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LC analysis of coumestrol incorporated into topical lipid nanoemulsions

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A simple, rapid, and sensitive LC method to determine coumestrol incorporated in the lipid nanoemulsions was validated. The analyses were performed at room temperature on a reversed-phase C18 column using a mobile phase composed of methanol/water with 0.1% trifluoracetic acid (70:30, v/v) at 0.8 mL min^{-1}. The detection was carried out on a UV detector at 343 nm. The linearity, in the range of 0.1–6.0 μg/mL, presented a determination coefficient (r^2) of 0.999, calculated by the least square method. No interferences of the oil core or the gelling excipients were detected. The R.S.D. values for intra- and inter-day precision experiments were lower than 2%. The recovery ranged from 99.42% to 100.72%. Finally, the proposed method was successfully applied to determine coumestrol incorporated in the proposed topical formulations.

1. Introduction

Coumestrol (Fig. 1) is a coumestan isoflavone found in many leguminous plants, such as in alfalfa, red clovers, and soybeans. Coumestrol has been receiving special attention due to its antioxidant activity. This activity is related to the ability of coumestrol in donating electrons from the hydroxyl groups present in the A and B-rings to free radicals, protecting cell components from oxidative damage. Early results reported by Mitchell et al. (1998) showed the antioxidant effect of coumestrol using different experiments. Coumestrol exhibits a high activity in comparison to other phytostrogens evaluated (genistein and daidzein), which was explained by a highly-extended π-electron system via the C-ring double-bond that enables conjugation to occur between A and B rings of coumestrol. The higher antioxidant activity of coumestrol, when compared with isolated isoflavones or in extracts from *Trifolium pretense* L. (red clover) and *Glycine max* (soybean), was also shown in other reports (Georgetti et al. 2003; Lee et al. 2006).

Topical application of natural antioxidants incorporated into nanosystems has been considered a promising strategy to prevent skin photoaging and carcinogenesis. The incorporation of antioxidants into nanoemulsions has been currently investigated (Silva et al. 2009; Almeida et al. 2010; Vargas et al. 2011) since such systems could increase the skin permeation rate and enhance the topical effect due to prolonged residence time in the uppermost skin layers, the large surface area and low surface tension of the oil droplets (Klang and Benita 1998). Such systems are dispersions of oil droplets stabilized by surfactants in an aqueous medium (Anton and Vandamme 2011).

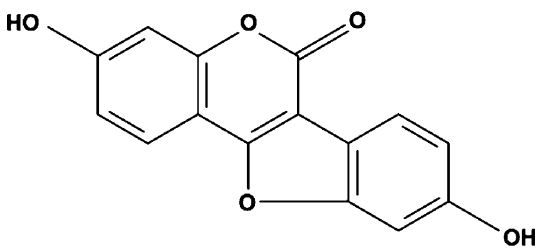

Fig. 1: Chemical structure of coumestrol

The purpose of this study was to validate an isocratic reversed-phase LC method, in accordance with ICH (2005), for determining coumestrol in nanoemulsions obtained in either the absence or the presence of hydroxyethyl cellulose added to adjust its viscosity to topical application. Even though literature describes the use of liquid chromatography methods to assay coumestrol (currently in mixture with other isoflavones in plant extracts) (Wang et al. 1990), to the best of our knowledge, no report on the determination of coumestrol in nanostructured systems is available in the literature.

2. Investigations, results and discussion

2.1. Method development

In the first set of experiments, different proportions of methanol and/or acetonitrile and water were tested as a suitable mobile phase for coumestrol assay. However, tailing and an irregular shaping of the coumestrol peak were detected for most solvent combinations. Such problems were solved by adding trifluoracetic acid into the mobile phase.

Under these acid conditions, a satisfactory peak resolution and symmetry could be observed, probably due to favoring of the non-ionized form of coumestrol as it has been demonstrated for phenolic compounds. Some chromatographic performance parameters were evaluated during this mobile phase selection, such as the number of theoretical plates (*N*), tailing factor (*T*), and retention factor (*k'*). Based on these preliminary results, the mobile phase composed of methanol/trifluoracetic acid at 0.1% (70:30) at pH 3.0 was the chosen setup for validation since *N* (2.907), *T* (1.09), *k'* (2.44), and R.S.D. for injection repeatability (0.11) were within the acceptance criteria, according to literature (ICH 2005; USP 2007).

