

**A SIMPLE AND ROBUST HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY
COUPLED TO A DIODE-ARRAY DETECTOR METHOD FOR THE ANALYSIS OF
GENISTEIN IN MOUSE TISSUES**

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ABSTRACT

A simple liquid-liquid extraction procedure and quantification by high-performance liquid chromatography (HPLC) method coupled to a diode-array detector (DAD) of genistein (GEN) was developed in various mouse biological matrices. 7-ethoxycoumarin was used as internal standard (IS) and peaks were optimally separated using a Kinetex C18 column (2.6 μ m, 150 mm X 2.10 mm I.D.) at 40 °C with an isocratic elution of mobile phase with sodium dihydrogen phosphate 0.01M in water at pH 2.5 and methanol (55:45, v/v), at a flow rate of 0.25 mL/min. The injection volume was 10 μ L. In all cases, the range of GEN recovery was higher than 61%. The low limit of quantification (LLOQ) was 25 ng/mL. The linearity of the calibration curves was satisfactory in all cases as shown by correlation coefficients >0.996. The within-day and between-day precisions were <15% and the accuracy ranged in all cases between 90.14 and 106.05%. This method was successfully applied to quantify GEN in liver, spleen, kidney and plasma after intravenous administration of a single dose (30 mg/Kg) in female BALB/C mice.

Keywords: Genistein, HPLC, Extraction Method, Biological Matrices, Method Validation

1. INTRODUCTION

Nowadays, biomedicine is returning to nature in order to find molecules that could have interesting applications both in human and in animal health. An example of these are polyphenolic compounds, which are the most abundant natural source of antioxidants (fruits, vegetables, legumes and plant leaves). Moreover, genistein (GEN) [1] is the principal soy isoflavone found in nature, together with daidzein and glycitein. Soy is a very important constituent in Asian and vegetarian nutrition [2], and represents a huge economic impact on society. Additionally, epidemiological studies suggest that GEN helps to protect against cancer [3-7], cardiovascular diseases [8], osteoporosis [9], age-related diseases [10] and inflammation processes [11]. Probably the most attractive activity of this bioflavonoid is its promising chemopreventive activity, which works by inducing either G₂/M or G₀/G₁ cell cycle arrest depending on the cell line [12-14]. Interestingly, this molecule does not interact with DNA, with the consequent absence of severe secondary effects when compared to current chemotherapy. Despite the high potential interest of GEN in antitumor therapy, further experiments are required before proceeding to clinical use, starting with cells, moving on to rodents and finally to humans.

Although GEN is a molecule that has been fully studied in other fields, such as agronomy or plant physiology, it is still novel in the biomedical field, and there are very few publications related to its quantification in biological matrices.

Regarding the agronomic or nutritional aspects, it is important to characterize soy content, explain how to harvest it [15-17] and how to enrich nutritional products designed for vegetarians [18], as nowadays there is a tendency in human dietetics towards vegetable-friendly food. Some analytical methods have been reported so far for the quantification of isoflavonoids, mainly based on liquid chromatography coupled to a tandem mass spectrometry (MS/MS) detector [16, 17].

Concerning the quantification of GEN in biological matrices, the few reported studies deal with its extraction and quantification in human plasma/serum and urine matrices based on HPLC-MS

using either complex solid-phase [19, 20] or liquid-liquid extraction procedures [21]. Liquid-liquid extraction methods were more or less time-consuming, since they involved agitation, evaporation and reconstitution steps. Other studies have proposed the usage of enzymes, such as β -glucuronidase, for the quantification of total (free and metabolized) GEN in mouse plasma and serum [22-25]. To the best of our knowledge, no validated quantification method of GEN has been reported so far regarding mouse tissues HPLC methods coupled to a DAD detector. However, Supko validated an HPLC-UV method for GEN quantification in plasma and urine with identification of GEN peaks by HPLC-MS in mouse matrices [26]. HPLC-MS has been used to quantify total GEN in rat serum [27, 28] and in rat endocrine-responsive tissues (brain, liver, mammary, ovary, prostate, testis, thyroid and uterus) [28], with a LOQ near to 5 ng/g. Apart from the previously mentioned methods, Feng studied the pharmacokinetic profile of GEN tablets in beagle dog plasma [29].

