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Article

Validation of an LC Method to Determine Skin Retention Profile of Genistein from Nanoemulsions Incorporated in Hydrogels

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Recent studies have shown the effect of soy isoflavones in preventing skin photoaging and photocarcinogenesis, especially for genistein (GEN). Nanoemulsions have been proposed as a delivery system for GEN administration due to the low water solubility of this isoflavone. This article describes the validation of an isocratic liquid chromatography method to determine GEN in porcine ear skin layers from nanoemulsions before and after incorporation into hydrogels. The analyses are performed on a reversed-phase C18 column using a mobile phase composed of methanol–water (70:30, v/v) under acid conditions (at pH 3.0) and UV detection at 270 nm. The method is linear in the range of 0.1–10 μg/mL ($r^2 > 0.999$) in the presence of skin extracts. The low limit of quantitation is estimated as 0.1 μg/mL. No interferences from formulation excipients or skin layer compounds are detected. The RSD values for intra- and inter-day precision are lower than 15%. Recovery ranged from approximately 90% to 110%. The method is applied to estimate GEN retention in the skin from formulations using Franz diffusion cells. The highest amount of GEN is detected in the epidermis (185 μg/cm^2). In conclusion, the method proved to be specific, precise, and accurate in determining GEN amounts from formulations in skin retention studies.

Introduction

Genistein (GEN) is a phytoestrogen, which belongs to the isoflavone class of flavonoids, naturally occurring in soybean seeds. The effect of GEN on the protection of skin photoaging and carcinogenesis has been extensively investigated (1–5). The main action mechanism involves protection of oxidative and photodynamically damaged DNA, down-regulation of ultraviolet-B activated signal transduction cascades, and antioxidant activities (6).

In spite of the increasing interest on the topical use of GEN, only a few reports have described its skin permeation profile. For instance, Minghetti et al. have evaluated in vitro the effect of different permeation enhancers on GEN permeation through the human skin using modified Franz type diffusion cells (7). PEG 400 was considered as the most effective vehicle for the transdermal delivery of GEN. More recently, Huang et al. have shown the pH effect on GEN permeation and retention on nude mouse skin using Franz cells (8). GEN in neutral conditions showed higher skin accumulation compared to the ionized condition, showing the lower penetration of the ionized form, due to the stratum corneum characteristics.

GEN can be efficiently incorporated into the oil core of nanoemulsions due to the poor water solubility of this isoflavone. Such formulations composed of a medium chain triglyceride or octyldodecanol stabilized by egg-lecithin exhibit droplet size in a 200 nm-range (9). Recent studies have described the permeation profile of GEN from these formulations, assessed in porcine ear skin using Franz diffusion cells (10). Irrespective of the oil core used, the overall results showed a slow permeation profile of GEN from formulations compared to the volatile reservoir.

However, GEN skin retention has not yet been assessed from these nanoemulsions. Even though recent literature describes the use of liquid chromatography (LC) methods to assay GEN (isolated or in mixture with other isoflavones in plant extracts) in permeation studies (7, 8, 11), a literature survey reveals the absence of validated methods for estimating GEN in skin layer samples. The validation of sensitive analytical methods is a key issue in skin penetration studies, especially when UV detection is used, due to the possible interference of UV absorbing compounds in skin samples and formulation excipients (12, 13). This article describes the validation of an LC method to assay GEN in porcine ear skin layers using Franz diffusion cells from nanoemulsions before and after the incorporation into hydrogels.

Experimental

Chemical and reagents

Egg-lecithin and medium chain triglycerides (MCT) were kindly donated by Lipoid GmbH (Ludwigshafen, Germany). Carbomer 940 and thrietanolamine were obtained from Delaware (Porto Alegre, Brazil). GEN (> 98% purity) was purchased from Sigma (São Paulo, Brazil). LC grade methanol and phosphoric acid were obtained from Merck (Darmstadt, Germany).