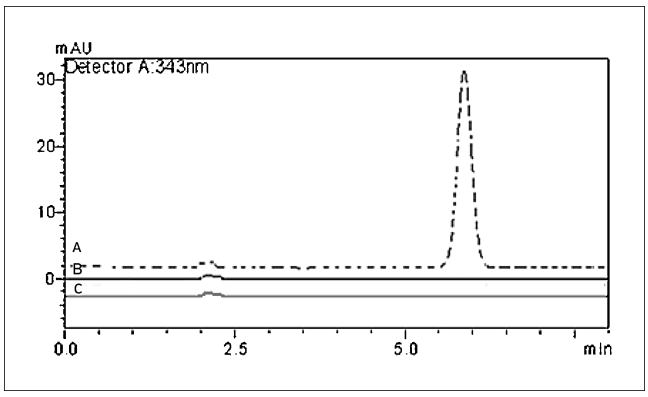

Fig. 2: Typical chromatograms of COU (A) and COU in the presence of excipients of H-NE (B) or H-CNE (C)

2.2. Method validation

The specificity of the method was performed to evaluate whether nanoemulsion excipients could interfere in coumestrol quantification since formulations are comprising a complex mixture of oil, surfactants, and thickening agents. The chromatographic conditions were chosen to obtain fast and efficient routine analyses. The specificity was determined by the comparison of the peak retention time of coumestrol and blank formulations. No interference was noticed since no peak was detected after injection of blank formulations at set wavelength (Fig. 2). Under the chromatographic conditions, coumestrol presented a retention time of approximately 6.0 minutes.

To assess linearity, three standard curves of coumestrol were constructed, plotting concentration (μg/mL) versus the corresponding mean peak area. The response for coumestrol was linear and the equation was $y = 152571\ x + 354.6$, showing a satisfactory determination coefficient ($r^2 = 0.999$) which was highly significant for the method ($P < 0.05$). The linearity data were analyzed by means of ANOVA, which demonstrated a significant linear regression (f calculated = 44707,01 > f critical = 4,24E-69; $P = 5\%$), no-significant linearity deviation ($P > 0.05$) and the confidence intervals for the intercept included zero (− 4803.6; + 5512.8). Such results confirmed the absence of a constant systematic error. From the linearity data, LOD (0.014 μg/mL) and LOQ (0.046 μg/mL) were estimated. Such values could be considered satisfactory for determining coumestrol incorporated in the formulations proposed in this study.

The accuracy of the method was assessed at three concentration levels (0.5, 2.5, and 3.0) on three different days (Table 1) for both H-NE and H-NEC. Regardless of the coumestrol concentration level, the overall results showed R.S.D. values lower than 1.96% in all experiments. The coumestrol recovery was approximately 100% under the same conditions. The difference between nominal and found concentrations of the standards demonstrated that the assay is accurate enough for its application. The precision of the method was assessed considering repeatability and intermediate precision at a single concentration of 2.5 μg/mL on three different days (Table 2). The intra- and inter-day precisions showed satisfactory R.S.D. values (lower than 2%). Such precision and accuracy results were within acceptable limits and in agreement with recommendations for analytical assays (ICH 2005).

2.3. Method application

Prior to the application of the proposed method for assaying coumestrol in formulations, a brief characterization of formulations was performed. Notwithstanding the composition, nanoemulsions exhibit an oil droplet size in a 200–240 nm-range. Concerning ζ-potential measurements, NE exhibits a negative value (−16 mV), which could be attributed to the presence of negatively-charged lipids in egg-lecithin, whereas formulations containing CTAB (CNE) displayed positive values of higher than + 55 mV, indicating the location of this cationic surfactant at the o/w interface of nanoemulsions. Such findings are in line with our previous results for the incorporation of flavonoids into topical nanoemulsions (Silva et al. 2009; Fasolo et al. 2009; Vargas et al. 2010).

