Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

Development and validation of a LC method for determination of genistein in topical nanoemulsions

A. P. Cappra Silva, L. S. Koester, P. Mayorga, V. Linck Bassani, H. Teixeira

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Prof. Dr. Helder Texeira, Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752/404A, 90610-000 Porto Alegre, RS, Brazil
helder@farmacia.ufrgs.br

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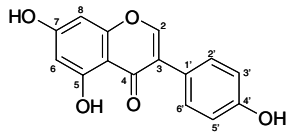
The aim of this study was to develop and validate an isocratic LC method for the quantification of genistein in topical nanoemulsions. The analyses were performed at room temperature on a reversed-phase C_{18} column using a mobile phase composed of methanol/water/acetonitrile (70 : 25 : 5, w/w/w) at 1.0 ml · min^{-1}. The detection was carried out on a UV detector at 327 nm. The linearity, in the range of 25–75 μg/ml, presented a determination coefficient (r^2) higher than 0.999, calculated by the least square method. No interferences from the excipients (egg-lecithin, octyldodecanol or medium chain triglycerides) were detected. The R.S.D. values for intra- and inter-day precision experiments were lower than 2.3%. The recovery of genistein from nanoemulsions ranged from 96.6% to 106.6%. The excellent performance of the method, its linearity, accuracy and precision, demonstrate that it can be readily used to quantify genistein incorporated in nanoemulsions.

1. Introduction

Ultraviolet radiation is responsible for causing various skin disorders including photoaging and cancer. In recent years, increasing evidence points that soybean isoflavon aglycones, especially genistein, show antiphotocarcinogenic and antiphotoaging effects (Wei et al. 2003). Genistein inhibits skin carcinogenesis and cutaneous aging induced by ultraviolet light in mice and photodamage in humans (Messina et al. 1994; Wei et al. 2003; Afaq and Mukhtar 2006). The main mechanism of action involves protection of oxidative and photodynamically damaged DNA, down-regulation of ultraviolet B activated signal transduction cascades, and antioxidant activities (Wei et al. 1995, 2002; Kang et al. 2003).

However, the use of isoflavones (as aglycones) in pharmaceutical products is compromised by their low water solubility. On the other hand, the use of nanoemulsions as colloidal carriers for the topical delivery of poorly-soluble drugs has received increasing attention (Piemi et al. 1999; Fernandez et al. 2000; Alves et al. 2005). Nanoemulsions are fine dispersions of oil-in-water (o/w) in which the poorly-soluble drugs could be dissolved in the oil core and/or adsorbed on the o/w interface (Tamilvanan and Benita 2004; Bouchemal et al. 2004). In fact, the incorporation of drugs in such systems could increase the skin permeation rate and enhance the topical effect due to prolonged residence time in the uppermost skin layers due to the large surface area and low surface tension of the oil droplets (Klang et al. 1998; Benita 1999).



In this context, the development of topical nanoemulsions containing genistein is currently under study by our research group. Recently, we have developed a sensitive LC method for the quantification of genistein using UV detection at 270 nm in view to evaluate its intrinsic skin permeation (Xavier et al. 2007). However, preliminary investigations have shown the interference of nanoemulsion excipients in the wavelength used. Then, the aim of the present study was to validate an isocratic LC method at 327 nm, in accordance with the ICH (2005), for the determination of genistein content in nanoemulsions.

2. Investigations, results and discussion

2.1. Physico-chemical properties

In a first step, physico-chemical properties of the nanoemulsions were evaluated (Table 1). The spontaneous emulsification procedure yielded monodisperse emulsions (IP < 0.2) with a typical droplet size in a range of 200–300 nm, in agreement with the results previously reported for other nanoemulsion systems (Yu et al. 1993; Bouchem-

Table 1: Physico-chemical properties of nanoemulsions containing genistein

	Medium chain triglycerides	Octyldodecanol
Droplet size (nm)	263 ± 6	270 ± 13
ζ-Potential (mV)	−44 ± 4	−42 ± 3
Viscosity (cP)	1.5 ± 0.1	1.8 ± 0.07
pH	6.8 ± 0.3	6.9 ± 0.2

Table 2: Within day and between day precision of the LC assay of genistein

Conc. (μg/ml)	Day 1 Peak area (mVs)[a]	Day 2 Peak area (mVs)[a]	Day 3 Peak area (mVs)[a]	Within day R.S.D.(%)[b]	Between day R.S.D.(%)
25.0	532316.7 ± 280.3	548212.3 ± 11103.2	536316.0 ± 2048.6	0.05–2.03	1.08
37.5	789345.0 ± 16403.4	826223.7 ± 903.0	816215.0 ± 5846.9	0.11–2.08	0.98
50.0	1077680.7 ± 1559.1	1077850.7 ± 1910.8	1066240.3 ± 8379.9	0.14–0.79	0.36
62.5	1322620.7 ± 22382.4	1346978.3 ± 2186.3	1322255.7 ± 43897.4	0.16–1.69	0.80
75.0	1597784.3 ± 13965.9	1598619.3 ± 31300.7	1625450.0 ± 37841.8	0.87–2.33	0.77

