Development and Validation of a Simple and Rapid Liquid Chromatography Method for the Determination of Genistein in Skin Permeation Studies

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Genistein (GEN) has potential advantages for topical skin delivery, but no literature data are available for its quantitation in different skin layers, such as the stratum corneum (SC). Therefore, a simple, rapid, selective and sensitive bioanalytical method was developed and validated for GEN quantitation in porcine skin samples following *in vitro* permeation studies. GEN was assayed by HPLC with UV-Vis detection (270 nm) using 0.5% acetic acid in water–*n*-propanol–acetonitrile (50 : 2 : 48, v/v/v) as mobile phase (flow-rate of 1.0 mL/min). Specificity was demonstrated since endogenous skin components did not interfere with GEN peak. Standard analytical curve was linear over the concentration range (0.1–60 μg/mL) and the lower limit of quantitation was determined for different skin layers (100 ng/mL). GEN recovery from skin layers ranged from 95.57 to 97.57%. Permeation studies were carried out using an automated vertical diffusion cell apparatus. No fluctuation on the amount of GEN retained in the SC was observed over time, but increasing amounts of the drug were found in deeper layers of the skin. The method was reliable and reproducible for the quantitation GEN in skin samples enabling the determination of the cutaneous penetration profile of this drug in permeation experiments.

Key words genistein; HPLC quantitation; bioanalytical validation; skin layer

Genistein (GEN) (Fig. 1) is the main isoflavone found in soybeans with several beneficial effects in cardiovascular diseases, osteoporosis and postmenopausal syndrome. Potential advantages of GEN have been reported in different types of cancer, such as breast and prostate, as well as skin cancer.[1] GEN acts in carcinogenesis through different mechanisms such as induction of differentiation, inhibition of topoisomerase II, protein tyrosine kinase activity and angiogenesis.[1,2]

Skin cancer arises predominantly from cells located within the epidermis.[3] Thus, to reach skin malignancies and achieve therapeutic benefits, antitumoral drugs, such as GEN, have to penetrate the stratum corneum and reach deeper layers of the epidermis.[4] In general, topical administration is a challenge in pharmaceutics.[5] Regardless, previous studies have demonstrated GEN's viability for topical[6] and transdermal permeation from different kinds of vehicles.[7] Thus, with the purpose of conducting percutaneous permeation studies, the quantitative determination of the drug in the skin is necessary in order to evaluate the efficiency of the proposed formulations.[8]

Due to the complexity of the skin matrices and the need to quantify low levels of the drug in the epidermis,[9] a highly selective analytical tool is required, since most samples usually contain several endogenous components of the skin. In addition, the analytical method must present enough sensitivity, due to possible very low concentrations of the drug present in each skin layer.[10] Considering the complexity and heterogeneity of the skin tissue, an effective extraction procedure to completely recover the analyte from the biological matrix is also required.[8]

GEN is typically determined using high performance liquid chromatography (HPLC) with mass spectrometry (MS), UV (UV) or electrochemical detection.[11–20] Nevertheless, little literature data are available for the determination of GEN in the skin[6] and, moreover, previous studies do not provide a method for the quantification of GEN in the stratum corneum as usually required for skin percutaneous penetration studies. In fact, some difficulties associated with significant interferences of skin components in the chromatographic method were reported.[21] Therefore, the challenge consists in extracting and determining the whole amount of drug accumulated in the stratum corneum and remaining skin layers.

In an attempt to: (i) extract total amounts of GEN from porcine ear skin, (ii) separate potential interferences such as skin components and (iii) quantify even low amounts of GEN in different skin layers, a simple and sensitive HPLC bioanalytical method was developed and validated in order to investigate GEN's percutaneous absorption and retention in the skin.