Preparation and characterization of nanoemulsions

Nanoemulsion composed of MCT, egg-lecithin, water, and GEN (0.96 ± 0.01 mg/mL) was obtained by means of spontaneous emulsification procedure as previous described (13). Formulations were characterized in terms of mean droplet size and ζ-potential using a Zetasizer - 3000HS (Malvern Instruments, England). GEN content and association efficiency was assessed according to Silva et al. (14). The final formulation

was incorporated in a carbomer-hydrogel at a final concentration of 0.5%.

Chromatographic conditions and apparatus

The LC apparatus consisted of a Shimadzu LC-10A system (Kyoto, Japan) equipped with a model LC-20AT pump, a SPD-20AV UV–vis variable wavelength detector (set at 270 nm), a degasser DGU-20A5, a CBM-20A system controller, and SIL-20A injection valve with a 100 μL loop. GEN was analyzed using a Shim-pack CLC-ODS (M) RP-18 column (5 mm, 250 mm Ÿ 4 mm i.d.).

Several proportions of acetonitrile and/or methanol and water mixtures, in the presence or not of increasing amounts of phosphoric acid (up to pH 2.5), were evaluated as a suitable mobile phase for the assay of GEN. The selected phase consisted of a methanol–water mixture (70:30 w/w) at pH 3.0 in isocratic flow. The LC system was operated at a flow rate of 1.2 mL/min, and the sensitivity was 0.5 AUFS at room temperature. All calculations were performed with external standardization by measurement of peak areas. For evaluation of the peak purity, a Waters 2690 Separation Module with autosampler, software Empower, coupled to a Waters 996 Photodiode array-UV Scanning Detector (Waters, Milford, MA) was employed.

Linearity

A stock solution of GEN was prepared by dissolving 5 mg of GEN in 50 mL of methanol in order to obtain a final concentration of 100 μg/mL. This solution was then diluted to obtain a series of standard solutions. The stock solution was further diluted to obtain seven concentrations (0.1 to 10.0 μg/mL) with appropriate volumes of receptor fluid and stratum corneum, epidemis, and dermis homogenates.

GEN extraction

To optimize the GEN extraction, stratum corneum, epidermis, and dermis samples were spiked with a methanol solution of GEN. After 1 h, to allow GEN penetration and solvent evaporation, increasing amounts of methanol (up to 10 mL) were used to extract GEN from samples, leading to the final theoretical GEN concentration of 12.5, 8.33, 5, and 2.5 μg/mL. The samples were shaken and maintained in an ultrasound bath for 30 min. Samples were then filtered through a 0.45-μm membrane filter, and the GEN content was assayed by LC.

Method validation

The LC method to determine GEN in both the fluid receptor and skin layers, based on recommendations from ICH for bioanalytical assays (13), was then validated as follows:

The specificity of the method was tested by analyzing skin samples or tape used in tape stripping experiments in the presence of GEN methanol solutions or blank formulations. The system response was examined for the presence of interference or overlaps with GEN response.

For linearity experiments, GEN solutions (in the presence of skin layer extracts) were prepared at seven concentrations within the range of 0.1–10 μg/mL, on three different days. The linearity was determined by plotting the detector response versus the nominal GEN concentration, which allowed for the evaluation of the linearity and coefficient of determination (r^2).

The low limit of quantification (LLOQ) was determined by assaying five independent samples of standards and determining the relative standard deviation. LLOQ was the lowest concentration of GEN that can be determined with precision and accuracy.

The intra-day precision (repeatability) of the method was determined by analyzing four samples of GEN at four levels (0.1, 1, 5, and 10 μg/mL) in the presence of skin layer extracts, during the same day under the same experimental conditions. Inter-day precision values were obtained by assaying GEN samples of the same concentrations levels on three different days. The results were expressed as relative standard deviation (RSD).

The accuracy was evaluated as the standardized correlation between the measured value and the theoretical value, as follows: RE% = [(mean calculated concentration − theoretical value)/theoretical value] x 100 (15).

Recovery was determined by comparing the response of GEN extracted from the skin with the response of GEN solutions not submitted to extraction, expressed as a percentage. The samples were analyzed at 0.1, 1, 5, and 10 μg/mL.

