

A SIMPLE AND FAST RP-UHPLC-PDA METHOD FOR DETERMINATION OF ISRADIPINE FROM POLYMERIC NANOCAPSULES

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ABSTRACT

Objective: A simple and fast analytical method of ultra-high performance liquid chromatography (UHPLC) was developed and validated to quantify isradipine (ISR) in poly(ϵ -caprolactone)/polyethylene glycol nanocapsules.

Methods: Experiments were performed by UHPLC on a C_{18} chromatographic column at 25°C, using a mobile phase composed by methanol and water (85:15 v/v) with a flow rate of 0.5 ml/min and UV detection at 327nm for achieving a total run time of 1.5 min. The UHPLC method was validated according to the guidelines set on The International Conference on Harmonisation.

Results: It proved to be selective, linear ($r=0.99962$), precise (relative standard deviation <4.1%), and accurate (recovery rates between 95.24% and 96.53%) at the range from 10 to 40 $\mu\text{g/ml}$. The performance was robust when slight changes in the flow rate, wavelength of detection, and mobile phase composition were tested. It was successfully applied to quantify ISR from nanoparticulate polymeric systems, showing a high loading efficiency rate, >98.55%.

Conclusion: These results provided an experimental basis to use this method for quantifying ISR with reliable results, besides being fast, easy to perform, and cheaper.

Keywords: Analytical method validation, Neuroprotective effect, Pharmaceutical quality control, Polymeric nanocapsules.

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INTRODUCTION

Quality control has been an essential tool for pharmaceutical industries since it ensures efficacy and safety for pharmaceutical products. Nevertheless, its results are only reliable when analytical methods employed are validated [1]. The validation of an analytical method is a major approach for assuring its quality, especially when the need to prove the quality of pharmaceutical analysis has been increasingly recognized and demanded since unreliable data can lead to wrong decisions and irrecoverable financial losses [2]. In this sense, ultra-high performance liquid chromatography (UHPLC) brought new opportunities in pharmaceutical industries to get faster analytical separation results while remaining the method quality. Its technique speed results in reduced consumption of solvent while presents a resolution and sensitivity greater than other conventional techniques [3].

The isradipine (ISR) or 3-methyl 5-propan-2-yl 4-(2,1,3-benzoxadiazol-4-yl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate is a calcium channel blocker which belongs to the dihydropyridine class and has demonstrated an interesting neuroprotective effect associated to its pharmacotherapeutic use [4]. In this case, the main challenge is to get this drug to cross the blood-brain barrier in therapeutic concentrations. The clinical study performed demonstrated that the dose of ISR as immediate-release formulation may have been insufficient to engage the target calcium channels associated with neuroprotective effects [5]. Thus, the research for strategies that make it possible to deflect the limitations related to the action of this drug in the central nervous system is growing and current.

In this context, polymeric nanocapsules have an interesting application for delivering drugs in the central nervous system, accompanied

by its controlled release at the site [6,7]. Before the advantages offered by nanoparticulate systems, there is a demand for a reliable method capable of quantifying the drug in these formulations. Some chromatographic methods to quantify ISR have been previously reported in the literature [8-12]. However, none of them is appropriate to quantify drug-loaded polymeric nanocapsules, besides presenting complexes steps and wasting more expensive products. Hence, the aim of this paper was to develop and validate a reversed-phase UHPLC-photodiode array (RP-UHPLC-PDA) method to quantify ISR loaded in poly(ϵ -caprolactone) (PCL)/polyethylene glycol (PEG) nanocapsules.

METHODS

ISR hydrochloride (European Pharmacopoeia reference standard, CAS number 75695-93-1), PCL ($M_w = 10\,000$ g/mol, Sigma-Aldrich, St. Louis, MO, USA), and PEG ($M_w = 6000$ g/mol, Fluka, St. Louis, MO, USA) were used as received. Methanol of gradient grade for liquid chromatography was purchased from Merck (Darmstadt, Germany). All other chemicals used were of analytical grade.

