Isoflavone metabolism in domestic cats (Felis catus): Comparison of plasma metabolites detected after ingestion of two different dietary forms of genistein and daidzein

K. M. Whitehouse-Tedd, N. J. Cave, C. E. Ugarte, L. A. Waldron, J. K. Prasain, A. Arabshahi, S. Barnes, W. H. Hendriks and D. G. Thomas

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Isoflavone metabolism in domestic cats (*Felis catus*): Comparison of plasma metabolites detected after ingestion of two different dietary forms of genistein and daidzein[1]

K. M. Whitehouse-Tedd,*[2,3] N. J. Cave,† C. E. Ugarte,‡ L. A. Waldron,§ J. K. Prasain,# A. Arabshahi,# S. Barnes,# W. H. Hendriks,‖¶ and D. G. Thomas*

*Center for Feline Nutrition, Institute of Food, Nutrition, and Human Health, Massey University, Palmerston North, 4410, New Zealand; †Institute of Veterinary, Animal, and Biomedical Sciences, Massey University, Palmerston North, 4410, New Zealand; ‡Central City Vets, Pitt Street, Palmerston North, 4410, New Zealand; §LWT Nutrition Ltd., Feilding 4740, New Zealand; #Targeted Metabolomics, and Proteomics Laboratory, Department of Pharmacology, and Toxicology, University of Alabama, Birmingham 35294; ‖Animal Nutrition Group, Department of Animal Science, Wageningen University, Wageningen, The Netherlands; and ¶Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

ABSTRACT: Some felid diets contain isoflavones but the metabolic capacity of cats toward isoflavones is relatively unknown, despite the understanding that isoflavones have divergent biological potential according to their metabolite end products. The objective of this study was to determine the plasma metabolites detectable in domestic cats after exposure to 2 different dietary forms of isoflavones, either as a soy extract tablet (n = 6) or as part of a dietary matrix (n = 4). Serial blood samples were collected after isoflavone exposure to identify the plasma metabolites of each cat. Genistein was detected in its unconjugated form or as a monosulfate. Daidzein was detected as both a mono- and disulfate as well as in its unconjugated form. Other daidzein metabolites detected included equol mono- and disulfate, dihydrodaidzein, and *O*-desmethylangolensin. No β-glucuronide metabolites of either isoflavone were detected. Equol was produced in markedly fewer cats after ingestion of a soy extract tablet as a single oral bolus compared with cats consuming an isoflavone-containing diet. The detectable metabolites of the isoflavones, genistein and daidzein, in domestic cat plasma after dietary ingestion has been described in the present study for the first time. The metabolic capacity for isoflavones by domestic cats appears to be efficient, with only minimal proportions of the ingested amount detected in their unconjugated forms. This has implications for the potential of isoflavones to exert physiological activity in the domestic cat when consumed at concentrations representative of typical dietary intake.

Key words: cat, diet, isoflavone, metabolite, soy

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INTRODUCTION

Isoflavones are nonsteroidal plant compounds commonly found in soy and soy products (Kurzer and Xu, 1997). In domestic cats (*Felis catus*), the isoflavones genistein and daidzein have elicited changes in sex steroid receptor expression (Bell et al., 2008), innate and acquired immunity, food intake, and

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[2]Present address: School of Animal, Rural, and Environmental Sciences, Nottingham Trent University, Southwell, Nottinghamshire, NG25 0QF, UK.

[3]Corresponding author: cheetah.research@yahoo.com.

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lean body mass (Cave et al., 2007b,c,d) whereas diets containing soy have also been associated with changes in serum thyroid hormone (T_4) concentrations (White et al., 2004).

Studies have shown that some domestic cat diets available in North America and New Zealand contain substantial concentrations of genistein and daidzein, with up to 249 μg genistein and 151 μg daidzein/g DM (Court and Freeman, 2002; Bell et al., 2006b). The isoflavone content of felid diets has been assumed to result primarily from the inclusion of soy products in the formulation of these diets (Setchell et al., 1987; Court and Freeman, 2002).

The potential for isoflavones to exert biological activity in felids depends initially on the ability of felid species to absorb and metabolize these compounds once ingested. Genistein and daidzein undergo considerable metabolism in the gut wall before entering the portal and peripheral circulation (Sfakianos et al., 1997; Prasain et al., 2006). Such metabolic modification may influence isoflavone binding to estrogen receptors or other proteins and may be important in explaining the diverse array of responses elicited by isoflavones under different study conditions (Barnes et al., 2000; Kinjo et al., 2004). However, detailed investigation of the manner that feline species metabolize dietary isoflavones has not previously been undertaken. The current study was designed to quantify the isoflavones and their metabolites present in the plasma of cats after ingestion of either a single oral bolus of soy extract or a commercially available feline diet known to contain isoflavones.