[a] Approximate mean ± S.D. ($n = 3$) [b] range of R.S.D. considering 3 days of validation

al et al. 2004). The highest droplet size and viscosity were detected for the formulation composed by octyldodecanol as oil core. These results could be related to the higher viscosity of this oil (58–64 cP) compared to medium chain triglycerides (25–33 cP) (Kibbe 2000; Melzer 2000). It is known that oil viscosity could influence the diffusion speed of organic phase on water phase during a spontaneous emulsification process and consequently the droplet size of particles. Wehrlé et al. (1995) have previously described that the migration speed of the organic solvent into the aqueous phase was the most important parameter affecting particle size of colloidal carriers obtained by solvent displacement methods. Concerning ζ-potential, nanoemulsions presented negative values due to the presence of negatively-charged phospholipids in egg-lecithin composition such as phosphatidylserine and phosphatidic acid at neutral pH of formulations, as previously reported in the literature (Yang and Benita 2000; Li and Tian 2002).

2.2. Validation

The first analytical experiments were performed in order to evaluate whether nanoemulsion excipients could interfere in genistein quantification since they are stabilized by egg-lecithin which is a complex mixture of phospholipids (mainly phosphatidylcholine) combined with other substances such as carbohydrates, fatty acids and triglycerides (Kibbe 2000). The mobile phase composition as well as the other chromatographic conditions were chosen in order to obtain efficient routine analysis. The specificity was carried out through the comparison of the peak retention time of the genistein and blank nanoemulsions. No interference of the excipients was noticed since no peak was detected after injection of blank nanoemulsion at the set wavelength (327 nm). In the employed chromatographic conditions, genistein presented a retention time of approximately 4.4 min.

The calibration curves of genistein, fitted by plotting concentration versus the corresponding mean peak area, were linear ($r^2 = 0.9997$) over genistein concentration range from 25 to 75 μg/ml. The linear regression for three replicate calibration curves was $y = 21470x - 4175.9$ where y is the peak area and x is the concentration of the analyte. The detection (DL) and quantitation (QL) limits were calculated based on the standard deviation of the response and the slopes. DL and QL were, 1.91 and 5.79 μg/ml, respectively. It must be pointed out that these DL and QL values, obtained for genistein assay at 327 nm, were significantly higher than those previously reported when analysis was performed at 270 nm (Xavier 2007). Indeed, these results could be attributed to the typical lower intensity of the band I (327 nm) of genistein compared to band II (270 nm) (Mabry et al. 1970). In the same way, precision of the method was assessed considering repeatability (intra-day analysis) and intermediate precision (inter-day analysis). The results were expressed as relative standard deviation of the mean values (R.S.D.) and are shown in Table 2. The intra- and inter-day precisions showed R.S.D. values lower than 2.33% for genistein, which can be considered acceptable for the purpose of the analysis (ICH 2005).

The accuracy of the method was determined as recovery test. As can be seen in Table 3, whatever amount of genistein was added in blank nanoemulsions, the recoveries ranged from 96.6 to 101.7% for nanoemulsion composed by medium chain triglycerides and egg-lecithin and from 103.3 to 106.6% for nanoemulsion composed by octyldodecanol and egg-lecithin, indicating a good agreement between amounts added and found.

Table 3: Accuracy of genistein in the presence of nanoemulsions excipients

Sample	Theoretical concentration (μg/ml)	Experimental concentration (μg/ml)	Recovery (%)	R.S.D. (%)
#1	25	24.17 ± 0.06	96.68	0.25
	50	50.85 ± 0.08	101.7	0.16
	75	75.23 ± 0.11	100.3	0.14
#2	25	26.09 ± 0.42	104.36	1.57
	50	53.31 ± 0.08	106.62	0.15
	75	77.51 ± 0.79	103.34	1.01

#1: blank nanoemulsion composed by egg-lecithin, medium chain triglycerides and water
#2: blank nanoemulsion composed by egg-lecithin, octyldodecanol and water

2.3. Determination of genistein content

The method was finally used to evaluate the genistein content in nanoemulsions composed by egg-lecithin and either medium chain triglycerides or octyldodecanol (Table 4). The determination of genistein content demonstrated R.S.D. $< 1.5\%$ from triplicate analysis, indicating the precision of the validated method. In a last step, we investigated the location of genistein in nanoemulsions by estimating its presence in the aqueous phase after ultrafiltration/centrifugation procedure (Michalowsky et al. 2004). No genistein was detected in the water phase of both formulations. Considering the QL described above, the association efficiency of genistein with the inner phase of both nanoemulsions was close to 100%.