The stability of GEN-spiked skin extracts was determined after 15 days storage at 4°C. Three replicates from SC-containing adhesive tapes and skin layers were spiked with GEN. After, GEN was extracted and assayed by LC.

GEN permeation/retention in porcine ear skin from formulations

GEN permeation was assessed using Franz type diffusion cells, which presented a surface area for diffusion of 1.77 cm^2 and a receptor volume of 10.0 mL. Excised circular pig ear skin was set between the donor and the receptor compartments, with the inner part facing the upper inside portion of the cell. Experiments were performed in sink conditions due to the presence of 30% ethanol in PBS as receptor phase. The bathing solution was kept under a controlled temperature (37 ± 1.0°C) and stirred. About 500 μg of GEN-loaded nanoemulsions were placed in the donor compartment, before and after their incorporation in hydrogels.

At the end of the experiment (8 h), samples of 1.0 mL were withdrawn and the skin was removed from the cell and cleaned using a cotton swab. The first stripped tape was discarded, while the following 15 tapes were used for GEN assay. Next, the epidermis was separated from the dermis by submerging skin slices in water heated to 60°C for 45 s, using a scalpel. The dermis was perforated into tiny pieces and the layers were weighed and placed in the test tube. Results are expressed as mean ± standard deviation of GEN retained per unit of area (μg/cm^2) of three independent experiments and were analyzed by the Student t-test with $p < 0.05$ significance.

Results and Discussion

In a preliminary set of experiments, different proportions of acetonitrile and/or methanol and water have been tested as a

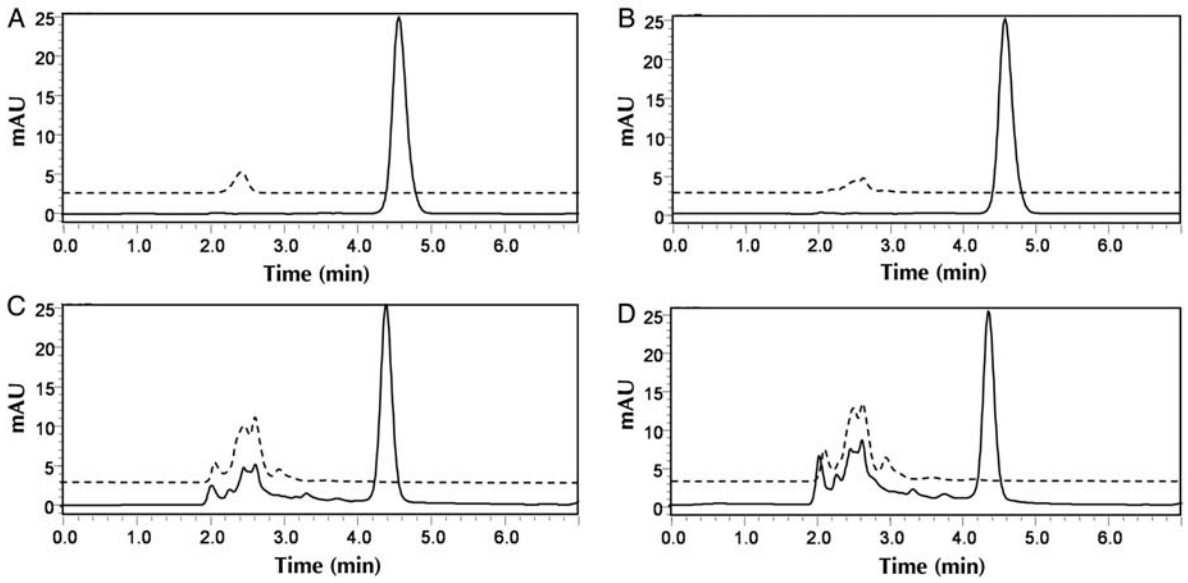

Figure 1. Representative chromatograms of specificity study. (A) Receptor fluid and GEN methanol solution, (B) Stratum corneum, (C) Epidermis, and (D) dermis-spiked with GEN-loaded nanoemulsion hydrogels. Dashed lines: non-spiked samples, solid lines: samples spiked with GEN-loaded nanoemulsion hydrogels.