MATERIALS AND METHODS

Reagents GEN (min 99%) was purchased from LC Laboratories (Woburn, U.S.A.). HPLC grade acetonitrile, methanol, *n*-propanol and acetic acid were purchased from J. T. Baker

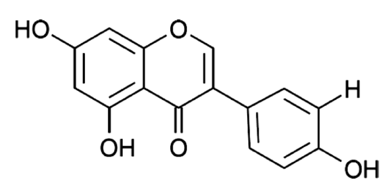

Fig. 1. Chemical Structure of GEN

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(Phillipsburg, U.S.A.). Water was purified using a Milli-Q system (Millipore, Billerica, U.S.A.) with a 0.22 μm pore end filter.

Skin Sample Preparation Full thickness porcine ear skin was used as a model for human skin.[4,21] Subcutaneous tissues were removed and the skin was stored at −80°C for a maximum period of 30 d before use.

Stratum corneum (SC) was separated from the remaining skin (RS) by the tape stripping technique.[22] For the experiments, the skin was tape-stripped 15 times with adhesive tapes (Durex Original 500, 3M, Sumaré, Brazil). The skin material removed by these 15 tapes represented the whole SC, and as such, the tapes were combined for quantitative determination of GEN in the pooled samples. The combined adhesive tapes containing the SC were placed in one single test tube for the extraction procedure. The same process was done with the RS.

GEN Extraction from Different Skin Layers Three blank samples of adhesive tapes with the SC were spiked with different amounts (50, 100, 200 μg) of GEN dissolved in methanol and dried under a N_2 stream. After evaporation of the solvent, tape strips were immersed in 5 mL of methanol for 20 min to obtain final concentrations of 10, 20 and 40 μg/mL. The mixture was vortexed for 2 min and analyzed by HPLC-UV. Three blank samples of RS were also spiked with the same amounts of GEN (50, 100, 200 μg) in methanolic solutions, followed by the evaporation of the solvent. RS was then dispersed in 5 mL of methanol and processed by a tissue homogenizer (T25 ULTRA-TURRAX®, Dispersing element S25KV-25G, IKA, Staufen, Germany) for 2 min at 6000 rpm followed by bath sonication (USC 1400, Unique, Indaiatuba, Brazil) for 20 min. The skin homogenate was then filtered and analyzed by HPLC-UV. Experiments were performed in triplicates.

Apparatus and Chromatographic Conditions Samples were analyzed with an HPLC system consisting of a quartenary pump (ProStar 240, Varian, Palo Alto, U.S.A.), autosampler (ProStar 410, Varian) and UV detector (ProStar 310, Varian). Separation was achieved in an Agilent ZORBAX SB-C18 column (250×4.6 mm, 5 μm), which was protected with an Eclipse XDB-C18 short guard column (12.5×4.6 mm). The mobile phase was 0.5% acetic acid in water (acidified water pH 3.0), acetonitrile and *n*-propanol 50 : 48 : 2 (v/v/v). Flow-rate was 1.0 mL/min, injection volume was 50 μL and UV detection was carried out at 270 nm (the wavelength of maximum UV absorbance determined by scanning the absorption spectra of GEN in a UV-Vis spectrophotometer Cary 50, Varian, Cary, U.S.A.). Data acquisition was performed using a Galaxie Chromatography Data System Software.

Method Validation The method was developed and validated for linearity, selectivity, accuracy, precision, recovery and quantitation limit in accordance to the Food and Drug Administration Guidelines[23] for validation of bioanalytical methods.

Linearity Blank samples of SC and RS were spiked with GEN to produce five concentration points between 0.1 and 60 μg/mL in triplicate. Peak areas were plotted against the concentration to obtain the analytical curve followed by linear regression analysis. The method was considered linear when error was lower than 15% and $r \geq 0.99$.

Selectivity The selectivity was investigated against skin endogenous components and adhesive tapes. Blank of skin homogenate and SC tape-stripped from six sources of skin were analyzed for the absence of interfering compounds at GEN retention time.

