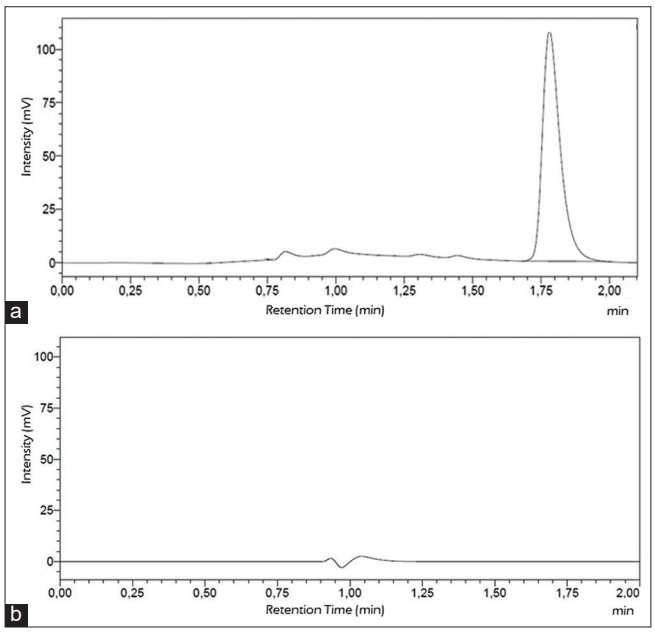

Fig. 1: Representative RP-UHPLC-PDA chromatograms: (a) Standard UA solution at 60.0 μg/ml, (b) non-loaded nanocapsules

Table 1: Linearity parameters of the RP-UHPLC-PDA method for quantifying ursolic acid from lipid-core nanocapsules

Parameters	Results
Linearity	
Linear range (μg/ml)	10.0–100.0 μg/ml
Detection limit (μg/ml)	1.35 μg/ml
Quantification limit (μg/ml)	4.10 μg/ml
Regression data*	
n	3
Slope	27,762.33
Standard deviation of slope	62.36
Relative standard deviation of slope (%)	0.77
Intercept (b)	–6096.13
Correlation coefficient (r)	0.9998

The LOD is the lowest concentration in which an analyte can be detected, while the LOQ is the lowest concentration that an analyte can be quantified with acceptable precision and accuracy [34]. Considering the proposed UHPLC-PDA method, the lowest concentration in which UA can be detected (LOD) and quantified (LOQ) was 1.35 and 4.10 μg/ml, respectively. These values infer that the background signal does not limit the sensitivity of the UHPLC-PDA peak.

The precision represents the contiguous occurrence of the results obtained from repeated measurements of the same sample [26]. The precision data were expressed as RSD obtained for repeatability and intermediate precision are described in Table 3. All the RSD values were below 5%, which confirm an appropriate precision for the evaluated method [35].

Regarding accuracy, the recovery rate of UA was between 96.16 and 100.30% for the different concentration levels evaluated. Table 4 describes the obtained data. These results demonstrate that the method is accurate since it is in agreement with the accuracy target limits of 95–105% [26].

The robustness was based on the RSD values obtained by changing analytical parameters, such as the flow rate, the mobile phase composition, and the column temperature [26]. An analytical method is classified as robust when it remains unchanged at small variations that eventually occur during the analysis process [26,35]. The RSD results depicted in Table 5 show that there was no significant difference in the peak area and the UA retention time after the proposed changes. Thereby, the RP-UHPLC-PDA method proved to be robust for analyzing UA even at small changes in chromatographic parameters.

Method applicability: Synthesis of PLA, preparation of ursolic acid-loaded lipid-core nanocapsules, and determination of EE

PLA was obtained as a white granular powder by the polycondensation method. The main advantage of polycondensation synthesis arises from the fact that it is a simple process and does not require additional organic solvents, which is essential for polymers that will be used for biomedical purposes [36]. PLA showed the main FTIR bands (Fig. 2) recorded at 3504 cm^{-1} for the –OH stretching, at 2995 cm^{-1} for the C–H stretching, at 1759 cm^{-1} for the C=O stretching, at 1197 cm^{-1} for the asymmetric O–C=O stretching, and at 1093 cm^{-1} for the symmetrical stretching of the C–O–C. These assignments were confirmed by the literature [37].

The obtained polymer showed a viscosity-average molecular weight of 1070 g/mol, which represents a low molar mass. This value is related to the polycondensation since the water obtained during the esterification causes the hydrolysis of the polyester, which leads to shorter polymer chains. In sequence, the nanocapsules were successfully obtained as nanosuspensions that showed a liquid aspect with a slightly bluish-white opalescent coloring, as typically reported in literature [38].