Preparation of polymeric nanocapsules containing ISR

Nanocapsule suspensions obtained from PCL/PEG polymer blend (80:20) containing isradipine (NC-ISR) were prepared in triplicate by the interfacial deposition procedure of a preformed polymer developed and described by Fessi *et al.* [13], according to the formulation described in Table 1. Besides the loaded formulation, nanocapsules without the drug were also produced in triplicate as a negative control (NC-0). Briefly, the polymer blend was solvated in acetone, along with sorbitan monooleate, ISR, and caprylic/capric triglycerides to obtain the organic phase. Then, the organic phase was dripped slowly into the aqueous phase, previously prepared with ultrapure water and polysorbate 80, and maintained under vigorous magnetic stirring and

Table 1: Formulations of the loaded and non-loaded nanocapsule suspensions

Formulation	Organic phase						Aqueous phase	
	ISR (mg)	PCL (mg)	PEG (mg)	CCT (mg)	Acetone (ml)	Sorbitan monooleate (mg)	Polysorbate 80 (mg)	Water (ml)
NC-ISR	20	80	20	300	27	77	77	53
NC-0	-	80	20	300	27	77	77	53

NC-ISR: Nanocapsules containing isradipine, NC-0: Negative control, PCL: Poly(ϵ -caprolactone), PEG: Polyethylene glycol, CCT: Capric triglycerides

temperature between 40°C and 50°C. In addition, the magnetic stirring was maintained for 10 min after the end of the drip and the solvent was eliminated by rotary evaporation at 50°C, reaching a final volume of 10 ml and a drug concentration of 2 mg/ml.

In sequence, the resulting nanosuspension was added with 5% of sucrose (w/v), as a cryoprotective agent, frozen at -80°C for 24 h, and lyophilized in a Terroni® lyophilizer model LD1500 for 48 h. To modify the surface of the nanocapsules [14], they were coated with polysorbate 80 after completing the conventional process for obtaining the nanoparticulate system. For this, the powder resulting from the freeze-drying was resuspended in 2 ml of a solution of polysorbate 80 (1%, w/v). The suspension remained incubated at room temperature for 30 min, was frozen again at -80°C for 24 h, and freeze-dried for 72 h. The final powder was stored in a desiccator at room temperature and protected from light.

Chromatographic conditions

Chromatographic conditions were tested and defined by the authors. Different composition, pH, and flow rate of the mobile phase were tested, as well as column temperature and detection wavelength.

Experiments were performed in a Shimadzu Nexera System® UHPLC, equipped with a quaternary pump (LC-30AD), degasser (DGU-20A), automatic sampler (SIL-30 AC), thermostatic column compartment (CTO-20AC), and a photodiode array detector (SPD-M20A). The analytical column used was a Shimadzu Shim-pack XR-ODS III (C18; 2.0 mm internal diameter × 75 mm long; particle size of 1.6 µm). The isocratic mobile phase consisted of methanol:water (85:15 v/v) with a flow rate of 0.5 ml/min; temperature of 25°C, injection volume of 10 µl, and wavelength detection at 327 nm. The total analytical run time was 1.5 min. The acquisition and processing of the data were obtained with the LabSolutions® Software (Shimadzu, Japan).

Preparation of standard solutions

A stock standard solution of 1 mg/ml of ISR was daily prepared by dissolving 10 mg of the drug in a 10 ml volumetric flask with methanol. This solution was further diluted in the mobile phase to prepare five different standard solutions ranging from 10 to 40 µg/ml. Final solutions were filtered through a poly(vinylidene fluoride) membrane filter (Durapore® membrane filter, 0.22 µm pore size) before injection into the UHPLC system.

Preparation of sample solutions

The final dosage form, a nanopowder, was resuspended in 10 ml of purified water. The nanosuspension (500 µl) was submitted to ultrafiltration in an Amicon® device (M_w cutoff = 10,000 g/mol, Merck Millipore) by centrifugation at 2200 rcf for 30 min. The amount of free ISR in ultrafiltration fraction was chromatographically quantified as proposed. All procedure was performed in triplicate.

Method validation

The UHPLC method was validated according to the guidelines set on The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use [15]. Parameters evaluated were selectivity, linearity and range, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and robustness.

Selectivity was investigated by comparing both chromatograms of the standard solution and the sample solution from NC-0, obtained from the

ultrafiltration of the nanosuspension prepared without ISR. Both solutions were chromatographed under previously defined conditions. The linearity of the method was assessed by preparing and analyzing of three analytical curves. For this, five concentration levels of ISR were obtained from dilutions of the standard solution in the mobile phase. The range was defined based on the intensity of the peaks produced in the chromatogram, including an interval from 10.0 µg/ml to 40.0 µg/ml. The analysis of linear regression was performed using the least-squares method and the slope was tested by ANOVA at a significance level of 0.05. LOD and LOQ were obtained based on the standard deviation of the response (s) and the slope (a) of three analytical curves (LOD = 3.3 s/a and LOQ = 10 s/a). Repeatability and intermediate precision were carried out by assaying six different samples at the same concentration (25.0 µg/ml) under the same experimental conditions during the same day (morning, afternoon, and night) and along three different days with different analysts, respectively. These results were expressed as relative standard deviation (RSD). Accuracy was determined by the recovery test. Different standard solutions were prepared and added to a known quantity of ISR. Final solutions were analyzed in the chromatographic system in triplicate. Robustness of the method was assessed from variations of the recommended conditions (mobile phase composition, flow rate, and wavelength of drug detection).

