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A novel, simplified and stability-indicating high-throughput ultra-fast liquid chromatography method for the determination of rosmarinic acid in nanoemulsions, porcine skin and nasal mucosa

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ABSTRACT

Currently, there is an increasing interest on the development of topical formulations containing rosmarinic acid (RA) due to its well-documented antioxidant activity. This study aimed to develop and validate a stability-indicating ultra-fast liquid chromatography (UFLC) method for the determination of RA in nanoemulsions, porcine skin and nasal mucosa intended to be applied in permeation/retention studies and for development of topical nanoemulsions. Chromatographic separation was carried out using a C18 column packed with 2.6 µm particle size in isocratic conditions using as mobile phase water:acetonitrile (83:17, v/v), acidified with 0.1% trifluoracetic acid (v/v), with a total time of analysis of 3.5 min and detection at 330 nm. RA analysis was specific in the presence of both non-biological (blank nanoemulsion and receptor fluid) and biological matrices (porcine ear skin and porcine nasal mucosa). No interference of degradation products of RA was verified after different stress conditions such as acidic, alkaline, oxidative, light exposure (UV-A and UV-C) and thermal demonstrating the method stability-indicating property. The analytical (0.1–10.0 $\mu g \cdot mL^{-1}$) and bioanalytical (0.5–10.0 $\mu g \cdot mL^{-1}$) linearity was proved by analysis of the calibration curves of RA and no matrix effect was observed. The method was sensitive, precise and accurate, and showed recovery higher than 85%. The method was considered robust as evaluated by a Plackett-Burman experimental design. In the validated conditions, the RA was determined in the nanoemulsions obtained by spontaneous emulsification procedure ($1.007 \pm 0.040\ mg \cdot mL^{-1}$), porcine ear skin ($1.13 \pm 0.19\ \mu g \cdot cm^{-2}$) and nasal mucosa ($22.46 \pm 3.99\ \mu g \cdot cm^{-2}$) after retention/permeation studies. Thus, a highly sensitive, simple, fast and stability-indicating method was developed for RA analysis during the development of topical nanoemulsions and bioanalytical assays in complex matrices.

1. Introduction

Rosmarinic acid (RA) is a polyphenolic compound, an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, commonly found in species from the *Boraginaceae* and *Lamiaceae* family [1,2]. It was first isolated from *Rosmarinus officinalis* L. and has various biological activities reported, including antiviral, antimicrobial, anti-tumoral, anti-inflammatory and antioxidant properties. Among these activities, a well-documented literature reports the ability of RA in protecting skin and brain against the damage caused by reactive species of oxygen [1–5]. Recently, the incorporation of RA into polymeric- and lipid-nanotechnology-based delivery systems has been proposed aiming to improve the RA stability and its permeability through biological membranes [6–10].

The development and validation of analytical methods for the accurate identification and quantification of bioactive compounds in analytical and/or bioanalytical samples with absence of interference of degradation products is a key consideration in the pharmaceutical development field [11,12]. Analytical assay of RA was mainly focused on its determination in plant extracts by high-pressure liquid chromatography (HPLC) [13–25], mainly due to advantages in terms of sensitivity, specificity and accuracy of this technique, although most of them operates in gradient mode and require a quite long period of time of analysis (normally upper than 14 min). The use of high-throughput liquid chromatography technologies has been proposed to circumvent these drawbacks, reducing the time of analysis, and consequently

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decreasing the environmental impact by reducing solvent consumption [26,27]. Only few approaches employing these technologies are available for RA assay in *Symphytum officinale* L. [28], Shengxiong glucose injections [29] and *Perilla frutescens* (L.) Britt. [30]. Bioanalytical assay of RA was also found in some studies mainly focused in beagle, pig, and rat plasma [15,29,31]. However, none stability-indicating method was found for RA.

Our research group has been recently interested in the development of topical nanoemulsions as delivery systems for RA. Nanoemulsions have been considered as a potential lipid-nanotechnology-based delivery system for topical administration and improvement of permeability and stability of poorly-soluble molecules. Such systems are nanodispersion of oily droplets in an external aqueous phase, stabilized by an appropriate surfactant system [32–35]. Thus, in this study we have described for the first time the development and validation of a fast and an isocratic stability-indicating UFLC analytical and bioanalytical method for RA assay in pharmaceutical nanoemulsions, as well as in porcine ear skin and nasal mucosa intended to be used in further retention/permeation studies for topical formulations development.

2. Material and methods

2.1. Materials and reagents

The reference standard of RA with a purity over than 98% was purchased from Carbosynth Ltd. (Berkshire, UK). Liquid Chromatography grade methanol, acetonitrile and trifluoracetic acid were obtained from Tedia (Rio de Janeiro, Brazil). Ultra-pure water used in UFLC was produced using a Milli-Q apparatus (Millipore, Billerica, USA). Medium chain triglycerides (MCT) and egg-lecithin (Lipoid E-80®) were purchased from Lipoid GmbH (Ludwigshafen, Germany). Potassium phosphate monobasic and dibasic were obtained from Nuclear (CAQ, São Paulo, Brazil). Porcine ears and porcine nasal mucosa were supplied from a local slaughterhouse (Ouro do Sul – Cooperativa dos Suinocultores do Caí Superior Ltda., Harmonia, Brazil).