MATERIALS AND METHODS

Two experiments were conducted to determine the metabolism of genistein and daidzein after 1) ingestion of a soy extract and 2) ingestion of a commercially prepared feline diet. Studies were approved by Massey University's Animal Ethics Committee (Anonymous, 2003).

Animals

All cats were born, raised, and housed at Massey University's Center for Feline Nutrition (Palmerston North, New Zealand) and had been maintained on a commercially prepared, isoflavone-free diet for 4 wk before initiation of the studies (washout period estimated from a half-life of 22 h for conjugated genistein; Cave et al., 2007a). Each cat had been vaccinated against feline herpes-1, calicivirus, and panleukopenia using a modified live vaccine (Felocell CVR; Norden Laboratories, München, Germany). Antibiotics had not been administered to any of the cats for at least 3 mo before the studies.

For the duration of the studies, cats were individually housed in polythene plastic metabolism cages (0.8 by 0.8 by 1.1 m) equipped with an elevated rest area, feed and water bowl area, and a litter tray (Hendriks et al., 1999). The cats were exposed to natural light cycles, provided with ad libitum access to water, and weighed weekly without restriction of food or water.

Plasma Analysis

A liquid chromatography (**LC**) electrospray ionization (**ESI**)-multiple reaction ion monitoring (**MRM**)-tandem mass spectrometry (**MS/MS**) assay was developed to measure simultaneously intact isoflavone sulfates and their aglycones in plasma. Isoflavone sulfate standards (genistein 4′-monosulfate, daidzein 4′-monosulfate, equol 7-monosulfate, equol 4′-7-disulfate, and daidzein 4′-7-disulfate; N. Botting, Dep. of Chemistry, St. Andrew's University, Fife, UK), genistein, daidzein, and equol (LC-Laboratories, Woburn, MA), and solvents (Fisher, Norcross, GA) were obtained accordingly.

A protein precipitation method was used for extraction. Samples were diluted 5-fold with methanol containing 0.1% acetic acid (vol/vol), vortexed, and centrifuged at 1,800 × *g* for 10 min at room temperature before the supernatant was extracted for the LC–MS/MS analysis. The liquid chromatography system consisted of a reversed-phase phenyl-hexyl column (100 by 2.0 mm i.d.; Phenomenex, Torrance, CA). The mobile phase consisted of a mixture of solvent A (water, containing 10 mmol/L ammonium acetate) and solvent B (acetonitrile, containing 10 mmol/L ammonium acetate) with a flow rate of 0.2 mL/min. A 15-min assay was established using a linear gradient from 10 to 100% B over the first 10 min; then the system was returned to the initial 10% B at 11 min to allow for equilibration.

Liquid chromatography-MS/MS analysis was performed using a system consisting of a refrigerated autosampler (Model SIL-HT; Shimadzu Scientific Instruments, Columbia, MD) and a mass spectrometer (API 4000; Applied Biosystems/MDS Sciex, Concord, ON, Canada), which was used for quantification and identification. Nitrogen was used as the nebulizing gas. Liquid chromatography-MS/MS was operated in the MRM mode. Mass transitions (mass-to-charge ratio [***m***/***z***]) used for MRM analysis were 333/117 (daidzein 4′-monosulfate), 413/333 (daidzein 4′-7-disulfate), 349/269 (genistein 4′-monosulfate), 401/321 (equol 4′-7-disulfate), 321/121 (equol monosulfate), 269/133 (genistein), 253/132 (daidzein), 255/149 (dihydrodaidzein), 241/119 (equol), and 257/108

[*O*-desmethylangolesin (***O*-DMA**)]. Additional transitions were monitored for isoflavone mono-β-glucuronides, m/z 429/253 (daidzein), 445/269 (genistein), and 417/241 (equol). Figure 1 illustrates the isoflavone structures. The quantification of isoflavone sulfates was based on a calibration curve of each isoflavone sulfate in the concentration range of 10 to 10,000 nmol/L. Apigenin (mass transition, m/z 269/149) was used as internal standard.

