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High-performance liquid chromatographic assay for genistein in biological fluids

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Abstract

A specific, sensitive and technically convenient HPLC method for assaying genistein in biological fluids has been developed. The compound and 4-hydroxybenzophenone, added as an internal standard, were efficiently isolated from both plasma and urine by extraction with *tert.*-butyl methyl ether. Following evaporation of the organic solvent, the extract was reconstituted with methanol–0.05 *M* ammonium acetate buffer, pH 4.7 (30:70, v/v), and loaded onto a 4 μm Nova-Pak C_8 column (15 cm × 3.9 mm I.D.). Chromatography was performed at ambient temperature using a mobile phase of acetonitrile–0.05 *M* ammonium formate buffer, pH 4.0 (27:73, v/v), at a flow-rate of 1.0 ml/min, with UV detection at 260 nm. Mean values of the t_R for the drug and internal standard, determined from chromatograms of the 1 μg/ml plasma standard during a 6 month period, were 8.27 ± 0.55 and 11.92 ± 0.71 min, respectively (S.D., $n = 29$). With a sample volume of 50 μl, the lowest concentration of genistein included in the plasma standard curve, 0.020 μg/ml, was quantified with a 10.7% R.S.D. over a 5 month period. Plasma standards having concentrations of 0.050 to 1.02 μg/ml exhibited R.S.D. values ranging from 2.3 to 6.1%. The drug was quantified in urine with similar reproducibility. The sensitivity of the assay was adequate for characterizing the plasma pharmacokinetics of genistein in the mouse and dog. However, a 10-fold improvement in sensitivity was afforded by increasing the sample size to 250 μl, without otherwise modifying the method. Thus, this procedure may prove suitable for determining plasma and urine levels of genistein in humans consuming dietary isoflavonoids in a much more convenient manner than permitted by existing methodology.

1. Introduction

The phytoestrogen genistein, 4',5,7-trihydroxyisoflavone (Fig. 1), has recently generated considerable interest as a potential agent for the prevention and treatment of cancer. Based upon information drawn from epidemiological studies [1] and collateral investigations [2–6], it has been proposed that long term systemic exposure to

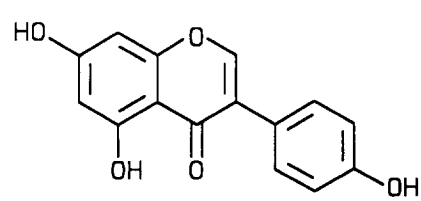

Fig. 1. Chemical structure of genistein.

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isoflavonoids such as genistein, from the dietary consumption of soy products [4,7,8], may prevent the development of breast cancer and delay progression of latent prostatic carcinoma [2,4,6,9–11]. Genistein is also known to suppress the growth of cultured human cell lines derived from a broad spectrum of solid tumors [9,10,12–19] and various forms of leukemia [17,20–25]. While a specific mechanism of action has not been identified, genistein modulates multiple processes involved in neoplastic transformation and the proliferation of cancer cells. These processes include the inhibition of tyrosine-specific protein kinases [21,26], DNA topoisomerase II [21,27,28], epidermal growth factor-induced phosphatidylinositol turnover [29] and S6 kinase activation [30], and angiogenesis [11].

With the development of specific methods for quantitative analysis [3,5,31,32], it was established that genistein and related compounds originating from dietary isoflavonoids occur predominantly as glucuronide and sulfate conjugates in the biological fluids of grazing farm animals [31–34] and human subjects [3–6]. Surprisingly, a search of the literature identified only two reports concerning the systemic levels of genistein provided from known doses of the pure compound. These studies involved the determination of free (i.e. unconjugated) [31,32] and conjugated [32] genistein in plasma specimens acquired from sheep at only a few time points following intramuscular [31] or intraruminal [31,32] administration. With a paucity of relevant pharmacokinetic information, characterizing the disposition and bioavailability of genistein in several species of animals will be necessary for its preclinical evaluation.

Detailed consideration of the methods previously used to assay genistein in biological fluids revealed their unsuitability for pharmacokinetic drug level monitoring in small animals, such as mice, rats and dogs. These procedures were developed for concurrently determining numerous dietary phytoestrogens and additional phenolic compounds in a single sample [3,5,31,32]. In each case, the free compounds were isolated before or after conjugate hydrolysis, and were then quantified as their trimethylsilyl ether derivatives by GC with flame ionization [31,32] or MS [3,5] detection. Both methods were very sensitive, allowing genistein to be measured at concentrations near 1–2 ng/ml. However, extremely large sample volumes were required to achieve this degree of sensitivity, ranging from 100 ml for the original GC assay [31,32] to 4 ml for the recently developed GC–MS method [3,5]. Even the smaller volume is approximately 10-times larger than that which would accommodate the analysis of plasma from an individual mouse or serial specimens from rats or dogs. Aside from the inherent complexities and labor-intensive techniques associated with multicomponent analyses, the procedures required to effectively remove endogenous components that severely interfere with the application of either analytical method renders them impractical for the routine analysis of a large number of samples. Furthermore, the GC–MS method suffers from several technical problems with regard to the quantitation of genistein, the most notable being incomplete derivatization of the compound and instability of the deuterated form of genistein employed as an internal standard [3,5].

The work presently described was undertaken to develop a technically convenient assay specifically for free genistein in plasma and urine employing a sample volume which would facilitate replicate analysis of specimens acquired from preclinical animal models. Reported herein is a procedure involving isocratic reversed-phase HPLC with UV detection following isolation of genistein from the biological matrix by extraction with an immiscible organic solvent. The assay has been thoroughly validated and proven highly reliable through extensive application during preclinical pharmacokinetic studies.