Accuracy, Precision and Recovery Accuracy and precision were analyzed by spiking GEN (0.1, 0.2, 0.4 mg/mL) into blank samples of SC and RS. Precision was determined by repeated analysis (5 replicates) of the samples in the same day and on three different days. Precision was expressed as the relative standard deviation (RSD%) of replicate measurements. Accuracy was calculated as the percentage difference between the measured value and the true value and expressed as the relative error of measurement (E%). Recovery was determined by computing the ratio of the amount of GEN extracted from spiked skin to the amount of GEN added. The comparison of the analytical results for extracted samples and the theoretical values (100%) resulted in the recovery data for the extraction procedure.

Limit of Quantification (LOQ) The LOQ for the proposed method was established through the analysis of blank samples (SC and RC) containing decreasing concentrations of drug to the lowest level quantified with suitable precision and accuracy (<20%).

Application of the Method to *in Vitro* Percutaneous Penetration Studies GEN penetration studies were carried out in a modified Franz-type diffusion cell (Microette; Hanson Research, Chatsworth, U.S.A.). Receptor and donor media consisted of an aqueous solution of 5% sodium lauryl sulfate. GEN solubility in the receptor medium, determined according to Higuchi and Connors,[24] was 1.48 mg/mL. To ensure sink conditions, the total amount of GEN in the donor compartment was 40 μg (200 μL of a sample containing 0.2 mg/mL of GEN). The available diffusion area between receptor and donor compartments was 1.86 cm^2. Stirring rate and temperature were kept, respectively, at 300 rpm and 37°C. Four different experiments were performed with permeation times of 6, 12, 18 and 24 h. After each experiment the skin surface was washed with deionized water in order to remove excess of GEN on the skin surface. Also, stratum corneum and remaining skin were separated as previously described and the amount of GEN in each layer was measured by HPLC-UV.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions Chromatographic conditions, particularly the composition of mobile phase, were crucial in the process of separating the interferences from skin endogenous compounds and quantitation of GEN with adequate precision.

It is interesting to note that the pH of the mobile phase clearly affected the symmetry of GEN peak.[25] A decrease in the pH value (pH 3.0) increased the peak symmetry (1.08) which indicates that the acidification of the mobile phase suppressed drug ionization and, consequently, improved peak symmetry. In addition, GEN degradation was accelerated in an alkaline environment.[26] As a result, the pH of the mobile phase was adjusted to 3.0 with acetic acid.

As an attempt to reduce the retention time for GEN, *n*-propanol was added to the mixture of acetonitrile–acidified water, due to the slightly stronger elution properties for *n*-propanol in reversed phase chromatography, consistent with its

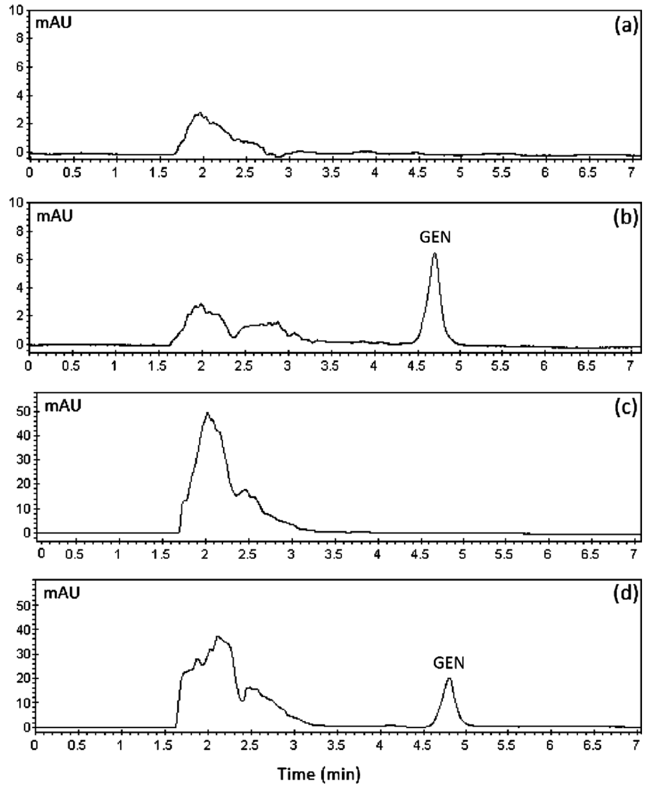

Fig. 2. Chromatograms Obtained from the Analysis of (a) Blank Stratum Corneum; (b) Stratum Corneum Spiked with 10 μg/mL GEN; (c) Blank Remaining Skin; (d) Remaining Skin Spiked with 10 μg/mL GEN

lower polarity index.