Method applicability: Determination of ISR loading efficiency

The amount of ISR was calculated and reported as loading efficiency following the equation below. Loading efficiency (%) was determined from the sample solution. Non-entrapped ISR was quantified in the ultrafiltrate by the proposed chromatographic method.

$$\text{Loading efficiency \%} = \frac{\text{Total drug added} - \text{Non entrapped drug}}{\text{Total drug added}} \times 100$$

RESULTS

Preparation of polymeric nanocapsules containing ISR

Nanocapsules from PCL/PEG polymer blend were successfully obtained by the proposed method. Formulations showed a powder aspect with a slightly yellowish-white coloring.

Method validation

The main parameters related to the proposed method for quantification of ISR are briefly described in Table 2.

The UHPLC method presented a total run time of 1.5 min and proved to be selective since the NC-0 formulation did not show relevant signal detection overlap at the same reading position of the drug (Fig. 1).

The linearity test was performed at five different concentrations level of the analyte and its mean analytical curve obtained is shown in Fig. 2.

Values of angular coefficient (slope of the line), linear coefficient (Y-intercept), Pearson's correlation coefficient (r), and coefficient of determination (r²) were obtained based on the linear regression analysis and were able to evidence the quality of the linear regression obtained. Results are described in Table 3.

Results showed that the Pearson's correlation coefficient statistically is equal to 1 and a non-zero slope (significance level of 0.05%).

To demonstrate that y really varies as a function of x, it has been shown that the variation due to the regression (model) is sufficiently greater

than that one due to the error (residuals). In this way, the residues were graphically plotted in Fig. 3.

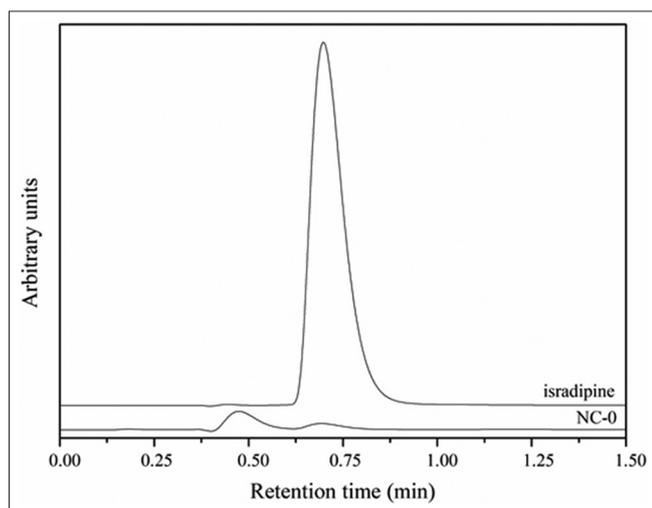


Fig. 1: Representative chromatogram of pure isradipine and NC-0 formulation

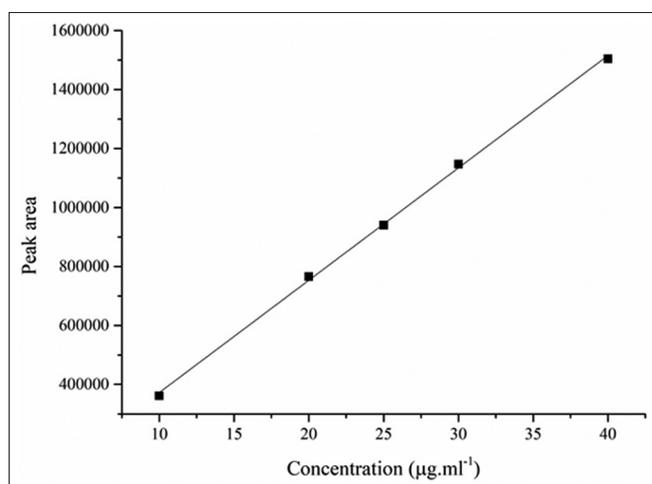


Fig. 2: Graphical representation of the mean analytical curve obtained to quantify isradipine by RP-UHPLC-PDA method at a concentration range from 10 to 40 µg/ml