Here we describe a simple liquid-liquid extraction procedure followed by a sensitive and accurate HPLC method coupled with a diode-array detector (DAD) to quantify GEN in different mouse biological matrices, including plasma, spleen, kidney and liver. Moreover, the pharmacokinetic and biodistribution of GEN in different organs were investigated in female BALB/C mice by using the method developed here.

2. EXPERIMENTAL

2.1. Chemicals and reagents

GEN was purchased from LC Laboratories (USA). The internal standard (IS), 7-ethoxycoumarin, was purchased from Sigma-Aldrich (Spain). All the reagents, including HPLC grade methanol, polyethylene glycol 400, dimethylsulfoxide, tert-butyl methyl ether and orthophosphoric acid were acquired from Merck (Germany). Sodium dihydrogen phosphate was acquired from Fagron (Spain). Phosphate buffer saline pH 7.4 1x (PBS) for organ homogenization was purchased from Gibco (USA).

2.2. Standard solutions, calibrator and quality control samples

Stock solutions of GEN and IS (concentration 1 mg/mL) were prepared by accurately weighing the required amounts into separate volumetric flasks and dissolving them in methanol. Standard solutions were prepared by diluting the stock solutions with methanol. For the calibrators and quality control samples (QC), serial dilutions were made with methanol to provide GEN intermediate stock solutions of 250, 500, 750, 1000, 2000, 2500, 5000, 10000, 12500, 20000 and 25000 ng/mL, and the IS was diluted up to 10000 ng/mL with methanol. Calibration samples were prepared in the different matrices using 10 μ L of 250, 500, 1000, 2000, 2500, 5000, 10000, and 25000 ng/mL GEN intermediate stock solutions and 90 μ L of each biological matrix to give a final range of 25–2500 ng/mL. QC were prepared in the same way using the intermediate stock solutions of 750 (QC2), 12500 (QC3) and 20000 (QC4) ng/mL. All the solutions were stored at -80 °C until use.

2.3. Sample pretreatment

2.3.1. Plasma samples

One hundred μ L of sample (calibrators, QC, and kinetic samples) were mixed with 90 μ L of IS solution followed by 1-min vigorous shaking. Then, 200 μ L of sodium dihydrogen phosphate 0.01 M pH 2.5 were added to the prior mixture, and mixed by a 10-sec homogenization. After this, 1 mL of tert-butyl methyl ether was added in order to extract GEN from the total sample and the mixture was vigorously vortexed during 1 min under the extraction hood. Later, the samples were centrifuged at 1 °C for 10 min at 300 g. The supernatant was carefully put inside glass tubes, which were later evaporated in a vortex evaporator (Labconco 4322000, Fisher Scientific, USA) at 40 °C for 15 min. Finally, 100 μ L of mobile phase were added to the prior evaporated sample tubes and vigorously stirred for 1 min. The final solution was put in 300 μ L HPLC glass vials for analysis.

2.3.2. Tissue samples: liver, spleen and kidney

Each organ was weighed and homogenized with 1 mL of PBS. Then, 100 μ L of the tissue sample (calibrator, QC or tissue sample) were mixed with 10 μ L of IS solution by 1-min of

vigorous shaking. The next steps were identical to those followed in plasma preparation samples (*see plasma samples section*).

2.4. Chromatographic system

GEN concentrations were determined using a HPLC system Agilent Technologies Series 1200 equipped with an Infinity Diode Array Detector and controlled by ChemStation for LC 3D systems (Agilent Technologies, USA). The analytical column was a Kinetex C₁₈ 2.6 μm, 150 mm x 2.10 mm I.D. (Phenomenex, USA) protected by guard cartridge precolumn with the same packing material.

The composition of the mobile phase was a mixture of sodium dihydrogen phosphate 0.01 M in water at pH 2.5 with methanol at a ratio of 55:45 (v/v). The flow rate was 0.25 mL/min and the injection volume was 10 μL. Methanol was used as needle wash after each injection. Column oven temperature and auto sampler temperature were set to 40±3 °C and 4±2 °C, respectively. GEN and IS were detected by UV absorbance at the wavelengths of 260 and 320 nm, respectively. The total analysis time was 13 min.

