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A bioanalytical HPLC method for coumestrol quantification in skin permeation tests followed by UPLC-QTOF/HDMS stability-indicating method for identification of degradation products

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ABSTRACT

Coumestrol is present in several species of the Fabaceae family widely distributed in plants. The estrogenic and antioxidant activities of this molecule show its potential as skin anti-aging agent. These characteristics reveal the interest in developing analytical methodology for permeation studies, as well as to know the stability of coumestrol identifying the major degradation products. Thus, the present study was designed, first, to develop and validate a versatile liquid chromatography (HPLC) method to quantify coumestrol in a hydrogel formulation in different porcine skin layers (stratum corneum, epidermis, and dermis) in permeation tests. In the stability-indicating test coumestrol samples were exposed to stress conditions: temperature, UVC light, oxidative, acid and alkaline media. The degradation products, as well as the constituents extracted from the hydrogel, adhesive tape or skin were not eluted in the retention time of the coumestrol. Hence, the HPLC method showed to be versatile, specific, accurate, precise and robust showing excellent performance for quantifying coumestrol in complex matrices involving skin permeation studies. Coumestrol recovery from porcine ear skin was found to be in the range of 97.07–107.28 μg/mL; the intra-day precision (repeatability) and intermediate precision (inter-day precision), respectively lower than 4.71% and 2.09%. The analysis using ultra-performance liquid chromatography coupled to a quadrupole time-of-flight high definition mass spectrometry detector (UPLC-QTOF/HDMS) suggest the MS fragmentation patterns and the chemical structure of the main degradation products. These results represent new and relevant findings for the development of coumestrol pharmaceutical and cosmetic products.

1. Introduction

Coumestrol (COU) is a phytoestrogen which belongs to the coumestan class. It is found in several species of *Fabaceae* family, like *Medicago sativa, Glycine max* and *Trifolium pratense*. The antioxidant [1,2] and estrogenic activities [3,4] of coumestrol revealing the potential of this molecule for topical skin anti-aging products, especially for post-menopause women. The estrogenic activity of coumestrol is related to its ability to be an estrogen agonist, in other words, it exhibits high binding affinity for the estrogen receptors (ER) [4]. In the human skin, two ER receptors are commonly distributed: ERβ and ERα [5]. ERβ is widely expressed in the epidermis, hair follicle, blood vessels and dermal fibroblasts, while ERα is especially in to the dermal papilla cells [5,6]. The binding of agonist to the ER is able to activate them, increasing the collagen content of the skin, thus delaying the aging and photo aging, important effect in women during and after menopause [6]. In addition, phytoestrogens have demonstrated to play an important role in wound healing, through increasing the production-level of transforming factor growth β1 (TGF-β1) by dermal fibroblasts [7].

Some chromatographic methods for quantifying coumestrol in different matrices have been published: in serum [8], urine [9], plasma [10] and also in drug delivery systems [11]. High performance liquid chromatography (HPLC) has been the most employed

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method for determining coumestrol concentration in these samples. However, these methods are not appropriated for coumestrol quantification in different skin layers during permeation studies. Thus the development of a HPLC method with high sensitivity reveals to be necessary to determine coumestrol in these complex matrices. Additionally, the method should present specificity enough to separate the analyte from interferents as constituents from the biological samples, formulation or even analyte degradation products.

In this context, the present study was designed to develop and validate a stability-indicating HPLC method in order to quantify the coumestrol delivered from a hydrogel in different layers of porcine ear skin in permeation studies. To the best of our knowledge there is no report in the literature related to the coumestrol stability test, as well as a method with sensibility or specificity enough to be applied to the permeation tests, what demonstrate the originality and the applicability of the proposed method. The results of linearity, specificity, accuracy, precision and robustness of the method are reported in order to quantify coumestrol in complex matrices. Moreover, in stability-indication UPLC-QTOF/HDMS analysis, the MS fragmentation patterns and the chemical structure of the main degradation products are suggested.

2. Material and methods

2.1. Chemicals

Coumestrol (95%, HPLC purity) was obtained by Sigma-Aldrich (Germany). Acetonitrile (HPLC grade, Tedia, USA), trifluoracetic acid (Vetec, Brazil) and purified water (Milli-Q^{TM} system, Millipore, USA) were used in the mobile phase of the liquid chromatography analysis. Hydroxypropylmethylcellulose (HPMC, Methocel® K4 M) was purchased from Blanver (São Paulo, Brazil). The polypropylene tape strip (Scotch 750, width 13 mm) was purchased from 3 M® and propyleneglycol from Synth (São Paulo, Brazil). Porcine ear skin samples were obtained from a local slaughterhouse (Rio Grande do Sul, Brazil). All the other reagents used were of analytical grade.