2. Experimental

2.1. Reagents and chemicals

Analytical reference samples of genistein (NSC 36586), equol (4′,7-dihydroxyisoflavan)

and formononetin (NSC 93360; 7-hydroxy-4'-methoxyisoflavone) were provided by the Pharmaceutical Resources Branch or Drug Chemistry and Synthesis Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Daidzein (4',7-dihydroxyisoflavone) was purchased from ICN Biomedicals (Costa Mesa, CA, USA); biochanin A (5,7-dihydroxy-4'-methoxyisoflavone) and 4-ethylphenol were obtained from Aldrich (Milwaukee, WI, USA). The following chemicals were used as supplied: ACS reagent grade ammonium acetate and ammonium formate (Eastman Kodak, Rochester, NY, USA); glacial acetic acid, formic acid and dimethyl sulfoxide (Sigma, St. Louis, MO, USA); 4-hydroxybenzophenone (Aldrich); "Baker Analyzed" HPLC reagent acetonitrile, methanol and *tert.*-butyl methyl ether (J.T. Baker, Phillipsburg, NJ, USA). Distilled water was deionized and stripped of dissolved organics by passage through mixed-bed resins and activated carbon (Hydro Water Systems, Rockville, MD, USA).

2.2. Analytical solutions

Stock solutions were made in class A borosilicate glass volumetric flasks with PTFE-lined septum screw tops (Kontes, Vineland, NJ, USA). Milligram quantities of genistein and the internal standard (I.S.), 4-hydroxybenzophenone, were accurately weighed on a Cahn C-31 microbalance (Cahn Instruments, Cerritos, CA, USA) and dissolved in dimethylsulfoxide (DMSO) to provide concentrations of 0.2 and 0.24 mg/ml, respectively. The solutions were routinely stored at 5°C.

Standard solutions were made by serially diluting the genistein stock solution with drug-free plasma or urine to provide concentrations of 1000, 800, 600, 400, 200, 150, 100, 50, 20 and 10 ng/ml. These solutions were prepared weekly and kept at −20°C when not in use. The solution used in the assay for sample extraction was made by diluting the I.S. stock solution with *tert.*-butyl methyl ether (TBME) in a volumetric flask to afford a concentration of 25 ng/ml.

2.3. Sample preparation

Samples were prepared for analysis in 10 ml (16 × 100 mm) borosilicate glass culture tubes (Sun Brokers, Wilmington, NC, USA) and centrifuge tubes (Fisher Scientific, Pittsburgh, PA, USA) with PTFE-lined phenolic screw caps. The tubes were washed with Alconox detergent (Alconox, New York, NY, USA), thoroughly rinsed with distilled water and oven dried. Surface deactivation of the glassware was found to be unnecessary.

Plasma or urine specimens (50 μl) and 3 ml of TBME containing the I.S. were pipetted into 10-ml centrifuge tubes and tightly capped. After vigorous mixing on a vortex-stirrer for 1 min, the samples were extracted for an additional 10 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, USA), then centrifuged for 3 min at 2500 *g*. The upper organic phase was carefully transferred to a round-bottomed culture tube and evaporated under a stream of nitrogen in a Meyer N-EVAP at 45–50°C (Organomation Assoc., Berlin, MA, USA). The residual material was reconstituted in 250 μl of methanol–0.05 *M* ammonium acetate buffer, pH 4.7 (30:70, v/v) with vortex-mixing for 10 s. The solution was then transferred into a borosilicate glass insert within a 12 × 32 mm autosampler vial and sealed with a PTFE-lined silicone septum crimp-top (Sun Brokers). A 100-μl aliquot of this solution was injected onto the chromatographic system.

2.4. Chromatographic conditions

Liquid chromatography was performed using an HP 1050 Series isocratic pump and autosampler fitted with a 100-μl sample loop (Hewlett-Packard, Palo Alto, CA, USA). Separations were conducted at ambient temperature under isocratic reversed-phase conditions on a 15 cm × 3.9 mm I.D. stainless steel column packed with 4 μm Nova-Pak C_8 (Millipore, Milford, MA, USA). The analytical column was protected by a 0.5-μm postinjector filter (Rainin Instrument, Woburn, MA, USA). A mobile phase composed of acetonitrile–0.05 *M* ammonium formate buf-

fer, pH 4.0 (27:73, v/v) was employed at a flow-rate of 1.0 ml/min. The solution was degassed in an ultrasonic bath for 15 min before use. UV absorbance of the column effluent was monitored at 260 nm (6.5 nm bandwidth) using an HP Model 79853C variable-wavelength detector fitted with a 14-μl flow cell (8 mm pathlength). The 1-V output of the detector was provided as the signal to an HP 3396 Series II integrator, configured to report peak area using a 0.2-min peak width at a threshold setting of 1 with baseline construction through each detected valley point.

The effluent from a similarly configured analytical system was introduced directly into a Hewlett-Packard 5989A mass spectrometer equipped with a thermospray LC–MS interface. As the interface functions most efficiently with an eluent flow-rate near 0.75 ml/min, the strength of the mobile phase was adjusted by increasing the acetonitrile content to 30% (v/v), which eluted genistein with a t_R time comparable to the 27% acetonitrile mobile phase at 1.0 ml/min. The thermospray ion source was operated in the buffer ionization-only mode with either positive- or negative-ion detection. The operating temperatures for the interface were as follows: ion source, 225°C; probe stem, 110°C; and probe tip, 210–220°C. Nominal resolution mass spectra (150–500 u) were acquired at the rate of 0.42 scan/s with the electron multiplier set at 2500 V. Selected-ion monitoring (SIM) was performed on either m/z 271 (positive-ion mode) or m/z 269 (negative-ion mode) using a mass width of 0.7 u and a dwell time of 2000 ms with the electron multiplier set at 2900 V.