2.5. Validation of the method

The assay was validated following the Food and Drug Administration (FDA) Guidelines on Bioanalytical Method Validation [30]. Specificity, linearity, precision and accuracy, recovery and stability were the parameters evaluated.

2.5.1. Specificity and selectivity

One lot of blank plasma and blank tissues, together with plasma and tissue samples from BALB/C mice, was tested for interferences. The data of the chromatograms were processed and the integrated response should not exceed 20% of the average integrated response of the LLOQ of GEN or 5% of the integrated response of IS.

2.5.2. Linearity and limit of quantification (LLOQ)

A total of 5 calibration curves were prepared in plasma and in the different tissues and measured during 5 runs. Calibration curves were obtained by fitting the peak area ratios to a weighted (1/x) least squares regression model. The calibration curve should have a correlation coefficient (r) equal or higher than 0.996. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value, except for LLOQ, which was set at 20%. At least 67% of non-zero standard should meet the above criteria including LLOQ and upper limit of quantification.

LLOQ was investigated by analyzing 5 replicates of spiked samples at a concentration of 25 ng/mL with acceptable precision. The signal-to-noise ratio (S/N) of LLOQ was at least 10 and the values for precision and accuracy were less than 20%.

2.5.3. Precision, accuracy and recovery

The accuracy and precision of the method were determined for the QC samples during 5 consecutive runs. In the first run, all QC concentration levels were analyzed 5 times (within-run accuracy and precision). During the 5 runs, a single sample of each level was analyzed (between-run precision). Mean accuracy and within-run precision (coefficient of variation) were calculated from the results ($n=5$) of the first run. Between-run accuracy precision was calculated from the results ($n=20$) of the samples of the first run and the samples of the second and third runs.

The acceptability criteria of the data were accuracy within $\pm 15\%$ S.D. (standard deviation) and precision $\pm 15\%$ R.S.D., as previously reported for analytical methods, except for the LLOQ, which reached a maximum of 20%.

The GEN recovered from the different matrices was determined by QC2 (75 ng/mL), QC3 (1250 ng/mL) and QC4 (2000 ng/mL), as well as the IS (1000 ng/mL) ($n=4$). Absolute recoveries were calculated by comparing the signal area (GEN/IS ratio) of the spiked samples obtained against the equivalent concentrations without extraction.

2.5.4. Dilution integrity

A dilution integrity validation of plasma samples was carried out on samples with GEN above the upper limit of quantification, the dilutions being 1:10 in plasma to obtain a theoretical GEN concentration of 2000 ng/mL. Precision and accuracy were determined for the samples by analyzing them against calibration curve standards. Dilutional integrity was considered acceptable if the precision and accuracy values of the replicate of the diluted samples ($n=4$) varied less than 15%.

2.5.5. Stability

The stability of GEN in QC2 and QC4 samples in all matrices was determined in the auto-sampler (4 °C) after 24 and 48 h and compared with the initial concentration. The analyte was considered stable in the extracts of each biological matrix if 85–115% of the reference concentration was obtained. No further stability experiments were performed because the conditions had already been tested in human serum [21] and mouse plasma [26, 31].

2.6. Applicability of the method

To demonstrate its applicability, the method was successfully applied to the pharmacokinetic evaluation of GEN in plasma, as well as its determination in kidney, spleen and liver. All experimental procedures were reviewed and approved by the Animal Experimentation Ethics Committee of the University of Navarra (Spain). The study was performed using female BALB/mice (6 to 8 weeks old; 20 ± 1 g body weight) purchased at Harlan (Harlan Ibérica, Spain). Mice were housed in cages and maintained at 22–25 °C and 20% relative humidity with a 12 h light/dark cycle. Remarkably, the mice feed had traces of GEN. Therefore, 12 h prior to the experiment start, mice were deprived from food ingestion and had drinking water *ad libitum*. A single dose of 100 μ L of a homogeneous GEN solution (30 mg/Kg) containing 10% dimethylsulfoxide, 25% polyethylene glycol 400 and 65% of water for injection was intravenously (i.v) administered to 6 animals. Blood samples were collected at 5, 15, 30, 60, 120 and 240 min in EDTA-coated tubes to avoid blood coagulation. Just after blood extraction, the samples were centrifuged at 5000 g for 10 min at 4 °C and plasma was kept at –80 °C until

sample analysis. Four hours after the start of the experiment, mice were sacrificed and their kidneys, spleens and livers were removed, homogenized with 1 mL of PBS pH 7.4 and kept at – 80 °C until sample analysis.