Table I
Linearity Data of LC Assay for GEN*

Parameter	Equation	r^2	Intercept
Receptor Fluid	$y = 97374x - 2576.4$	0.9993	[−1978.07;7130.94]
Stripped Skin	$y = 109111.9x + 1805.3$	0.9993	[−8893,48;5282.92]
Epidermis	$y = 117426.9x + 1050.6$	0.9991	[−8305.39;6203.97]
Dermis	$y = 113388.0x + 1693.1$	0.9995	[−2962.32;5232,32]

* GEN at 0.1 to 10.0 μg/mL.

Table II
GEN Recovery Using Different Amounts of Methanol

Methanol (mL)	GEN (μg/mL)	Recovery (%)		
		Stratum corneum	Epidermis	Dermis
2	12.5	88.9 ± 3.2	55.4 ± 8.5	69.0 ± 5.6
3	8.33	102.3 ± 5.7	70.1 ± 9.6	93.8 ± 11.9
5	5.00	100.1 ± 2.6	96.7 ± 4.7	95.6 ± 3.8
10	2.50	102.1 ± 8.3	96.5 ± 5.1	95.3 ± 7.2

suitable mobile phase for GEN assay. However, in most cases, the base peak was broad with a low purity peak. Increasing amounts of phosphoric acid were added to the mobile phase in order to avoid peak tailing and to reduce retention time. These results allowed us to optimize a suitable isocratic reversed phase LC method using methanol–water 70:30 (v/v) at pH 3.0 as a mobile phase. The retention time of GEN was approximately 4.5 min as can be seen in Figure 1A. In such acid conditions, a satisfactory peak resolution and symmetry could be observed, most likely due to a favoring of a non-ionized form of GEN (16).

The specificity was assessed through the comparison of the GEN peak retention time in the presence of nanoemulsion components and skin layer extracts. Figure 1 exhibits typical chromatograms of a GEN methanol solution (Figure 1A) and GEN-loaded formulation components in the presence of porcine skin layers (Figure 1B–1D). The chromatograms showed that the method is specific because no interference of formulation and skin components (skin layers and tapes used for the tape stripping) could be observed and because no peak was detected in the GEN retention time at set wavelengths.

The linearity of the method was evaluated in the presence of both fluid receptor and skin layer extracts (Table I). The linearity experiments were carried out in different media (i.e., receptor fluid, stripped skin, epidermis, and dermis). Satisfactory linearity could be observed in the evaluated concentration ranges. The response for the drug was linear and the linearity equations showed excellent determination coefficients ($r^2 > 0.999$), which were highly significant for the method ($p < 0.05$). The confidence intervals for the intercept included zero in all cases, confirming the absence of a constant systematic error. The lowest concentration of the linearity was used as the limit of quantification because the analyte peak was reproducible with a satisfactory precision and accuracy, according to FDA recommendations for bioanalytical method validation (12). In this study, the LLOQ was set as 0.1 μg/mL, indicating a satisfactory sensitivity of the proposed method for retention studies.

Table II shows the effect of increasing amounts of methanol on GEN extraction from skin samples. Methanol was chosen because GEN is freely soluble in this solvent (7). As can be seen, a progressive GEN extraction was observed with the progressive addition of methanol until reaching a plateau. Regardless of the sample, it was necessary to add at least 5 mL methanol to extract more than 95% of GEN from the samples. Such GEN extraction conditions were used in subsequent studies because this involves only few steps and a satisfactory time period. In addition, the GEN recovery after storage remained quite similar, indicating that the freezing process affected neither GEN stability nor the efficiency of the procedure (data not shown).