Validation Study

Stock solutions of each analyte were prepared in dimethyl sulfoxide, and standard working solutions containing chrysin (internal standard, 5 μmol/L) were prepared by appropriate dilution in 80% aqueous methanol. The analytical method was validated to demonstrate the specificity, lower limit of quantification, accuracy, and precision of measurements. Specificity was established by the lack of interference peaks at the retention time for the internal standard and isoflavone standards. Linearity was tested at 9 levels of concentrations, covering a range of 0.01 to 10 μmol/L. The calibration curves were established by plotting the peak area ratio of the analytes to the internal standard (chrysin) compared with analyte concentrations in the calibration samples. The regression estimates, slope, intercept, and correlation coefficient were calculated by linear least-square regression ($1/x^2$ weighting).

The accuracy and precision (presented as % CV) of this analytical method were determined using quality control (**QC**) samples ($n = 3$) in 5 to 6 replicates of 0.02, 0.2, and 2 μmol/L of analytes. For determination of the intrabatch accuracy and precision, replicate analysis of all analytes was performed on the same day. The run consisted of calibration curves and 6 replicates of QC samples 0.02 (lower), 0.2 (middle), and 2 μmol/L (higher). All these samples were analyzed on 3 different batches to determine intrabatch and interbatch accuracy and precision. Accuracy was determined by comparing the calculated concentration using calibration curves with known concentration. The limit of quantification (**LOQ**) was defined as precision and accuracy within 15%. The lower limit of quantification was defined as the smallest amount of the analyte that could be measured in a sample with sufficient precision and accuracy (within 20% for both terms) and was chosen as the lowest concentration on the calibration curve. The LOQ for dihydrodaidzein and *O*-DMA was previously determined to be 1 to 5 n*M* (Prasain et al., 2010).

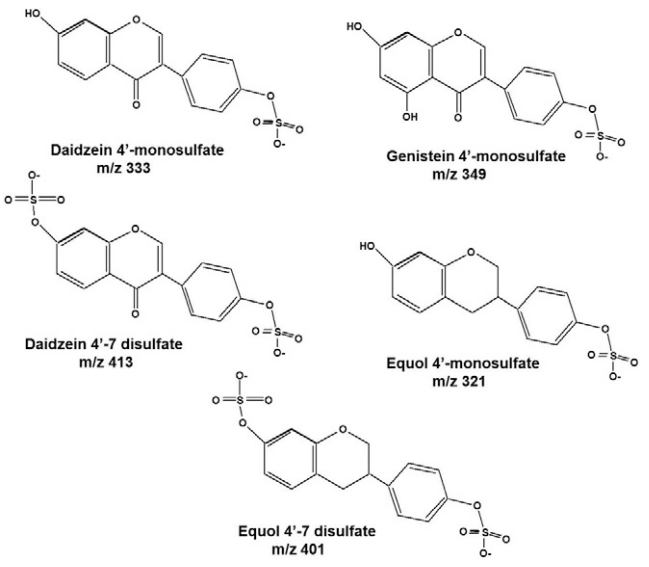

Figure 1. Structure of isoflavone metabolites. Top: daidzein disulfate [mass-to-charge ratio (m/z): 413/333], middle: daidzein monosulfate (m/z: 333/117), and bottom: genistein monosulfate (m/z: 349/269).

Experiment 1

This study aimed to determine isoflavone metabolites present after ingestion of a single oral bolus of soy extract. Six adult castrated male, domestic short-haired cats with a mean age of 3.75 ± 0.65 yr and an average BW of 4.58 ± 0.12 kg were used in this study. Twenty-four hours before the start of the study, indwelling catheters (central venous catheter; Shoof International, Cambridge, New Zealand) were placed in a jugular vein of each cat according to standard aseptic veterinary procedures (Seldinger technique; Seldinger, 1953). Food was withheld 18 h before oral administration of 1 soy extract tablet (approximately 0.218 g, Phytolife1 One a Day; Healthy Living, Palmerston North, New Zealand). Analysis by HPLC (Bell et al., 2006b) showed that on average, 1 tablet contained 10.17 mg genistein and 13.12 mg daidzein (as-fed) as aglycone equivalents. One-half of the daily allowance (293 kJ·kg $BW^{-1}·d^{-1}$; NRC 1986) of an isoflavone-free diet (Table 1) of each cat was offered 5 min before administering the tablet. The diet was a nutritionally complete and balanced, moist commercial diet, which met the maintenance nutrient requirements of adult cats (AAFCO, 2009) and contained the following ingredients: selected meat byproducts and meat derived from beef, lamb, and chicken; fish byproduct; vegetable protein; gelling agents; calcium; coloring; and necessary vitamins. This diet was previously analyzed (Bell et al., 2006b), and no detectable isoflavones (genistein, daidzein, formononetin, and biochanin A) were found.