As can be seen in Table 3, the determination of coumestrol in formulations (in the absence or the presence of hydroxyethyl cellulose) demonstrated a R.S.D. < 4.3% from triplicate analysis, indicating the precision of the validated method. The location of coumestrol in nanoemulsions was estimated by investigating its presence in the aqueous phase after ultrafiltration/centrifugation procedure. Coumestrol detection in the water phase of the nanoemulsions was below LOQ (0.046 μg/mL), which means that coumestrol seems to be incorporated into nanoemulsions. These findings could be related to the lipophilic character of coumestrol (log P = 2.41, estimated by means of ChemAxon version 5.4 available in chemicalize.org) promoting its incorporation (encapsulation and/or adsorption) into the oil core of nanoemulsions.

In conclusion, this article shows a useful LC method for the quantification of coumestrol in nanoemulsions in the presence of various excipients used in the composition of formulations. In the validated conditions, the method proved to be linear, precise, accurate, and specific.

3. Experimental

3.1. Chemical and reagents

Egg-lecithin (Lipoid E-80^R) and medium chain triglycerides were kindly donated by Lipoid GmbH (Ludwigshafen, Germany). Polysorbate 80 was obtained from Vetec (Rio de Janeiro, Brazil). Hydroxyethyl cellulose was obtained from Delaware (Porto Alegre, Brazil). Cetyl trimethylammonium bromide (CTAB) and coumestrol (≥ 95%) were purchased from Sigma-Aldrich (São Paulo, Brazil). Ultrapure water was obtained from a Milli-Q apparatus (Millipore, Billerica, USA). Methanol LC grade was obtained from Tedia (Fairfield, USA). Trifluoracetic acid was obtained from Merck (Hoherbrunn, Germany).

3.2. Preparation and characterization of the nanoemulsions

Nanoemulsions containing coumestrol were prepared using a spontaneous emulsification procedure. Briefly, the method consists of injecting an organic phase containing components of the oil core into the water phase under magnetic stirring. Subsequently, the organic solvent was removed by evaporation under reduced pressure at 40–45 °C. The final composition of formulations is presented in Table 4. Typically, two kinds of formulations were prepared in the absence (NE) and in the presence of the cationic surfactant CTAB (CNE).

Hydroxyethyl cellulose was added as thickening excipient (H-NE or H-CNE) to adjust the viscosity for topical application. Coumestrol was added to the ethanol phase to produce a final concentration in nanoemulsions of 1 mg/mL. Blank nanoemulsions were prepared under the same conditions, but in the absence of coumestrol as control formulations.

The mean droplet size and ζ-potential of the nanoemulsions were determined at 25 °C by photon correlation spectroscopy (PCS) and electrophoretic mobility, respectively (3000HS Zetasizer, Malvern Instruments, England). The samples were adequately diluted in water for size determinations or in 1 mM NaCl solution for ζ-potential measurements.

3.3. Apparatus and chromatographic conditions

The LC system consisted of a Shimadzu LC-20A system (Kyoto, Japan) equipped with a model LC-20AT pump, a SPD-20 AV UV-VIS variable–wavelength detector, SIL-20A auto injector and a degasser module. The data were acquired and processed by Shimadzu LCSolution GPC software (Shimadzu, Kyoto, Japan). The column used was a C18 Phenomenex Gemini (150 x 4.6 mm, i.d., 5 μm particle size). The optimized mobile phase consisted of a mixture of methanol/water with 0.1% trifluoracetic acid (70:30, v/v), filtered and degassed by suction-filtration through

Table 1: Accuracy of coumestrol in the presence of formulation excipients

Formulation	Coumestrol amount		Recovery (%)[a]	R.S.D. (%)
	Added (μg mL^{-1})	Found (μg mL^{-1})		
H-NE	0.5	0.51	101.65	1.00
	2.5	2.48	99.42	0.30
	3.0	3.02	100.72	0.03
H-CNE	0.5	0.50	100.07	1.81
	2.5	2.50	100.06	0.90
	3.0	3.01	100.37	1.29