3. RESULTS AND DISCUSSION

3.1. Development of the analytical method

A simple and accurate HPLC method has been developed for the quantification of GEN in different biological matrices. Here, different parameters that influence effective separation of the molecule of study from the biological matrices will be further discussed, such as the structure of GEN and IS, the composition and pH of the mobile phase, the temperature and the analytical column.

When regarding the separation and quantification techniques, despite the HPLC-MS procedure previously reported for the analysis of GEN in biological samples, including plasma, no HPLC methods coupled to DAD detection have been reported so far for GEN quantification in mouse plasma and tissues.

GEN and IS (Figure 1) are two molecules containing double bonds in their structure, being possible their detection in HPLC-UV. Both molecules exhibit a similar chemical structure containing a benzopyranone fraction, as well as, comparable shape and rigidity. Their aromatic cycles bestow a lack of spin due to their rigid conformation.

A very important parameter in the method development was the pH of the mobile phase, as GEN possessed three hydroxyl groups that could be deprotonated depending on the pH value (GEN $pK_{a1}=7.2$; $pK_{a2}=10.0$; $pK_{a3}=13.1$) [32]. In order to avoid the presence of partially deprotonated forms of GEN, pH of the mobile phase was fixed to $pH < 4$.

Several compositions of the mobile phase were tested, including some mixtures of methanol/sodium phosphate dibasic solution, methanol/formic acid and acetonitrile/formic acid, using both isocratic and gradient elutions. An efficient separation of IS and GEN for all the matrices studied was achieved, using isocratic elution of methanol/sodium phosphate dibasic solution. In order to determine the optimal separation, different proportions of sodium

phosphate dibasic solution/methanol were used. The selectivity of the method was strongly influenced by the sodium phosphate dibasic solution/methanol ratio, as higher amounts of methanol (58% v/v) were associated with GEN as the first peak, whereas lower amounts of methanol (45% v/v) were associated with IS as the first peak.

Alongside the mobile phase, temperature was a crucial parameter for obtaining an optimal separation. Different temperatures (40, 50 and 60 °C) were studied together with various mobile phase proportions. For instance, the method selectivity of GEN and IS with the selected mobile phase methanol/sodium phosphate dibasic solution 45:55 (v/v) changed dramatically with temperature. Although higher temperatures lead to shorter analysis time, the selected method was set at 40 °C. This lower temperature allows a more efficient separation of the many endogenous compounds contained in biological matrices, avoiding interference with the GEN and IS compounds.

To find out the best separation method, several columns were tested, including RP-C₁₈ 3 µm Gemini NX reverse phase 150 x 2.0 mm (Phenomenex) and RP-C₁₈ 5 µm Durashell 150 mm x 4.6 mm (Agela Technologies Inc). Recently, an efficient separation of several isoflavones has been reported by using the Gemini column using a flow rate of 0.15 mL/min in 80-min analysis [33]. The Gemini column led to suitable separation of GEN in 15-min chromatograms when looking at the plasma gradient method (GEN retention time ~ 3.3 min). A good peak resolution in convenient times (retention time ~ 6 min) was also possible using the Durashell column, which has a larger diameter (4.6 vs. 2.0 µm), larger particle size (5 vs. 3 µm) and works at 6-fold higher flow rates than the Gemini column. Although both columns were well-adapted to separate GEN mixtures in biological matrices, the use of a core-shell technology package enabled us to obtain robust symmetrical peaks, as well as being a more sensitive method, approximately 10 times better than the Durashell column and 5 times better than the Gemini column, reaching a LLOQ of 25 ng/mL.