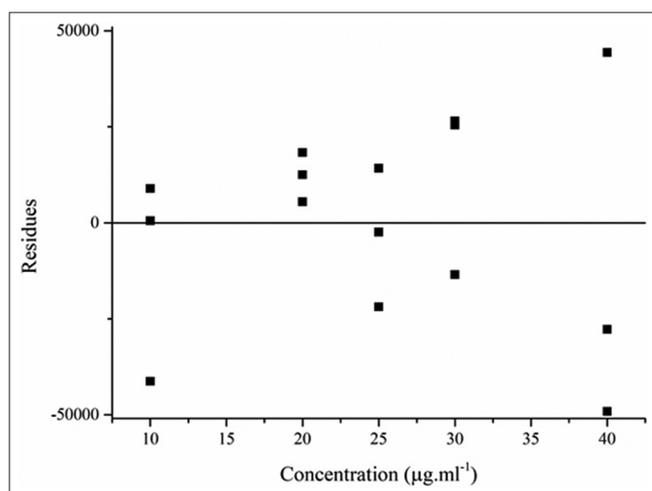


Fig. 3: Graphical representation of the residues obtained from replicates of each concentration level of the analytical curves

The visual analysis indicated that the residues are independent and homogeneously distributed according to the mean.

The developed method showed a LOD of 2.9 µg/ml and a LOQ of 8.78 µg/ml.

The results of repeatability and intermediate precision obtained for this UHPLC-PDA method are shown in Table 4.

The obtained values of RSD were <5.0%.

Regarding accuracy, the recovery rate of ISR is between 95.24% and 96.53% for different concentration levels evaluated. Table 5 describes the obtained data.

Recovery rates obtained for robustness are summarized in Table 6. These values showed a variation <5% due to small changes in the flow rate, wavelength of detection, and the organic phase concentration in the mobile phase.

Method applicability: Determination of ISR loading efficiency

Loading efficiency of ISR from nanocapsules was performed by the purposed RP-UHPLC-PDA method.

The chromatograms obtained from the ultrafiltrate samples showed no peaks; that is, there is no detectable concentration of free ISR in

Table 2: Main parameters of the RP-UHPLC-PDA method for quantifying isradipine from polymeric nanocapsules

Parameters	Results*
Retention time (min)	0.699±0.001
Width at 5% of the peak height (min)	0.208±0.001
Tailing factor	1.485±0.006

*Mean±standard deviation. RP-UHPLC-PDA: Reversed-phase-ultra-high performance liquid chromatography-photodiode array

Table 3: Linearity parameters of the RP-UHPLC-PDA method for quantifying isradipine from polymeric nanocapsules

Parameters	Results
Linearity range	10–40 µg/ml
Linear equation ($y = ax + b$)	$y = 38\,100x - 8723.8$
Slope (a)	38 100.0
Y-intercept (b)	-8723.8
Pearson's correlation coefficient (r)	0.99962
Coefficient of determination (r^2)	0.99899

RP-UHPLC-PDA: Reversed-phase-ultra-high performance liquid chromatography-photodiode array

Table 4: Results of precision assays of the RP-UHPLC-PDA method for quantifying isradipine from polymeric nanocapsules

Parameters	Sample concentration (µg/ml)	RSD* (%)
Repeatability (n=6)	25	4.02
Intermediate precision (n=12)	25	3.70

*RSD means relative standard deviation. RSD: Relative standard deviation, RP-UHPLC-PDA: Reversed-phase-ultra-high performance liquid chromatography-photodiode array

Table 5: Results of accuracy assays of the RP-UHPLC-PDA method for quantifying isradipine from polymeric nanocapsules

Theoretical sample concentration (µg/ml)	Recovery rate (%) (n=3)
20	95.95
25	95.24
30	96.53

RP-UHPLC-PDA: Reversed-phase-ultra-high performance liquid chromatography-photodiode array

Table 6: Results of robustness assays of the RP-UHPLC-PDA method for quantifying isradipine from polymeric nanocapsules

Parameters		Theoretical sample concentration (µg/ml)	Recovery rate (%) (n=3)
Flow rate (±0.02 ml/min)	0.48 ml/min	25	104.21
	0.52 ml/min	25	96.21
Wavelength of detection (±2 nm)	325 nm	25	99.98
	329 nm	25	99.94
Mobile phase composition (±2% of methanol)	83:17 (methanol:water)	25	100.41
	87:13 (methanol:water)	25	100.78

RP-UHPLC-PDA: Reversed-phase-ultra-high performance liquid chromatography-photodiode array

the nanosuspension. Thus, it is possible to assert that the prepared polymeric system was able to incorporate ISR in its interior, with efficiency >98.55%, considering the value that meets to the LOD of the method.