2.5. Quantitation

Standard curves were constructed by plotting the peak-area ratio of genistein to the I.S. against the concentration of genistein. Linear least squares regression was performed using a weighting factor of $1/y_{obs}$, without inclusion of the origin, to determine the slope, y-intercept and correlation coefficient of the best fit line. Concentrations of the drug in unknown samples were calculated using results of the regression analysis. Biological samples acquired from animals treated with genistein were initially assayed in duplicate. Additional analyses were performed if the replicate determinations deviated from the average by more than 10%. Specimens with an analyte concentration exceeding the standard curve were reassayed upon appropriate dilution with drug-free plasma or urine.

2.6. Relative recovery and reproducibility

Precision and accuracy of the assay were evaluated by analyzing the backcalculated sample concentrations and regression parameters from standard curves of genistein in plasma or urine prepared and analyzed on separate days. The R.S.D. of the mean-predicted concentration for the independently assayed standards provided a measure of precision. The lower limit of quantitation was defined as the minimum concentration of genistein amenable to analysis with a R.S.D. not exceeding 10%. Relative recoveries of genistein were calculated by comparing the mean predicted concentration to the known concentration in the standard solutions.

2.7. Absolute recovery

Reference solutions with concentrations similar to standard solutions that had been processed for analysis were made by diluting appropriate volumes of the respective stock solutions with methanol–0.05 M ammonium acetate buffer, pH 4.7 (30:70, v/v). Five replicate plasma or urine standards, ranging in concentration from 0.02 to 1.0 μg/ml, were prepared for analysis and sequentially chromatographed with the corresponding reference solution. Absolute recovery was calculated by comparing the chromatographic peak area of the drug or I.S. observed in the standard solution to the paired reference solution.

2.8. Stability studies

At initial concentrations of 0.1, 0.5 and 1.0 μg/ml, the stability of genistein in plasma and

urine was evaluated at −70, −20 and 37°C. For the experiments at −70 and −20°C, 20 μl of a DMSO stock solution of genistein was added to 4.0 ml of ice-cold plasma or urine. After mixing on a vortex stirrer, 50-μl aliquots of the solution were transferred into forty 10-ml glass centrifuge tubes and flash frozen in a dry ice–isopropanol bath. The tubes were divided between standard laboratory freezers maintained at approximately −20 or −70°C. Two aliquots of each solution were removed from the freezers every 3 to 4 days during five successive weeks for analysis. Freezer temperature was continuously monitored with a Model 51K/J digital thermocouple thermometer (Fluka Manufacturing, Everett, WA, USA) and recorded on the assay days.

Stability studies at 37°C were conducted in tightly capped polypropylene microcentrifuge tubes (1.9 ml) placed in an Eppendorf Model 5436 Thermomixer (Madison, WI, USA) shaking at 1000 rpm. Plasma or urine (1.5 ml) was pipetted into the tubes and allowed to equilibrate to 37°C, as indicated by a thermocouple probe within an adjacent tube. Kinetic runs were initiated by adding 7.5 μl of genistein stock solution to the reaction medium. Subsequently, at 9 time intervals ranging from 1 min to 6 h, 50 μl of the reaction mixture was removed, immediately quenched by flash freezing in a 10-ml centrifuge tube, and stored at −20°C until assayed.

2.9. Dosing and sample collection

Male $CD2F_1$ mice (National Cancer Institute, Frederick, MD, USA) were treated with 25 mg/kg of genistein by bolus i.v. injection without anesthesia. The injectable was formulated by dissolving the drug in neat DMSO to provide a concentration of 25 mg/ml. Before treatment and at postinjection times of 3, 60 and 150 min, the mice were anesthetized with methoxyfluorane and bled by retro-orbital puncture. The plasma afforded by immediate centrifugation (12 000 *g*, 25°C, 5 min) of the whole blood was flash frozen and stored at −20°C until assayed.

3. Results and discussion

3.1. Preparation of biological fluids for analysis

The solubility of genistein in water-immiscible organic solvents prompted the assessment of liquid–liquid extraction for isolating the compound from plasma proteins and other endogenous components prior to chromatographic analysis. While the drug was efficiently recovered from plasma and urine upon direct extraction with either ethyl acetate or TBME, the latter solvent afforded markedly cleaner chromatograms. Following evaporation of the organic solvent, the extract was dissolved in a vehicle with a sufficiently weaker solvent strength than the chromatographic mobile phase as to allow injection volumes of at least 100 μl without adversely affecting the separation. The simplicity of the procedure allowed a series of 15 to 20 unknown samples to be prepared in duplicate together with a 10-point standard curve in about 4 h. The prepared samples exhibited adequate stability at ambient temperature for overnight analysis with an autosampler.