Blood samples (1.5 mL) were collected via the indwelling catheter into sterile, heparinized vacutainers (containing 34 IU heparin) at 0, 2, 4, 6, and 12 h after isoflavone ingestion. Catheter patency was maintained by flushing with 1 mL sterile heparinized saline (10 IU/mL) after each sample collection. Within 10 min of

collection, the blood was centrifuged at 1,130 × *g* for 10 min at room temperature, and plasma was collected, transferred to labeled vials, and stored at –20°C until analysis. After the final sampling period, the catheters were removed, and cats were returned to their normal group housing facilities.

Experiment 2

This study aimed to determine isoflavone metabolites present after ingestion of an isoflavone-containing, commercially prepared diet. Four adult castrated male, domestic short-haired cats with a mean age of 4.80 ± 1.83 yr and BW of 4.55 ± 0.55 kg were gradually adapted over a 10-d period before the start of the trial to a moist, commercially prepared meat and gravy formula diet containing (as aglycone equivalents; Bell et al., 2006b) 151 μg genistein and 1,984 μg daidzein/g DM (Table 1). During the 10 d, cats were offered an amount of food in 2 distinct meals (0900 and 1700 h) to meet their maintenance energy requirements (293 kJ·kg $BW^{-1}·d^{-1}$; NRC, 1986). The dietary ingredients were meat byproducts and meat (including beef), cereal byproducts, vegetable protein, gelling agents, calcium, starch, coloring, and essential vitamins. The proximate composition of the diet is described in Table 1.

Placement of indwelling catheters and blood collection were the same as those described in Exp. 1. After an 18-h fast, a baseline blood sample was collected from the catheter of each cat (time 0), and then each cat was given an amount of food equivalent to 50% of their individual daily ME requirements. Cats were allowed 10 min to consume the food, and any remaining food was weighed. The time that the meal was consumed was recorded, and serial blood samples were taken at 0.75, 1.5, 2.5, 4, 6, 8, and 12 h after food ingestion. After obtaining the final blood sample, the catheter was removed and cats were returned their normal group housing conditions.

Statistical Analyses

All absorption and excretion variables were normalized to the individual dose for each cat before averaging. All values are expressed as mean ± SEM unless otherwise stated. The maximum plasma concentration ($\mathbf{C_{max}}$) and time to achieve C_{max} ($\mathbf{T_{max}}$) were determined using noncompartmental modeling software (WinNonLin Professional, version 4.1; Pharsight Corp., Cary, NC). Noncompartmental methods using the linear trapezoidal rule with extrapolation to infinity was used to estimate the area under the curve ($\mathbf{AUC_{0–12}}$) to estimate isoflavone metabolite absorption over the 12-h sampling period. Curves were plotted for each cat before average T_{max} and C_{max} values were calculated.

RESULTS

Liquid Chromatography-Tandem Mass Spectrometry Analysis of Plasma Isoflavones

The calibration curves for the isoflavone standards were generated at different concentrations ranging from 0.01 to 10 μmol/L. The standard curve was linear over this 1,000-fold concentration range. The MRM chromatograms of genistein, daidzein, and equol and their sulfate conjugate standards are shown in Fig. 2. The assay method for quantification of plasma isoflavone metabolites was demonstrated to be very sensitive and specific, and a linear response was obtained over a range of 0.01 to 10 μmol/L with a correlation coefficient of 0.99. The LOQ was 0.01 μmol/L.

The accuracy and precision of this assay for isoflavone sulfates and aglycones were determined at various concentrations (0.02, 0.2, and 2 μmol/L) using QC samples, and the within-assay accuracies and precisions for analytes were in the range of 84 to 121% and 3 to 17%, respectively. For the lowest quality control (0.02 μmol/L), the interbatch (between assay) precisions for the analytes were less than 14%, and the accuracies were between 91 and 103%. The method was sensitive, reproducible, and reliable, and the LOQ was 0.02 μmol/L.