2.2. Stock and reference solutions

Three stock solutions (20 μg/mL) were prepared dissolving 2.0 mg of coumestrol in methanol into a 100 mL volumetric flask. These coumestrol stock solutions were transferred to individual glass flask protected from light and stored under refrigeration (2–8 °C). The reference solutions of coumestrol were prepared in appropriate concentrations by dilution of the stock solutions in mobile phase prior to the analysis.

2.3. Complex matrices

2.3.1. Porcine ear skin

Full thickness porcine ear skin with approximately 1 mm of thickness was used as membrane in the permeation study. The whole skin on the back of the ear was removed from the underlying cartilage and subcutaneous fat using a scalpel. The circular skin discs were stored at −20 °C, and used within a month. For a purpose of validation the circular skin discs were spiked with different concentrations of coumestrol reference solutions. The skins were left to dry at room temperature, then, the skin samples were cut into pieces, put into tube-tests and sonicated with 4 mL of methanol for 30 min.

2.3.2. Formulation

The hydrogel was prepared dispersing 3.5% (w/w) of HPMC in water 24 h prior to the incorporation of the active compound. Coumestrol was dispersed in 1% propylene glycol (w/w) and it was incorporated on the preformed hydrogel. The final coumestrol concentration was 0.1% (w/w) used in skin permeation studies. A blank hydrogel was prepared, without the presence of coumestrol. The hydrogel formulation was weighed (0.500 g) and spiked with different concentrations of coumestrol. The extraction of coumestrol was carried out by sonication of 30 mL of methanol for 30 min.

2.3.3. Tape striping

The coumestrol retention within stratum corneum was evaluated applying the hydrogel topically on the skin and the permeation test run in Franz-type diffusion cells. Further, the corneocytes layers were removed with the assistance of adhesive tape. The coumestrol content was measured in cumulative amount present in all the skin strips [12,13]. For validation of the tape stripping step, the skin was similarly prepared. In this case, 14 pieces of tapes, used in tape stripping process, were spiked onto the adhesive part of the tape with predetermined concentrations of the reference solutions. After, these 14 pieces of tapes were put into a test-tube and sonicated with 4 mL of methanol for 30 min. The supernatant was filtered and the coumestrol concentration determined using HPLC method.

2.4. Apparatus and procedures

2.4.1. HPLC analysis

The development and validation of the HPLC method were performed using a Shimadzu HPLC-20A equipment (Kyoto, Japan) composed of HPLC-20AT pump, a SIL-20A autosampler and a UV/vis variable-wavelength SPD-20AV system detector. Data acquisition and treatment were performed with Shimadzu HPLC Solution GPC software (Shimadzu, Japan). A C18 Phenomenex Gemini column (150 × 4.6 mm, i.d., 5 μm) linked to a C18 pre-column (20 × 3.9 mm i.d.; 10 mm) (Waters, USA) were used. The mobile phase consisted of a mixture of acetonitrile/water with 0.1% trifluoracetic acid (40:60, v/v), filtered through a 0.45 μm pore size membrane filter and degassed for 30 min, eluted in isocratic mode. The flow rate, detection wavelength, injection volume, and column temperature were, respectively, 0.8 mL/min, 343 nm, 20 μL and 30 °C. All samples were filtered through Millipore PVDF membrane (0.45 μm of nominal pore diameter) before injection.

2.5. Validation of the analytical method

The HPLC method was validated according to the official guidelines in the concentration range of 0.04–10.0 μg/mL. For coumestrol quantification in the hydrogel formulation it was used official ICH guideline [14], while for biological matrices (tape strip samples, and porcine ear skin samples) it was used the FDA recommendation [15]. The statistical analysis was performed using Student's *t*-test and analysis of variance (ANOVA), with significance level of 0.05. The possible interference of degradation products generated from forced stability indicating test of coumestrol was also evaluated in the validation test.

2.5.1. Assessment of the matrix effect

The matrix effect was evaluated as described by Watanabe et al. [16] and Yatsu et al. [17] using the comparison of the slopes of analytical curves of reference coumestrol dissolved in the mobile phase or into three complex matrices (porcine ear skin, hydrogel formulation and adhesive tape samples). Three analytical curves were obtained, in three consecutive days, by plotting the peak area versus the concentration of the coumestrol standard (0.04, 0.5, 2.5, 5.0, 7.5, 10.0 μg/mL) in acetonitrile 40% (v/v) and in the complexes matrices solutions. Each concentration level was analyzed in five replicates.