[a] The mean of three independent experiments for each one of the three spiking levels

Table 2: Intra- and inter-day precision of LC assay of coumestrol

		H-NE		H-CNE	
		Measured (μg/mL)	R.S.D. (%)	Measured (μg/mL)	R.S.D. (%)
Intra-day[a]	Day 1	2.37	1.77	2.28	1.96
	Day 2	2.35	0.82	2.29	1.08
	Day 3	2.38	0.69	2.26	1.19
Inter-day[b]		2.37	1.50	2.28	1.90

[a] The value represents the mean of six individual experiments
[b] The value represents the mean of eighteen individual experiments

Table 3: COU content in formulations

	Droplet size (nm)	ζ-potential (mV)	Coumestrol (%)
NE	234 ± 22	−16 ± 2	91.7 ± 1.5
H-NE	–	–	100.2 ± 2.6
CNE	196 ± 2	55 ± 4	92.8 ± 4.0
H-CNE	–	–	99.8 ± 1.9

0.45 μm nylon membrane, in isocratic flow. The LC system was operated at room temperature, sample injection volume of 20 μL, flow rate of 0.8 mL min^{-1} and detection at 343 nm.

3.4. Method validation

The method was validated based on the ICH guidelines (ICH 2005), taking into account the characteristics required for assaying dosage forms, as follows:

Specificity: The specificity of the method was evaluated by analyzing solutions of the blank formulations obtained in the absence of coumestrol. The system response was evaluated through the presence of interference or overlaps with coumestrol response.

Linearity, Limit of Detection (LOD) and Limit of Quantitation (LOQ): Solutions of coumestrol were prepared at seven concentrations within the range of 0.1–6.0 μg/mL on three different days. LOD and LOQ were determined based on the standard deviation of the response and slope of the calibration curve.

Precision: The precision of the method was determined by measuring the repeatability (intra-day precision) and the intermediate precision (inter-day precision), both expressed as relative standard deviation (R.S.D. %). The repeatability was evaluated by assaying six samples of formulations, at the same concentration (2.5 μg/mL) during the same day. The intermediate precision was studied by comparing the results obtained on three different days.

Accuracy: This parameter was determined by the recovery test, which consisted of adding known amounts of coumestrol to the blank nanoemulsions. The analyses were made in three different levels: low, medium and high concentration, corresponding to 20, 100 and 120%. Samples were appropriately diluted and analyzed. Accuracy was calculated as the percentage of the coumestrol recovered from three independent samples.

Table 4: Final composition of formulations (%, w/w)

	NE	H-NE	CNE	H-CNE
Coumestrol	0.1	0.1	0.1	0.1
MCT	8.0	8.0	8.0	8.0
Egg-lecithin	2.0	2.0	2.0	2.0
Polysorbate 80	1.0	1.0	1.0	1.0
CTAB	–	–	0.5	0.5
Hydroxyethyl cellulose	–	2.5	–	2.5
Purified water to	100.0	100.0	100.0	100.0

MCT: medium chain triglycerides; CTAB: Cetyl trimethylammonium bromide

3.5. Determination of coumestrol content

The coumestrol content analysis was determined after direct solubilization of formulations in pure methanol. Appropriate aliquots of the nanoemulsions obtained in both absence (NE or CNE) and presence of the thickening agent (H-NE or H-CNE) were diluted up to a final coumestrol concentration of 5 μg/mL. These solutions were filtered and analyzed under the validated conditions presented below. For nanoemulsions (NE or CNE), free coumestrol was also assayed in a clear ultrafiltrate obtained through the separation of the water phase using an ultrafiltration/centrifugation procedure (Fasolo et al. 2009). Samples of nanoemulsions were added to ultrafiltration membranes (100,000 Da cut off, Ultrafree MC, Millipore, U.S.A.) and centrifuged at 15000 rpm for 15 min. The association efficiency (%) was estimated by the difference between the total and free coumestrol concentration.

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