Table III
Precision, Accuracy, and Recovery Data for Skin Samples

Sample	GEN (μg/mL)	Intra-day Precision* (RSD)	Intra-day Accuracy (%)†	Inter-day Precision (RSD)*	Inter-day Accuracy (%)†	Recovery‡ (%)
Receptor fluid	0.1 (LLOQ)	1.09	−8.02	1.57	−6.33	93.67 ± 1.57
	1.0	0.34	10.88	0.44	10.65	110.65 ± 0.44
	5.0	0.91	−0.79	0.48	−0.96	99.04 ± 0.48
	10.0	0.22	1.06	1.60	0.62	100.62 ± 1.60
SC	0.1 (LLOQ)	3.21	14.05	5.49	13.43	113.43 ± 5.49
	1.0	3.31	0.46	11.97	4.13	104.13 ± 11.97
	5.0	2.34	14.06	7.92	9.52	109.52 ± 7.92
	10.0	6.44	1.52	13.10	5.64	105.64 ± 13.10
Epidermis	0.1 (LLOQ)	2.07	−10.51	8.29	−9.91	90.09 ± 8.29
	1.0	0.66	−2.36	3.85	−6.28	93.72 ± 3.85
	5.0	0.21	−1.72	2.35	−4.62	95.38 ± 2.35
	10.0	0.11	−4.54	7.39	4.49	104.49 ± 7.39
Dermis	0.1 (LLOQ)	1.03	−10.09	9.42	−10.48	89.52 ± 9.42
	1.0	0.15	2.64	5.54	−1.29	98.71 ± 5.54
	5.0	0.86	−2.22	8.18	−0.76	99.24 ± 8.18
	10.0	0.19	−3.42	11.15	9.05	109.05 ± 11.15

* Precision was expressed as relative standard deviation (RSD) of replicate measurements.
† Accuracy was expressed as relative error of measurement.
‡ The mean absolute recovery for each level was calculated from three GEN spiked replicates.

Table IV
GEN Detected in the Porcine Ear Skin Layers After 8 h Permeation

	GEN (μg/cm2)		
	Stratum Corneum	Epidermis	Dermis
Nanoemulsion	2.52 ± 0.43	143.69 ± 39.11	11.26 ± 2.29
Hydrogel	0.44 ± 0.21	185.53 ± 29.76	21.09 ± 2.44

The precision of the method was assessed considering the repeatability and intermediate precision at three concentration levels (1.0, 5.0, and 10.0) as well as at the LLOQ (estimated as 0.1 μg/mL) on three different days (Table III). Whatever the GEN concentration level, the overall results showed the RSD values lower than 13% in all experiments. Under the same conditions, the method proved to be accurate since GEN recovery was comprised between approximately 90% and 115%, even at the LLOQ. The difference between nominal and found concentrations of the standards demonstrated that the assay is accurate enough for its application and that the values were within acceptable limits. Such precision and accuracy results were in agreement with recommendations for bioanalytical assays (13).

In the validated conditions, GEN permeation profile was evaluated from nanoemulsions before and after their incorporation in hydrogels (Table IV). After 8 h of kinetics, GEN released from formulations was assayed in skin samples (stratum corneum, epidermis, and dermis).

Regardless of the formulation, the GEN released reached all skin layers; however, only low amounts were detected in the stratum corneum ($<2.5\ \mu g/cm^2$). Higher amounts of GEN were detected in the other skin layers, especially in the epidermis. GEN retention in the epidermis was significantly higher (t-test, $p < 0.05$) from the hydrogel ($185\ \mu g/cm^2$) as compared to that from nanoemulsion before thickening ($143\ \mu g/cm^2$), suggesting the positive effect of the vehicle in the penetration of GEN through the skin layers.

Conclusion

This article shows a useful LC method to determine, accurately and precisely, GEN released from formulations through the porcine ear skin layers. The GEN extraction from skin layers using methanol under ultrasonication allowed the recovery of up to approximately 100% of GEN from samples. Within the validated conditions, neither the excipient formulations nor the skin layer compounds interfered with the determination of GEN. In this light, this method allowed the detection of differences among GEN skin retention profiles from different formulations.

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