For the mobile phase composed of 0.5% acetic acid in water–ACN–*n*-propanol (50 : 48 : 2, v/v/v) and the flow rate of 1.0 mL/min, the analytical run was 6 min with a complete separation of GEN from the impurity peaks. The separation factor (R_s) of GEN and interfering peaks was greater than 1.94. The retention time for GEN was 4.65 min.

The conditions established for the HPLC method resulted in a short run time, which is suitable for routine analysis of a large number of samples during *in vitro* skin permeation studies.

Typical chromatograms of different skin layers (stratum corneum and remaining skin) either blank or containing GEN are presented in Fig. 2. The proposed method was able to quantify GEN without interference from endogenous compounds of the skin, including a large numbers of UV-absorbing nucleotides and nucleosides,[13] demonstrating the specificity of the method. Skin proteins are more polar,[27] thus eluting from 1.6 to 3.3 min (Fig. 2).

Table 2. Recovery of the Method for Determining GEN Concentrations in Both Skin Layers (n=3)

Sample	Amount of GEN (μg/mL)	Absolute recovery	
		Mean (%)	RSD (%)[a]
Stratum corneum	10	100.02	2.60
	20	98.85	4.02
	40	93.85	2.51
Remaining skin	10	96.28	2.32
	20	95.10	2.08
	40	95.35	2.66

a) Relative standard deviation.

Analytical Results. Linearity The analytical curve was linear in the concentration range of 0.1–60 μg/mL. The equation of a typical calibration curve obtained by analyzing GEN samples extracted from stratum corneum was $y=185.53x+38.06$ with a correlation coefficient (r) of 0.9997, whereas the equation of a curve obtained by assaying GEN from remaining skin was $y=166.86x+29.41$ ($r=0.9996$).

Accuracy, Precision and Recovery Results for accuracy and precision are presented in Table 1. All values of accuracy and precision were within acceptable limits, with E% and RSD no greater than 15%.[23] The mean recovery rates were 97.57% and 95.57% for the stratum corneum and remaining skin, respectively, which demonstrated an efficient extraction of GEN from skin samples, as shown in Table 2.

Limit of Quantification (LOQ) The LOQ for GEN in both SC and RS was 100 ng/mL. Previous reports in the literature present an LOQ of 5.79 μg/mL[28] for an analytical method applied to a nanoemulsion topical formulation. Thomas *et al.*[17] obtained a LOQ for GEN of 2 ng/mL and 22 ng/mL in plasma and urine samples, respectively. However, the LOQ for GEN in skin samples has not been reported. A low LOQ in skin layers is important when investigating topical drug delivery, since several drugs hardly penetrate into deeper layers of the skin. Thus, the LOQ obtained in this work points toward the sensitivity of this method for the quantitation of GEN in skin layers.

Table 1. Accuracy, Between-Day and Within-Day Variability of the HPLC Method for Determining GEN Concentrations in Both Skin Layers (n=5)

Theoretical concentration (μg/mL)	Between-day variability		Within-day variability		Accuracy
	Real concentration (μg/mL) (mean±S.D.)	RSD (%)[a]	Real concentration (μg/mL) (mean±S.D.)	RSD (%)[a]	E%[b]
Stratum corneum					
10	9.38±0.61	6.47	10.00±0.26	2.90	0.02
20	20.25±1.46	7.20	19.77±0.80	4.55	1.15
40	37.05±0.78	2.11	37.54±1.00	2.99	6.15
Remaining skin					
10	9.99±0.51	5.10	9.63±0.23	2.69	3.72
20	18.73±0.39	2.02	19.02±0.42	2.44	4.90
40	37.71±0.92	2.44	38.14±1.06	3.11	4.65

a) Relative standard deviation, *b*) Relative error.