3.2. High-performance liquid chromatography

Reversed-phase HPLC has been utilized extensively for the qualitative and quantitative analysis of genistein and other naturally occurring isoflavones in crude plant material and food products [8,35–38]. Nevertheless, the technique has not been applied to the quantitative determination of genistein in mammalian biological fluids. With regard to the present application, an isocratic separation was desired to maximize sensitivity, minimize analysis time and simplify instrumental requirements [39]. In addition, isocratic conditions would provide direct compatibility with each of the detection methods that have been used to monitor genistein during reversed-phase HPLC, namely, UV absorption, electrochemical oxidation [35,38,40] and thermospray ionization MS (TSP-MS) [35,37,38,41–43].

HPLC with UV detection performed using isocratic conditions similar to those previously described [35–38,40,42,43] proved unsatisfactory

for the analysis of genistein isolated from biological fluids by extraction with TBME. Thus, a variety of bonded and polymeric stationary phases were evaluated with eluents composed of methanol or acetonitrile and ammonium acetate, ammonium formate or potassium phosphate buffers ranging from pH 4.0 to 7.2. The best separation was achieved using a Nova-Pak C_8 column (4 μm, 15 cm × 3.9 mm I.D.) with a mobile phase of acetonitrile–0.05 *M* ammonium formate buffer, pH 4.0 (27:73, v/v) at a flow-rate of 1.0 ml/min. UV absorption of the column effluent was monitored at 260 nm, which is the wavelength of maximum absorbance of genistein in the mobile phase. Under these conditions, genistein was completely resolved ($R_s \geq 1.5$) from 4-ethylphenol, its only known phase I metabolite [44–46], and all related compounds of dietary origin that have been identified in mammalian biological fluids [5,43,45] (see Table 1).

Liquid chromatograms of drug-free human plasma and urine prepared for analysis without addition of the I.S. are shown in Fig. 2A,B, respectively. Comparison with chromatograms of plasma to which genistein was added at concentrations of 0.05 μg/ml (Fig. 2C) and 1.02 μg/ml (Fig. 2D) clearly demonstrates the absence of endogenous components which may interfere with UV detection of the drug or I.S. Similarly, no interfering peaks in chromatograms of dog plasma or urine (data not shown) and mouse plasma (Fig. 3A) were observed. Genistein eluted prior to the I.S. and there were no strongly retained endogenous peaks. Thus, the run time for a single sample was 15 min. Retention times (mean ± S.D., $n = 29$) determined from the chromatogram of the 1.02 μg/ml plasma standard, run on a single column during a 6 month period, were 8.27 ± 0.55 min for genistein and 11.92 ± 0.71 min for the I.S. Several thousand biological samples have been analyzed

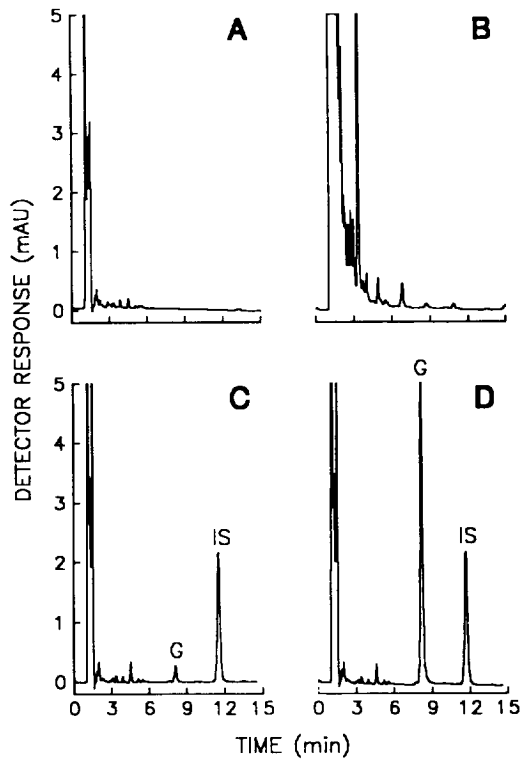

Fig. 2. Representative chromatograms of drug-free human plasma (A) and urine (B), assayed without addition of the internal standard, and standard curve samples prepared by adding genistein to plasma to afford concentrations of 0.05 μg/ml (C) and 1.0 μg/ml (D). Chromatographic conditions as in Table 1. Peaks: G = genistein; I.S. = internal standard (4-hydroxybenzophenone).

Table 1
Parameters characterizing the HPLC separation of genistein and related compounds[a]

Compound	k'	t_R (min)	$W^b_{1/2}$ (min)	R_s
Daidzein	1.96[c]	3.67	0.099	16.6[d]
Genistein	5.19	7.69	0.186	–
Equol	5.90	8.45	0.180	2.5
4-Ethylphenol	6.83	9.73	0.207	6.1
Formononetin	9.50	13.04	0.293	13.2
Biochanin A	28.01	36.02	0.835	23.5

[a] Individual reference samples of each compound (100 ng), dissolved in acetonitrile (0.1 mg/ml), were separated isocratically under the following conditions: column, Nova-Pak C_8 (4 μm, 15 cm × 3.9 mm I.D.); eluent, acetonitrile–0.05 *M* ammonium formate buffer, pH 4.0 (27:73, v/v); flow-rate, 1.0 ml/min; temperature, ambient; detection, UV at 260 nm.

[b] $W_{1/2}$, bandwidth at half-height.

[c] The centroid of the solvent front was used as t_0 for calculating k' values.

[d] Resolution of the chromatographic band relative to genistein.