3.2. Chromatographic conditions

Good separations were obtained under the chromatographic conditions indicated in the Experimental Section. Figure 2 shows the chromatograms of blank samples, spiked plasma and tissue samples with IS and GEN at the LLOQ analyzed by the HPLC-DAD technique. The retention times for GEN and IS were: 9.65 ± 0.01 and 8.72 ± 0.01 min for plasma, 9.19 ± 0.01 and 8.35 ± 0.01 min for liver, 10.43 ± 0.13 and 9.19 ± 0.03 min for spleen and 10.25 ± 0.13 and 8.98 ± 0.07 min for kidney.

3.3. Sample preparation

A crucial step in determining GEN from biological matrices concerns the previous sample treatment since a good extraction procedure would lead to high recovery yields together with easier separation and therefore, quantification. Also, an optimal sample preparation lengthens the life of an analytical column and leads to lower drug interferences with other compounds of the biological matrix.

The recovery yield of the sample extraction procedure is good. It might be thought that a method with recoveries ranging from 61.25-88.15% could be improved. However, when compared to other liquid-liquid extraction methods, our recovery range is similar. Concretely, previous studies in human plasma showed mean recovery values of 57.58% [21] and between 40-60% [34], values slightly lower to the here described method, in which the lowest recovery values were of 61%. However, Supko achieved mean recovery values of almost 100% in mouse plasma after an exhaustive extraction method [26] when compared to ours. Solid-phase extraction methods without containing enzymes for the quantification of GEN and other isoflavone metabolites have shown recoveries around 78.8% in human plasma [31].

3.4. Validation

3.4.1. Specificity and selectivity

Biological matrices were tested with the aim of observing any possible interference with GEN and the compounds already present in the sample. The chromatographic conditions developed for each matrix contributed to a significant resolution of GEN and IS peaks, together with a high

specificity and selectivity. Moreover, the data showed that the peaks were reproducible due to a robust and reliable separation method. As shown in Figure 2, the chromatograms did not exhibit any peak interfering with GEN or IS in any matrix.

3.4.2. Linearity and LLOQ

The linearity of the method was checked for all mouse matrices. All the biological matrices studied in this article were linear from 25 to 2500 ng/mL of GEN, the r values ranging from 0.996-0.998. The regression curve equations for the different mouse matrices ($n=5$) are shown in Table 1. Also, the back-calculated calibrator concentrations from these regression curve equations did not exceed 15% of the theoretical value. For all the biological matrices tested here, the LLOQ was 25 ng/mL, paving the way for the development of future GEN pharmacokinetic and biodistribution tests at therapeutic doses of GEN. In this case, the previously validated method by Supko in mouse plasma is comparable to the here-developed method [26]. However, no data regarding HPLC-UV validation methods in mouse tissues have been found, being the present study only comparable to already published HPLC GEN quantification methods coupled to a MS detector [23, 31]. In these cases, LLOQ values of 0.1 ng/mL [19] and 1 ng/mL [21] in human serum, 8.5 ng/mL in human plasma [20] were reached. The method here developed coupled to a DAD reached an acceptable LLOQ (25 ng/mL), even when it was compared with methods previously developed using HPLC coupled to a MS/MS detector and UV.

3.4.3. Precision, accuracy and recovery

The between- and within-day precision and accuracy values for the 4 biological matrices are displayed in Table 2. The precision values ranged from 2.36 to 10.24% in the case of plasma, 6.27 to 10.37% in the case of liver, 5.87 to 11.20% in the case of spleen and 5.35 to 9.87% in the case of kidney. Alongside these, the accuracy values ranged from -6.92 to 5.17% in the case of plasma, 0.81 to 6.05% in the case of liver, 9.86 to 4.62% in the case of spleen and -8.30 to 3.76% in the case of kidney. All the data were within the FDA acceptance criteria (<15%).

The range of recoveries of GEN found in plasma, liver, spleen and kidney ranged between 61.25-88.15% for GEN, obtaining the highest values in plasma and the lowest in spleen. In the case of the IS, the recoveries ranged from 63.66-78.26% (Table 3).

3.4.4. Dilution integrity

Dilution integrity was evaluated at one dilution factor (1:10) for plasma samples ($n=4$). The values for precision and accuracy were respectively 6.78 and -4.99%, being within the acceptance limit of 15%.