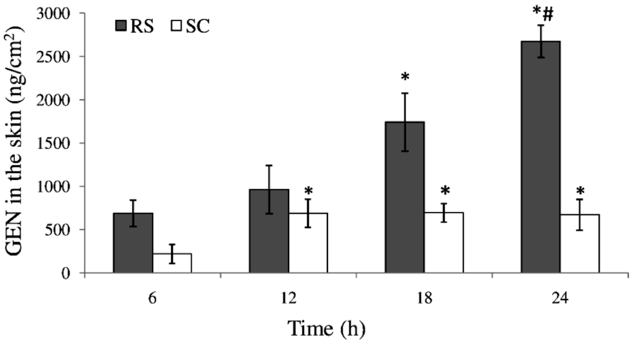

Fig. 3. GEN in Different Skin Layers (SC: Stratum Corneum and RS: Remaining Skin) after 6, 12, 18 and 24 h of Permeation Experiment

Data are the mean±S.D. of six replicates. Symbols indicate significant difference ($p<0.05$) on the amount of GEN permeated in each skin layer with time.

Application of the Analytical Method Topical chemotherapy could be an interesting alternative to treat skin cancer with reduced systemic toxicity.[29] Thus, topical delivery of drugs has attracted attention from different researchers.[5,30]

In order to evaluate drug delivery to the skin, tape stripping technique is extensively used to separate the stratum corneum and the remaining skin.[28] This method consists of subsequent removal of the stratum corneum by adhesive tapes and represents a simple and efficient method to evaluate the skin penetration performance of new formulations.[22] Due to the growing interest in topical and transdermal formulations, a bioanalytical method was applied to quantify GEN in stratum corneum and remaining skin in percutaneous penetration studies. Data in Fig. 3 represent the amount of GEN found in each skin layer (RS and SC, represented by the total of GEN in the 15 tapes stripped) in 6, 12, 18 and 24 h of the permeation experiment. For the quantification of GEN the skin layers, the drug was extracted as previously described ('GEN Extraction from Different Skin Layers').

Results shown in Fig. 3 demonstrate that the amount of GEN in the RS increases with time. Conversely, no fluctuations in the amount of GEN were detected in the SC from 12 to 24 h of the experiment ($p<0.05$). Quantitative data for GEN in skin layers with less than 3 h of contact were lower than LQ and did not meet validation criteria.

GEN quantitation in the receptor medium of the Franz diffusion cells was also performed. For the receptor compartment of the diffusion cells, a calibration curve ($y=65.138x+96.718$, $r=0.9982$) and the quantitation limit (100 ng/mL) were determined. The presence of GEN was detected in the receptor solution of Franz diffusion cells after the first 6 h of the permeation experiment, however in a concentration below the LOQ. Even after 24 h of the experiment, the amount of GEN in the receptor solution was lower than the quantitation limit.

It seems that GEN has great affinity for the skin layers, and did not permeate across the skin into the receptor solution over 24 h. Studies have reported that lipophilic drugs could form a depot in skin and can be utilized for sustained drug delivery.[31,32] Investigations with primaquine demonstrated that both binding to corneocyte keratin as well as the miscibility of the drug with the lipid domains contribute to the horny layer reservoir.[33] Furthermore, a higher amount of GEN retained in the skin could be advantageous to ensure continuous drug levels in skin tissues and/or to reduce frequent administrations.[31]

CONCLUSION

A simple HPLC bioanalytical method was developed and validated for the precise and accurate determination of genistein in different skin layers, applicable to skin permeation studies. The high sensitivity and the possibility to quantify genistein without interferences from skin components make this technique particularly attractive for this purpose. Thus, the method was successfully applied to evaluate the *in vitro* depth of penetration of GEN in porcine skin layers and its concentration in the skin. Due to the growing interest in topical and transdermal formulations, the quantitation of GEN through skin layers could be interesting in the assessment of kinetics and drug delivery of topical formulations containing this drug.

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