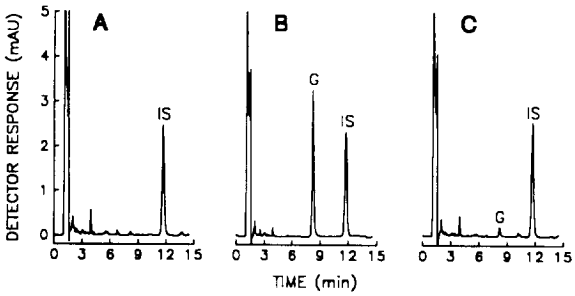

Fig. 3. Chromatograms determined with UV detection at 260 nm of plasma specimens obtained from an untreated mouse (A) and at 3 min (B) and 2.5 h (C) after 25 mg/kg of genistein was administered by rapid i.v. injection. The sample acquired 3 min after dosing was assayed upon 50-fold dilution with drug-free plasma. The plasma concentration of genistein decreased from 30.8 μg/ml at 3 min to 0.036 μg/ml during 2.5 h after dosing. Chromatographic conditions and peak assignments are as in Fig. 2.

with no detectable degradation in column performance. Selection of 4-hydroxybenzophenone as the I.S. involved careful evaluation of its chromatographic behavior, solubility properties, chemical stability and detectability by each of the methods used for monitoring genistein during HPLC [38]. The use of an I.S. served primarily to correct for variability introduced during sample preparation and injection.

3.3. Standard curves and assay validation

The genistein-to-I.S. chromatographic peak-area ratio was directly proportional to the drug concentration for standard solutions encompassing a 50-fold range from 0.020 to 1.02 μg/ml. The standard curves exhibited excellent linearity, as regression analysis performed with a weighting factor of reciprocal peak-area ratio typically afforded correlation coefficients greater than 0.999, with intercept values that did not deviate significantly from the origin. There was no significant difference between the mean slopes for the calibration curves of genistein in plasma (1.73 ± 0.06 ml/μg, $n = 18$) and urine (1.67 ± 0.08 ml/μg, $n = 8$), prepared with a single stock solution of the drug during a 3 to 5 month period. Furthermore, the R.S.D. values of the average slopes, which were 3.5% for plasma and 4.8% for urine, demonstrated that the assay was highly consistent during this time.

Relative recoveries of genistein from urine and plasma calculated for selected concentrations encompassing the range of the standard curves are summarized in Table 2. The data was compiled from the backcalculated concentrations of the standard curves that were assayed over 3 to 5 months, as indicated above. In general, the relative recovery of genistein was found to be independent of the sample matrix and concentration, exhibiting mean values that ranged from 97.1 to 101.2%. The R.S.D. for replicate determinations of the standard solutions with concentrations of 0.05 to 1.02 μg/ml were 2.3 to 6.1% ($n = 18$) for plasma and 1.1 to 6.5% ($n = 8$) for urine. Employing a sample size of 50 μl,

Table 2
Absolute and relative recovery of genistein from biological fluids

Amount added (μg/ml)	Absolute recovery (%)[a]		Relative recovery (%)[b]	
	Plasma	Urine	Plasma	Urine
1.02	90.2 ± 1.2	91.2 ± 3.9	101.2 (3.5)	101.1 (1.1)
0.61	92.0 ± 6.8	90.8 ± 3.2	99.6 (2.3)	99.3 (1.6)
0.20	95.1 ± 1.9	93.7 ± 6.1	100.2 (4.3)	100.9 (3.9)
0.11	96.2 ± 1.8	88.2 ± 6.6	98.7 (3.8)	97.1 (5.3)
0.020	100.3 ± 2.1	93.2 ± 3.1	99.9 (10.7)	98.1 (12.0)
Mean[c]	94.8 ± 3.9	91.4 ± 2.2	99.9 ± 0.9	99.3 ± 1.7

[a] Tabulated values are the mean ± S.D. of 5 replicate determinations.
[b] Number of replicates: plasma, 18 (20 weeks); urine, 8 (12 weeks). Numbers in parentheses, percent R.S.D.
[c] Aggregate mean recovery ± standard error.

genistein was readily detectable in samples with concentrations as low as 10 ng/ml, but could not be quantified with good reproducibility. At the lowest drug concentration included in the calibration curves, 0.020 μg/ml, R.S.D. values were 10.7% and 12.0% for plasma and urine, respectively. Accordingly, the analytical method is considered accurate and reproducible for quantifying genistein in plasma and urine at concentrations ranging from 0.02 to 1.0 μg/ml.

3.4. Extraction efficiency

Genistein was efficiently extracted from both plasma and urine throughout the range of concentrations included in the standard curve (Table 2). Determinations of the absolute recovery (mean ± S.D., $n = 5$) ranged from 90.2 ± 1.2 to 100.3 ± 2.1% for human plasma and 88.2 ± 6.6 to 93.7 ± 6.1% for human urine. Aggregate mean values (± standard error) for the absolute recovery of genistein at the five concentrations were 94.8 ± 3.9% from plasma and 91.4 ± 2.2% from urine. The I.S. was also efficiently extracted from plasma and urine, with absolute recoveries of 92.9 ± 2.8% and 93.6 ± 4.3% (mean ± S.D., $n = 25$), respectively. The drug and I.S. were similarly recovered from the plasma and urine of mice and dogs (data not shown).

3.5. Stability of genistein in plasma and urine

Information on the temperature dependence of genistein stability in plasma and urine was necessary for development of a sample acquisition and storage protocol for use during pharmacokinetic studies. The results of these studies are summarized in Table 3. There was no observable loss of genistein from solutions in human plasma or urine maintained at 37.1 ± 0.1°C during a 9-h period. Thus, blood specimens obtained from subjects treated with the drug may be directly centrifuged at physiological temperature, thereby avoiding the need to separate plasma from blood at lower temperatures, which could influence the plasma concentration of the drug if temperature dependent partitioning with red blood cells occurs. Similarly, there was no change in the concentration of genistein in plasma or urine during 31 days at −17 or −69°C. Therefore, plasma samples acquired during pharmacokinetic studies may be stored in a standard freezer for at least one month prior to analysis.