Table 2: ANOVA results for linearity of the RP-UHPLC-PDA method for quantifying ursolic acid from lipid-core nanocapsules

Ursolic acid	SS	DF	MS	F	Fcrit
Model	1 126 210E+12	1	1.12 6210E+12	24 314.34	2.990
Residual	8 800 568E+08	19	4.631 878E+07	Linear	
Lack of fit	380 576 702	5	76 115 340	2.1334	2.307
Pure error	499 480 095	14	35 677 150	No lack of fit	

SS: Sums of squares, DF: Degrees of freedom, MS: Mean squares, F: F value of the test, Fcrit: Critical F value

Table 3: Repeatability and intermediate precision data of the RP-UHPLC-PDA method for quantifying ursolic acid from lipid-core nanocapsules

Precision	Theoretical concentration (μg/ml)	Experimental concentration (μg/ml, mean±SD*)	RSD** (%)
Repeatability			
n=6	60	60.39±0.25	0.42
n=3	20	19.95±0.40	1.99
n=3	50	49.48±0.67	1.35
n=3	90	91.20±0.89	0.98
Intermediate precision			
Intraday			
n=6	60	60.35±0.30	0.49
n=3	20	19.80±0.32	1.60
n=3	50	49.73±0.84	1.68
n=3	90	91.30±0.92	1.00
Interday			
n=6	60	60.49±0.24	0.40
n=3	20	20.05±0.28	1.41
n=3	50	49.97±0.89	1.78
n=3	90	91.66±0.78	0.85
Different analyst			
n=6	60	60.60±0.28	0.46
n=3	20	19.93±0.28	1.41
n=3	50	49.57±0.74	1.50
n=3	90	91.17±0.94	1.03

*SD: Standard deviation, **RSD: Relative standard deviation

Table 4: Accuracy data of the RP-UHPLC-PDA method for quantifying ursolic acid from lipid-core nanocapsules

Level of concentration	Final ursolic acid concentration (μg/ml)	SD (%±SD*)	RSD** (%)
Low	25	100.30±2.48	2.47
Medium	55	98.60±2.80	2.84
High	95	96.16±1.24	1.29

*SD: Standard deviation, **RSD: Relative standard deviation

Table 5: Robustness data of the RP-UHPLC-PDA method for quantifying ursolic acid from lipid-core nanocapsules

Initial parameter	Change	RSD* (%)
Flow rate	0.75 μg/ml	1.29
0.80 μg/ml	0.85 μg/ml	1.85
Mobile phase proportion	91:9 (v/v)	1.08
92:8 (v/v)	93:7 (v/v)	1.15
Temperature	48°C	0.54
50°C	52°C	2.00

*RSD: Relative standard deviation

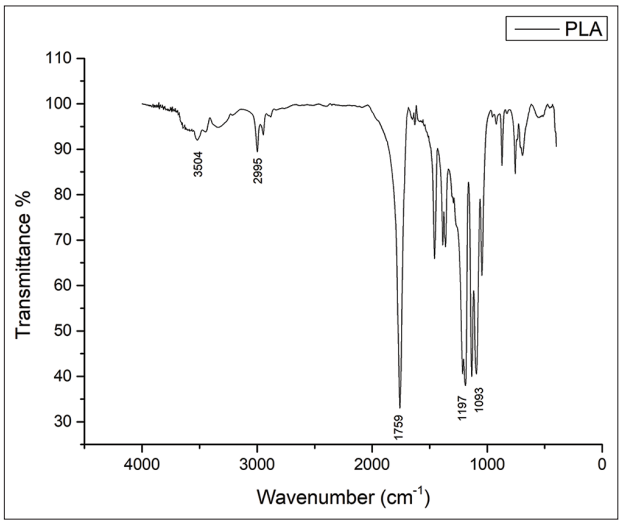

Fig. 2: FTIR spectrum of poly(L-lactic acid)

The previously validated method was then used for the UA quantification from the loaded formulation. The lipid-core nanocapsules presented a suitable EE of 99.89±0.01%. This value is entirely expected due to the high lipophilicity of the drug (water solubility of 0.102 ng/l at 25°C) [12], which leads to its high incorporation in the lipid-core of this nanosystem.

CONCLUSION

The reverse-phase UHPLC-PDA method was developed and validated for quantifying UA from lipid-core PLA nanocapsules. This analytical method is simple, specific, linear, sensitive, precise, accurate, and robust. In addition, it provides advantages over the methods described in the literature since it is fast and easy to apply in the pharmaceutical daily routine of quality control. It requires simple sample preparation, uses a low volume of reagents, shows faster analysis time, and decreases the waste amount, which is very important for the pharmaceutical industry.

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AUTHORS' CONTRIBUTIONS

Bruna Carletto, Amanda Martinez Lyra, and Paulo Vitor Farago contributed substantially to the concept and study design; Bruna Carletto, Amanda Martinez Lyra, and Rubiana Mara Mainardes performed the data acquisition; Amanda Martinez Lyra, Andressa Novatski, and Bruna Carletto carried out the data analysis/interpretation; Bruna Carletto and Paulo Vitor Farago drafted the manuscript; Paulo Vitor Farago provided a critical revision of the manuscript for important intellectual content; Adriana Yuriko Koga and Leandro Cavalcante Lipinski were responsible for the statistical analysis; Paulo Vitor Farago performed the supervision; all authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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