In conclusion, this paper shows a useful LC method to estimate genistein incorporated in nanoemulsions. In spite of the lower sensitivity of the proposed method, in the wavelength

Table 4: Genistein content in nanoemulsions at 1 mg/mL

Nanoemulsion	Genistein assayed (μg/mL)	R.S.D. (%)
Medium chain triglycerides	1.03 ± 0.01	1.07
Octyldodecanol	1.05 ± 0.02	1.48

of 327 nm, compared to that previously reported (Xavier et al. 2007), this method allows to accurately and precisely estimate genistein incorporated in nanoemulsions.

3. Experimental

3.1 Materials

Egg-lecithin (Lipoid E-80®) and medium chain triglycerides were kindly donated by Lipoid GmbH (Ludwigshafen, Germany). Octyldodecanol was obtained from Delaware (Porto Alegre, Brazil). Genistein was purchased from Sigma (São Paulo, Brazil). Ultrapure water was obtained from a Milli-Q apparatus (Millipore, Billerica, USA). Acetonitrile and methanol LC grade were obtained from Merck (Darmstadt, Germany).

3.2 Preparation and characterization of nanoemulsions

Nanoemulsions were prepared using the spontaneous emulsification procedure (Bouchemal et al. 2004). This method consists of injecting an organic phase containing components of the oil core into the water phase under magnetic stirring (15 min). Subsequently, the organic solvent was removed by evaporation under reduced pressure at 40–45 °C. A typical formulation consisted of genistein, egg-lecithin, octyldodecanol or medium chain triglycerides and water. The final concentration of genistein in nanoemulsions was 1mg/mL. Blank nanoemulsions were obtained, under the same conditions, in the absence of genistein.

The mean droplet size of the nanoemulsions was determined by quasi-elastic light scattering after proper dilution in water and the electrophoretic mobility was measured with a zetasizer (HAS 3000, Malvern, England). The pH values of nanoemulsions were directly determined in samples (Micronal B374 potentiometer). The viscosity of the nanoemulsions was measured using a capillary viscosimeter. The measurements were performed at room temperature.

3.3 Chromatographic conditions and apparatus

The LC apparatus consisted of a Shimadzu LC-10A system (Kyoto, Japan) equipped with a model LC-10AT pump, a SPD-10AV UV-VIS variable-wavelength detector (set at 327 nm), a SCL-10Avp system controller, a Rheodyne 7725 injection valve with a 50 μL loop. Genistein was analyzed using a Shim-pack CLC-ODS (M) RP-18 column (5 μm, 250 mm × 4 mm i.d.), connected to a Waters RP-18 precolumn (10 μm). The mobile phase consisted of a methanol : water : acetonitrile mixture (70 : 25 : 5 w/w/w), filtered and degassed, in isocratic flow. The LC system was operated at flow-rate of 1.0 mL · min^{-1} and the sensivity was 0.5 AUFS, at room temperature. All calculations concerning the quantitative analysis were performed with external standardization by measurement of peak areas.

3.4 Method validation

The validation was performed based on the ICH (2005), taking into account the characteristics required for assaying dosage forms.

The specificity of the method was evaluated by analyzing solutions of the blank nanoemulsions obtained in the absence of genistein. The system response was examined through the presence of interference or overlaps with genistein response.

For linearity experiments, solutions of genistein were prepared at five concentrations within the range of 25–75 μg/mL, on three different days. The results were represented graphically, which allowed the evaluation of the calibration curve and coefficient of determination. Detection and quantitation limits were determined based on the standard deviation of the response and the slope, using the calibration curve data.

The intra-day precision (repeatability) of the method was determined by analysis of three samples of genistein for each point of the calibration curve, during the same day under the same experimental conditions. Inter-day precision values were obtained by assaying freshly prepared solutions of genistein calibration curve on three different days.

Accuracy was evaluated as recovery of the method. The accuracy experiments were performed applying the method to quantify genistein in the presence of formulation excipients. Then, blank nanoemulsions were spiked with known amounts of genistein at different levels: low, medium and high, corresponding, respectively, to 25, 50, and 75 μg/mL. Samples were appropriately diluted and analyzed. The results represent the mean recovery for three independent samples.

3.5 Genistein content

The determination of genistein content in the nanoemulsions was carried out under the conditions previously described. Nanoemulsion aliquots of 0.25 ml containing genistein were appropriately diluted in methanol, filtered and analyzed. For association efficiency study, sample of nanoemulsions were added to ultrafiltration membranes (Ultrafree-MC 10,000 MW, Millipore) and centrifuged at 5,000 rpm (Michalowski et al. 2004). Free genistein was determined in a clear ultrafiltrate obtained by separation of the water phase. The association efficiency (%) was estimated by the difference between the total (genistein content) and free drug concentrations.

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