3.6. Application and specificity of the assay

Fig. 3 shows liquid chromatograms of plasma samples obtained before and at selected times after mice were treated by rapid i.v. injection

Table 3
Stability of genistein in plasma and urine

Medium	Temperature (°C)	Time interval	n[b]	Mean concentration found (μg/ml)[a]		
				0.10 μg/ml[c]	0.51 μg/ml[c]	1.02 μg/ml[c]
Plasma	−68.7 ± 1.6	31 d	10	0.108 ± 0.005	0.54 ± 0.02	1.05 ± 0.04
	−16.7 ± 4.9	31 d	10	0.110 ± 0.005	0.55 ± 0.03	1.06 ± 0.05
	37.1 ± 0.1	6.2 h	9	0.110 ± 0.003	0.57 ± 0.02	1.06 ± 0.04
Urine	−68.7 ± 1.6	31 d	10	0.113 ± 0.012	0.60 ± 0.06	1.14 ± 0.07
	−16.7 ± 4.9	31 d	10	0.111 ± 0.011	0.59 ± 0.04	1.11 ± 0.10
	37.1 ± 0.1	6.2 h	9	0.129 ± 0.005	0.53 ± 0.01	1.28 ± 0.06

[a] In the absence of a discernable trend toward lower concentrations, indicative of degradation, stability has been expressed by reporting the mean ± S.D. of the assayed concentrations during the course of each experiment.
[b] Number of time points in each kinetic run.
[c] Concentration added.

with 25 mg/kg of genistein. The plasma concentration of drug at 3 min postinjection was found to be substantially in excess of the 1.0 μg/ml upper range of the standard curve. Consequently, the specimen was diluted with drug-free plasma and reassayed, affording a value of 30.8 μg/ml for the sample concentration (Fig. 3B). Plasma levels decreased by nearly three decades during the subsequent 150 min (Fig. 3C). Thus, in accord with inferences concerning the disposition of genistein in sheep [31], the compound appears to be eliminated very rapidly from systemic circulation by mice. In addition, peaks consistent with drug metabolites were not observed in the chromatograms.

TSP-MS detection was employed to confirm the identity of the peak that possessed the same k' as authentic genistein in chromatograms of plasma acquired from mice following drug administration. Positive- and negative-ion mass spectra (150–500 u) were acquired during chromatographic analysis of a 1-μg reference sample of genistein (Fig. 4). The soft-ionization characteristic of the TSP technique was aptly demonstrated by abundant formation of the molecular species without discernible fragmentation. As previously demonstrated for the positive-ion detection of genistein [35,38,41–43], the base peak in the mass spectrum, m/z 271, corresponded to the protonated molecular ion, $[M+H]^+$ (Fig. 4A). Complementary to this, the base peak in the negative-ion mass spectrum occurred at m/z 269, consistent with the expected $[M-H]^-$ species (Fig. 4B).

Genistein was not detectable by LC–TSP-MS, operated in the scanning mode, in plasma specimens acquired from mice 60 min after receiving a 25 mg/kg i.v. dose. Thus, SIM was implemented to enhance sensitivity, whereby the chromatographic peak corresponding to 5 ng of genistein was detected with a signal-to-noise ratio of approximately 10 whether monitoring positive ions at m/z 271 or negative ions at m/z 269. Reexamination of the 60-min murine plasma sample under SIM conditions clearly showed a chromatographic band eluting with the same t_R as authentic genistein (Fig. 4C,D). Observing ions consistent with both molecular species,

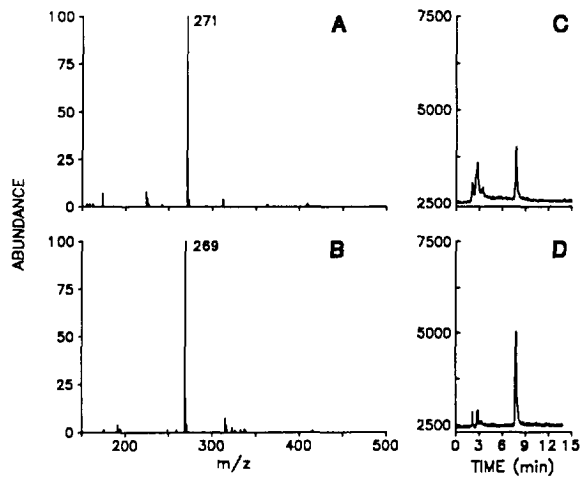

Fig. 4. Averaged TSP mass spectra acquired during the elution of a 1-μg genistein standard by continuous scanning over a mass range of 150–500 u with positive- (A) and negative-ion (B) detection. Chromatograms of plasma collected from a mouse 60 min after dosing i.v. with 25 mg/kg of genistein determined with TSP-MS detection in the SIM mode monitoring positive ions at m/z 271 (C) and negative ions at m/z 269 (D). Chromatography was performed at 25°C on a 4-μm Nova-Pak C_8 column (15 cm × 3.9 mm I.D.) eluted with acetonitrile–0.05 *M* ammonium formate buffer, pH 4.0 (30:70, v/v) at a flow-rate of 0.75 ml/min.