DISCUSSION

Pharmaceutical quality control requires that its results are reliable and reproducible. For this, it is imperative to use methods that meet these demands besides being fast, easy to perform, and inexpensive. In this sense, the method developed in this work is very fast and simple and presents a lower cost when compared to the other methods that have already been described for quantification of ISR, whereas the mobile phase is composed only by methanol and ultra-purified water [8-12].

Analytically, we can say that the peak width at the base is adequate to guarantee the quality of the developed method, on the other hand, the peak tailing factor shows that it is not perfectly symmetrical since the ideal symmetry is represented by values equal to 1. However, Petersson *et al.* [16] describe that the UHPLC, which has columns with particle sizes smaller than 2 µm, produces tailing factor values significantly higher than the equivalent HPLC, which uses columns with 3 µm particles when the same amount of sample is injected. Expressions have shown that the tailing factor values change with the particle size and the number of theoretical plates, and even so, UHPLC produces more efficient separations when considered adjacent peaks. Thus, the tailing factor value must be analyzed, not singly, but rather associated with the peak width values at the base, to obtain a better parameter for measuring the quality of the method.

The validation process is responsible for ensuring that the results obtained are befitting. Moreover, once the parameters recommended by official agencies are strictly respected, the validation is able to prove that the analytical method employed produces reliable results, and it is suitable for the purpose for which it was intended [15,17].

The method developed for quantification of ISR proved to be selective since no excipient of the formulation showed absorption of electromagnetic radiation at the selected wavelength [18]. In this way, it ensures the ability of the method to quantify the drug before any other component. It generates an exclusive response to ISR allowing ensuring a positive result for the analyte and negative for the other substances.

The linearity test showed the method ability to get replies proportionally equivalent to the concentration of the drug within the stated range. It is known that the closer to 1 the Pearson's correlation coefficient is, the smaller the dispersion among the points and the greater the safety of the results [19]. Thus, it is clear the relevance of using a calibration curve instead of a single point for standardization in the analysis routine. In addition, the visual analysis indicated that the residues are independent and homogeneously distributed according to the mean, ensuring the reliability of the reply generated by the method [20].

The LOD, which refers to the lowest concentration of the analyte that the method is able to detect, and the LOQ, which is the smallest amount of substance that the method is able to quantify with precision and accuracy, confirmed the feasibility of applying the method within the stated concentration range [21].

Official agencies describe that precision must be able to assess the closeness among the values found in the experiments and must be expressed as repeatability and intermediate precision [15]. In this work, repeatability showed the agreement among the results found when the method was performed under the same conditions, in a short time interval. When the intermediate precision was tested, the method performance showed a low variation when the analysis were accomplished in different days and with different analyzers, at the same laboratory [22].

The results also showed the agreement between the data obtained against the actual reference value when employing the quantification method of ISR, proving to be accurate [23].

In addition, the method was evaluated for the ability to withstand slight variations in the conditions of analysis, without hazarding its accuracy. In view of the recovery values obtained, the method was considered robust. Its robustness values the results obtained from this method enforcement since we assume that it can be reproduced without major problems [21].

When the UHPLC method was assayed to quantify the ISR nanoencapsulated in polymeric nanocapsules, it results in a high encapsulation rate. This rate its completely expected due to the high lipophilicity of the drug (water solubility <10 mg/l at 37°C) [24], which optimizes its incorporation in the oily core of this nanosystem.

In summary, according to the presented results, the method was considered selective, linear for the proposed application range, precise, accurate, and robust; therefore, it is properly validated. In addition, it was successfully used for quantifying the ISR nanoencapsulated in polymeric nanocapsules, providing reliable results.

CONCLUSION

The RP-UHPLC-PDA analytical method proposed above showed to be selective, linear, precise, accurate, and robust at the tested range. It is suitable for determining ISR loaded in PCL/PEG nanocapsules. The obtained nanoformulation presented high loading efficiency. These results provided an experimental basis to use this method for quantifying ISR from polymeric nanosystems, providing reliable results, besides being fast, easy to perform, and cheaper.

AUTHORS' CONTRIBUTIONS

Conception and design: Fernanda Malaquias Barboza, Amanda Martinez Lyra, Paulo Vitor Farago.

Sample preparation: Fernanda Malaquias Barboza, Guilherme do Anjos Camargo.

Analytical method performance: Fernanda Malaquias Barboza, Guilherme do Anjos Camargo, Amanda Martinez Lyra.

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Drafting of the article: Fernanda Malaquias Barboza, Jessica Mendes Nadal, Paulo Vitor Farago.

Critical revision of the article for important intellectual content: Paulo Vitor Farago.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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