3.4.5. Stability

The stability of GEN was analyzed after the extraction process. QC of 75 and 2000 ng/mL were studied at 24 and 48 h after storage in the autosampler (4 °C). As Table 4 shows, all the precision and accuracy values were within the acceptance limit of 15%. Previous studies confirmed GEN stability of human serum samples in methanol/water (80:20; v/v) for 72 h at 4 °C and for two months at -20 °C [21]. Also, the stability of GEN was confirmed in mouse plasma samples in methanol/0.05 M ammonium acetate buffer pH 4.7 (30:70; v/v) for 6.2 h at 37 °C and for one month at -16 and -68 °C [26] and in acetonitrile/water (15:85; v/v) for 4 h at 25 °C, 8 h at 20 °C, one week at -20 °C and after 3 freeze/thaw cycles (-20 to 25 °C) [31].

3.4.6. Application of the method: pharmacokinetic and biodistribution studies

The applicability of the method was initially demonstrated *in vivo* by determining GEN concentrations in plasma, liver, spleen and kidney after the i.v. administration of 30 mg/Kg to female BALB/C mice ($n=6$). Figure 3 depicts the levels of GEN in plasma. A typical i.v. drug administration profile was observed, showing a biphasic profile. The C_{max} (8500 ng/mL) was immediately achieved after i.v. administration ($t=5$ min), followed by a dramatic decrease in the levels until $t=45$ min, obtaining a concentration of approximately 750 ng/mL. After that time, GEN levels underwent a slower decrease until $t=240$ min, where the concentration observed was approximately 250 ng/mL. These results are in agreement with those obtained by Yang *et*

al., in which immediately after i.v. administration of a dose of 20 mg/Kg, GEN showed a C_{max} of 15600±5900 ng/mL, followed by a rapid and pronounced decrease of GEN levels until a plateau of 27 ng/mL that was reached after 6 h of the i.v. administration. The same profile was also observed by Andrade *et al.*, the GEN levels being lower due to a smaller dose (1.2 mg/Kg) [35]. Moreover, Penza *et al.*, studied the profile of GEN after the oral administration of a dose of 50 mg/Kg to mice while maintaining their normal food. In that study, it was observed that, after that dose was administered for three continuous days, the levels of GEN increased from 25 to 459 ng/mL [22].

Regarding the biodistribution of GEN, as seen in Figure 4, all the levels of the soy isoflavone were detectable in liver and kidney. However, in the case of spleen, in only one case were the levels quantifiable, being very close to the LLOQ of the method. Nonetheless, the mean levels seen in liver (2000 ng/g) were twice those of the mean levels in kidney (1000 ng/g), probably due to the hepatic metabolism of GEN.

4. CONCLUSION

A simple, accurate and robust HPLC method coupled to a DAD for the analysis of GEN was developed and validated. The method showed good extraction yield recoveries exceeding 61% in all matrices, being specific, accurate, precise and reproducible for the analysis of this soy isoflavone in mouse plasma, kidney, spleen and liver. Remarkably, it was possible to reach a LLOQ of 25 ng/mL using a HPLC coupled to a DAD system. Moreover, this method is valuable for the pharmacokinetic behavior of this bioflavonoid and its distribution in BALB/C mice.

ACKNOWLEDGEMENTS

The authors thank Hugo Lana for his technical support with the *in vivo* experiments. C. Tamames-Tabar would like to acknowledge her fellowship grant from the Asociación de Amigos de la Universidad de Navarra (ADA). This work was supported Fundación Universidad de Navarra (FUN), by CNRS and UVSQ funding.

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Figure Legends

Figure 1. Chemical structure for genistein and 7-ethoxycoumarin.

Figure 2. Chromatograms of genistein (GEN) at $\lambda = 260$ nm and 7-ethoxycoumarin (IS) at $\lambda = 320$ nm, for all the mouse biological matrices, being blank plasma (A1), blank liver (B1), blank spleen (C1) and blank kidney (D1) when compared to the LLOQ (25 ng/mL) in plasma (A2), liver (B2), spleen (C2) and kidney (D2).

Figure 3. Pharmacokinetic profile of genistein (D=30 mg/Kg) in BALB/C female mice after intravenous administration.

Figure 4. Biodistribution of genistein (D=30 mg/Kg) in kidney, spleen and liver in BALB/C female mice 240 min after intravenous administration.