$[M+H]^+$ and $[M-H]^-$, aided in establishing the identity of the peak as genistein. Furthermore, the sample concentration estimated from the TSP-MS response, 0.14 μg/ml, was in excellent agreement with the value determined by UV detection.

3.7. Sensitivity enhancement

With 20 ng/ml as the lower limit of quantitation, the assay was found adequate for characterizing the disposition of genistein in mice and dogs. Whereas the concentration of unconjugated genistein in human biological fluids has not been reported, plasma levels determined as the free compound plus sulfate conjugates were only 1 to 5 ng/ml in a group of Japanese subjects consuming a high-soy diet [6]. Therefore, alternate methods of detection were explored in an

attempt to increase the sensitivity of the HPLC assay.

As compared to UV detection, substantially lower detection limits for genistein were reported through the use of electrochemical oxidation on glassy carbon electrodes [38,40]. However, this technique proved highly inferior to UV detection during LC analysis of the compound upon isolation from plasma by extraction into TBME. Undoubtedly, oxidizable substances transparent to UV detection at 260 nm were present in the plasma extract, thereby leading to a condition of decreased sensitivity. TSP-MS proved extremely useful for substantiating the presence of genistein in plasma specimens acquired from mice treated with the drug. However, monitoring elution of the chromatographic peak for 0.4 ng of pure genistein by UV absorption at 260 nm yielded a signal-to-noise ratio that was approximately 50-times greater than that realized for a 5-ng sample with TSP-MS detection.

Unable to identify a more sensitive method of detection than UV absorption, the extent to which the sample volume could be increased in order to enhance sensitivity, without otherwise modifying the assay procedure, was evaluated. Plasma samples ranging in size from 100 to 250 μl were extracted with 3 ml of TBME as previously described. Chromatographic analysis of drug-free samples showed no endogenous peaks eluting in the presence of the drug or I.S. The absolute recovery of drug from these larger volumes of plasma differed insignificantly from values determined with a 50-μl sample. Using a plasma volume of 250 μl, linear and reproducible standard curves of genistein were obtained in the concentration range extending from 2 to 100 ng/ml, improving the lower limit of quantitation by a factor of 10. Thus, the present HPLC method with UV detection can be used to reliably and specifically quantitate genistein in 250 μl of plasma at concentrations as low as 2 ng/ml (R.S.D., 9.4%; $n = 5$). This approaches the sensitivity of the existing GC–MS method, which requires 16 times more sample for determining 0.75 ng/ml of genistein with a 15.7% R.S.D. [5].

4. Conclusions

A specific, sensitive and facile assay based upon isocratic reversed-phase HPLC has been developed for the determination of genistein in biological fluids. The preparation of samples for analysis simply involves a single extraction with TBME. Following removal of the organic solvent, the reconstituted sample is directly analyzed by liquid chromatography with UV detection. Employing only 50 μl of plasma or urine, a volume which permits replicate determinations of specimens obtained from a single mouse and serial plasma samples from a rat or dog, the lowest concentration of genistein included in the standard curves was 0.020 μg/ml. Without any modification to the method, increasing the sample size to 250 μl afforded a 10-fold improvement in sensitivity. The assay has been shown to be specific, accurate and reproducible. The ease of sample preparation and utilization of commonly available chromatographic instrumentation renders the procedure well-suited for drug level monitoring during preclinical pharmacokinetic studies.

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References

[1] H. Adlercreutz, *Scand. J. Clin. Lab. Invest.*, 50 (Suppl. 201) (1990) 3.
[2] S. Barnes, C. Grubbs, K.D.R. Setchell and J. Carlson, *Prog. Clin. Biol. Res.*, 347 (1990) 239.
[3] H. Adlercreutz, T. Fotsis, C. Bannwart, K. Wahala, G. Brunow and T. Hase, *Clin. Chim. Acta*, 199 (1991) 263.
[4] H. Adlercreutz, H. Honjo, A. Higashi, T. Fotsis, E. Hamalainen, T. Hasegawa and H. Okada, *Am. J. Clin. Nutr.*, 54 (1991) 1093.
[5] H. Adlercreutz, T. Fotsis, J. Lampe, K. Wahala, T. Makela, G. Brunow and T. Hase, *Scand. J. Clin. Lab. Invest.*, 53 (Suppl. 215) (1993) 5.
[6] H. Adlercreutz, H. Markkanen and S. Watanabe, *Lancet*, 342 (1993) 1209.