2.2. Instrumentation and UFLC conditions

The RA analyses were performed using a Shimadzu Prominence series UFLC system equipped with a temperature controlled automatic injector, a photodiode array (PDA) detector and an LC solutions software for data processing (Kyoto, Japan). Chromatographic separation was carried out in a Kinetex C18 column (100 × 2.1 mm i.d.; particle size, 2.6 µm; Phenomenex, USA) guarded by a pre-column filter in-line Ultra (Phenomenex, USA) at 55 °C. The mobile phase was composed of water and acetonitrile (83:17, v/v) with pH approximately 2.5, acidified with 0.1% trifluoracetic acid (v/v), filtered through a 0.22 µm porous nylon membrane and degassed under vacuum, set at isocratic mode up to 3.5 min. The samples were kept at 4 °C in the auto-sampler, and a volume of 3 µL was injected. Eluent system was pumped at a flow rate of 0.55 mL·min^{-1} and detection wavelength was set to 330 nm.

2.3. Solutions

2.3.1. Preparation of standard solutions

A RA stock solution (1 mg·mL^{-1}) was obtained by weighting accurately 10 mg of rosmarinic acid into a 10 mL calibrated volumetric flask, dissolved in methanol. Standard solutions were obtained from the dilutions of an adequate aliquot of stock in water/acetonitrile mixture (80:20; v:v). The stock and diluted standard solutions were kept in darkness at 4 ± 2 °C.

2.3.2. Preparation of matrices solutions

2.3.2.1. Nanoemulsions. RA loaded-nanoemulsions (NE_{RA}) were prepared by spontaneous emulsification procedure as previously described by our research group [35]. The final formulations were composed by RA 0.1% (w/v), medium chain triglycerides 8.0% (w/v), egg lecithin 2.0% (w/v), and water up to 100%. The oil components were dissolved in ethanol, and after were poured into the water phase under magnetic stirring. Subsequently, a process of evaporation at 40 °C under reduced pressure removed the organic solvent and concentrated RA up to 1 mg·mL^{-1}. RA amount in nanoemulsions were determined diluting appropriate aliquots of nanoemulsion in a water/acetonitrile mixture (80:20; v:v), filtered by 0.22 µm nylon syringes filter. Blank nanoemulsions (NE_B) were prepared in similar conditions in the absence of RA.

2.3.2.2. Porcine ear skin and nasal mucosa. The porcine ear skin (PES) was excised from the outer region of the porcine ear and stored at −20 °C until use, for up to a maximum of one month. To the extraction procedure, the PES (surface area 1.80 ± 0.02 cm^2, thickness 1.0 ± 0.1 mm) was reduced to tiny pieces and placed in different test tubes, 2 mL of methanol were added on the tubes and the samples were maintained in an ultrasound bath for 45 min.

The porcine nasal mucosa (PNM) was carefully removed from the nasal turbinates obtained by an incision along the nasal septum of the porcine heads, as previously described by Barbi et al. [36]. The PNM was stored at −20 °C until use, for up to a maximum of one month. For extraction procedure, the PNM (surface area 1.80 ± 0.02 cm^2) was reduced to tiny pieces and placed in different test tubes, 4 mL of methanol were added on the tubes and the samples were maintained in an ultrasound bath for 120 min. Appropriate aliquots of PES and PNM solutions were filtered by 0.22 µm nylon syringes filter, and then analyzed.

2.3.2.3. Receptor fluid for retention/permeation studies. Potassium phosphate buffer pH 7.4 and 5.8 were chosen to be the receptor fluid (RF) for the retention skin and nasal permeation studies, respectively. Appropriate aliquots of RF were filtered by 0.22 µm nylon syringes filter, and then analyzed.

2.4. UFLC method validation

The UFLC developed method was fully validated in terms of specificity/selectivity, linearity, matrix effect, precision, accuracy, extraction recovery, and stability, according to the official guidelines, that is: ICH specifications [37] for the validation of analytical procedure in non-biological matrices (nanoemulsion and receptor fluid), and the EMA and FDA recommendations [38,39] for the validation of bioanalytical procedure in biological matrices (porcine skin and nasal mucosa tissue). Analysis of variance (ANOVA) and Student's *t*-test were used to analyze the results using a significance level of $\alpha = 0.05$.

2.4.1. System suitability

System suitability tests were performed to verify the adequacy of the chromatographic system for the analysis. The parameters of peak area, retention time, theoretical plates and tailing factor of RA were measured and evaluated [40].

2.4.2. Specificity and forced degradation

The specificity was determined by comparing chromatograms obtained for pure standard RA solution, matrices spiked with RA at the concentration of 10 µg·mL^{-1} and solutions containing only the matrices (blank samples). In order to verify the presence or absence of interferences from matrices, the peak corresponding to RA was analyzed and identified in each spiked matrix by UV spectra between 250 and 370 nm, peak purity and retention time.

Forced degradation protocols were also performed. The stock RA standard solutions were submitted to acid and alkaline hydrolysis, oxidative stress, photolytic and temperature degradation. Briefly, acid hydrolysis was investigated by adding Hydrochloric acid (HCl) to the standard solutions to achieve the final concentration of 1.0 M. Alkaline