Experiment 1

Cats ingested an average of 2.22 ± 0.06 mg genistein and 2.86 ± 0.08 mg daidzein/kg BW. Unconjugated daidzein and genistein were detected in the plasma of 5 of the 6 cats. No β-glucuronide metabolites were detected in any of the plasma samples. The metabolites, daidzein monosulfate, daidzein disulfate, and genistein monosulfate, were detected in the plasma of all cats (Fig. 3). Both unconjugated genistein and daidzein

Table 1. Proximate composition of diets

Component	Exp. 1	Exp. 2
DM, % as is	17.7	15.5
CF, % DM	25.2	23.3
CP, % DM	35.7	47.0
CHO,[1] % DM	29.2	20.0
Ash, % DM	6.90	7.68
ME,[2] kJ/g DM	18.9	18.4
Genistein, μg/g DM	0.00	151
Daidzein, μg/g DM	0.00	198

[1]Carbohydrate calculated by difference (100 – CP – crude fat – ash).

[2]Calculated using modified Atwater factors (CP × 3.5, crude fat × 8.5, and carbohydrate × 3.5).

were no longer detectable in plasma at 12 h after isoflavone ingestion although all other metabolites were still detectable at this final sampling point. Equol (monosulfate) was only detected at the final sampling point (12 h after dose) in 1 cat (Fig. 3). No other forms of equol were detected in any of the plasma samples. Dihydrodaidzein and *O*-DMA were not detected in any of the plasma samples. Plasma appearance and disposition curves for the 3 major metabolites are shown in Fig. 4.

Unconjugated genistein and daidzein exhibited the earliest T_{max} whereas daidzein disulfate was the last to reach its maximum concentration. The greatest C_{max} was achieved by genistein monosulfate, and unconjugated genistein and daidzein exhibited the least C_{max}. The C_{max} and T_{max} of detected metabolites are shown in Table 2.

The proportion of administered genistein recovered in the plasma as a sulfate conjugate (estimated from the $AUC_{0–12h}$), was similar at all sampling points (i.e., mean 99.41 ± 0.00% at 2 h and 99.98 ± 0.00% at 12 h). Likewise, the proportion of administered daidzein recovered in the plasma as a sulfate conjugate was 99.03 ± 0.00% on average at 2 h and 99.93 ± 0.00% at 12 h. No difference was detectable between the extent of conjugation for genistein or daidzein.

Experiment 2

Cats consumed an average of 0.62 ± 0.06 mg genistein and 0.82 ± 0.08 mg daidzein/kg BW, providing a total mean isoflavone dose of 1.44 mg/kg BW. Three of the 4 cats consumed less (by an average of 21.1%) than that predicted from their average intake per meal from the previous 5 d whereas 1 cat consumed slightly more than anticipated (15.7%).

Daidzein monosulfate, genistein monosulfate, and equol monosulfate were present in the plasma of all cats (Table 3; Fig. 5). Daidzein disulfate and equol disulfate were detected in 3 of 4 cats. The disulfate metabolites of daidzein and equol and the monsulfate of genistein were present in the baseline plasma sample of cats. Unconjugated equol was not detected in the plasma of any cat. Dihydrodaidzein and *O*-DMA were detected in 1 cat. No β-glucuronide metabolites were detected in any cat. The T_{max} and C_{max} of isoflavone metabolites are shown in Table 3.

Plasma appearance and disposition curves for the 3 major metabolites are shown in Fig. 6. One cat (number 10) appeared divergent in its plasma metabolite profile (qualitatively and quantitatively), and as such, its plasma data are reported separately (Table 3). This cat was the only cat observed to have detectable concentrations of unconjugated genistein and daidzein and had simultaneously less than average concentrations of the monosulfate conjugates of these isoflavones. Likewise, this cat did not produce any disulfate metabolite whereas the other 3 cats did. Equol monosulfate was detected in concentrations (0.16 μmol/L at only 1 time point) less than observed in other cats (mean, 1.43 ± 0.71 μmol/L). This cat produced detectable concentrations of dihydrodaidzein and *O*-DMA whereas the other 3 cats did not. Excluding the apparent outlier (cat number 10), genistein and daidzein monosulfate exhibited the