[7] A.C. Eldridge, *J. Agric. Food Chem.*, 30 (1982) 353.
[8] L. Coward, N.C. Barnes, K.D.R. Setchell and S. Barnes, *J. Agric. Food Chem.*, 41 (1993) 1961.
[9] G. Peterson and S. Barnes, *Biochem. Biophys. Res. Commun.*, 179 (1991) 661.
[10] L. Schweigerer, K. Christeleit, G. Fleischmann, H. Adlercreutz, K. Wahala, T. Hase, M. Schwab, R. Ludwig and T. Fotsis, *Eur. J. Clin. Invest.*, 22 (1992) 260.
[11] T. Fotsis, M. Pepper, H. Adlercreutz, G. Fleischmann, T. Hase, R. Montesano and L. Schweigerer, *Proc. Natl. Acad. Sci. USA*, 90 (1993) 2690.
[12] K. Kiguchi, A.I. Constantinou and E. Huberman, *Cancer Commun.*, 2 (1990) 271.
[13] G. Kaur, M. Stetler-Stevenson, S. Sebers, P. Worland, H. Sedlacek, C. Myers, J. Czech, R. Naik and E. Sausville, *J. Natl. Cancer Inst.*, 84 (1992) 1736.
[14] M.C. Amaral, S. Miles, G. Kumar and A.E. Nel, *J. Clin. Invest.*, 92 (1993) 848.
[15] Y. Matsukawa, N. Marui, T. Sakai, Y. Satomi, M. Yoshida, K. Matsumoto, H. Nishino and A. Aoike, *Cancer Res.*, 53 (1993) 1328.
[16] Y. Mousavi and H. Adlercreutz, *Steroids*, 58 (1993) 301.
[17] M.C. Pagliacci, F. Spinozzi, G. Migliorati, G. Fumi, M. Smacchia, F. Grignani, C. Riccardi and I. Nicoletti, *Eur. J. Cancer*, 29A (1993) 1573.
[18] G. Peterson and S. Barnes, *Prostate*, 22 (1993) 335.
[19] K. Yanagihara, A. Ito, T. Toge and M. Numoto, *Cancer Res.*, 53 (1993) 5815.
[20] J. Nishimura, H. Takahira, K. Shibata, K. Muta, M. Yamamoto, H. Ideguchi, T. Umemura and H. Nawata, *Leuk. Res.*, 12 (1988) 875.
[21] A. Constantinou, K. Kiguchi and E. Huberman, *Cancer Res.*, 50 (1990) 2618.
[22] Y. Honma, J. Okabe-Kado, T. Kasukabe, M. Hozumi and K. Umezawa, *Jpn. J. Cancer Res.*, 81 (1990) 1132.
[23] M. Makishima, Y. Honma, M. Hozumi, K. Sampi, M. Hattori, K. Umezawa and K. Motoyoshi, *Leuk. Res.*, 15 (1991) 701.
[24] F. Traganos, B. Ardelt, N. Halko, S. Bruno and Z. Darzynkiewicz, *Cancer Res.*, 52 (1992) 6200.
[25] M. Makishima, Y. Honma, M. Hozumi, K. Nagata and K. Motoyoshi, *Biochim. Biophys. Acta*, 1176 (1993) 245.
[26] T. Akiyama, J. Ishida, S. Nakagawa, H. Ogawara, S. Watanabe, N. Itoh, M. Shibuya and Y. Fukami, *J. Biol. Chem.*, 262 (1987) 5592.
[27] A. Okura, H. Arakawa, H. Oka, T. Yoshinari and Y. Monden, *Biochem. Biophys. Res. Commun.*, 157 (1988) 183.
[28] J. Markovits, C. Linassier, P. Fosse, J. Couprie, J. Pierre, A. Jacquemin-Sablon, J. Saucier, J. Le Pecq and A.K. Larsen, *Cancer Res.*, 49 (1989) 5111.
[29] M. Imoto, T. Yamashita, T. Sawa, S. Kurasawa, H. Naganawa, T. Takeuchi, Z. Bao-quan and K. Umezawa, *FEBS Lett.*, 230 (1988) 43.
[30] C. Linassier, M. Pierre, J. Le Pecq and J. Pierre, *Biochem. Pharmacol.*, 39 (1990) 187.
[31] H.R. Lindner, *Aust. J. Agric. Res.*, 18 (1967) 305.
[32] D.A. Shutt, A. Axelsen and H.R. Lindner, *Aust. J. Agric. Res.*, 18 (1967) 647.
[33] D.A. Shutt, R.H. Weston and J.P. Hogan, *Aust. J. Agric. Res.*, 21 (1970) 713.
[34] W.H. Braden, R.I. Thain and D.A. Shutt, *Aust. J. Agric. Res.*, 22 (1971) 663.
[35] H.D. Dewald, S.A. Worst, J.A. Butcher Jr. and E.F. Saulinskas, *Electroanalysis*, 3 (1991) 777.
[36] G. Wang, S.S. Kuan, O.J. Francis, G.M. Ware and A.S. Carman, *J. Agric. Food Chem.*, 38 (1990) 185.
[37] R.J. Barbuch, J.E. Coutant, M. Welsh and K.D.R. Setchell, *Biomed. Environ. Mass Spectrom.*, 18 (1989) 973.
[38] K.D.R. Setchell, M. Welsh and C.K. Kim, *J. Chromatogr.*, 386 (1987) 315.
[39] L.R. Snyder, J.L. Glajch and J.J. Kirkland, *Practical HPLC Method Development*, John Wiley and Sons, New York, NY, 1988, p. 191.
[40] Y. Kitada, Y. Ueda, M. Yamamoto, M. Ishikawa, H. Nakazawa and M. Fujita, *J. Chromatogr.*, 366 (1986) 403.
[41] Y. Arai, T. Hanai, A. Nosaka and K. Yamagichi, *J. Liq. Chromatogr.*, 13 (1990) 2449.
[42] M.S. Lee, D.J. Hook, E.H. Kerns, K.J. Volk and I.E. Rosenberg, *Biol. Mass Spectrom.*, 22 (1993) 84.
[43] P.E. Juniewicz, S.P. Morell, A. Moser and L.L. Ewing, *J. Steroid Biochem.*, 31 (1988) 987.
[44] T.J. Batterham, N.K. Hart and J.A. Lamberton, *Nature*, 206 (1965) 509.
[45] W.H. Braden, N.K. Hart and J.A. Lamberton, *Aust. J. Agric. Res.*, 18 (1967) 335.
[46] L.A. Griffiths and G.E. Smith, *Biochem. J.*, 128 (1972) 901.