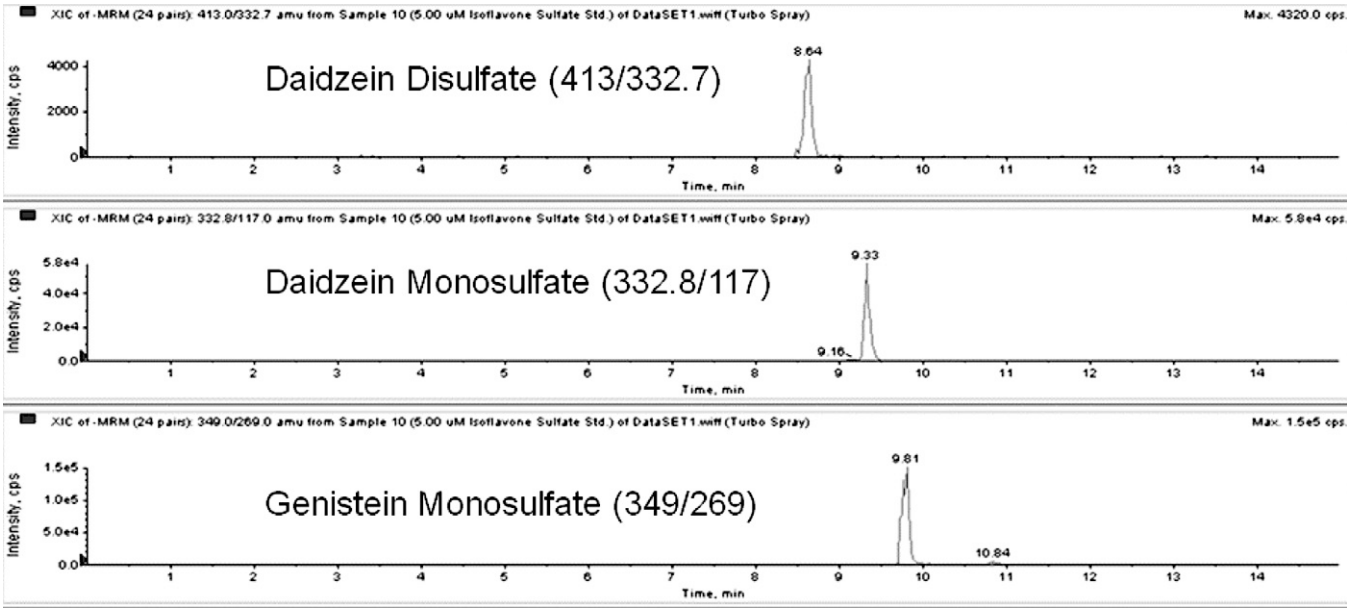

Figure 2. Multiple reaction ion monitoring chromatograms of genistein, daidzein, and equol and their sulfate conjugate standards. Mass transitions used were mass-to-charge ratio (*m/z*): 333/117 (daidzein 4′-monosulfate), 413/333 (daidzein 4′,7-disulfate), 349/269 (genistein 4′-monosulfate), 401/321 (equol 4′,7-disulfate), 321/121 (equol monosulfate), 269/133 (genistein), 253/132 (daidzein), and 241/119 (equol). MRM = multiple reaction ion monitoring; cps = counts per second.

earliest T_{max}, and equol disulfate was the last to reach its maximum concentration. Genistein monosulfate achieved the greatest C_{max} whereas the lowest C_{max} was observed for equol disulfate.

The amount of administered genistein recovered in the plasma as a sulfoconjugate did not vary according to sampling time, ranging from 99.60 ± 0.00% at 1.5 h up to 100.00 ± 0.00% at 6 and 12 h after dose. The amount of administered daidzein recovered in the plasma as a sulfate conjugate also did not vary, ranging from 99.60 ± 0.00% at 1.5 h after dose to 100.00 ± 0.00% at 4, 6, and 12 h after dose.

Comparison of Experiment 1 and Experiment 2

Table 4 presents the incidence of detecting each isoflavone metabolite at any time point in plasma of

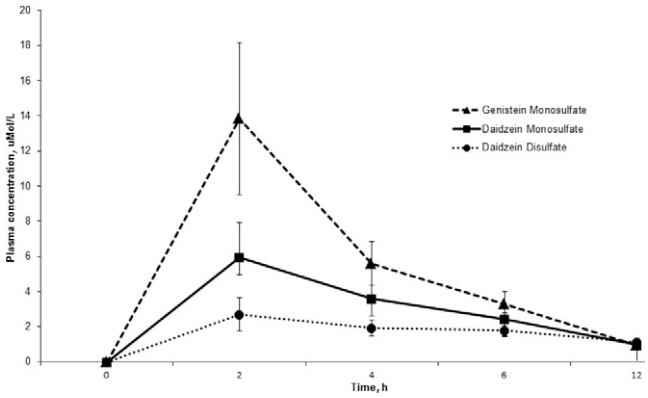

Figure 4. Mean plasma concentration of 3 major metabolites identified in domestic cats (n = 6) after exposure to soy extract tablets containing 10.17 mg genistein and 13.12 mg daidzein (as-fed) providing 2.22 mg genistein and 2.86 mg daidzein/kg BW.

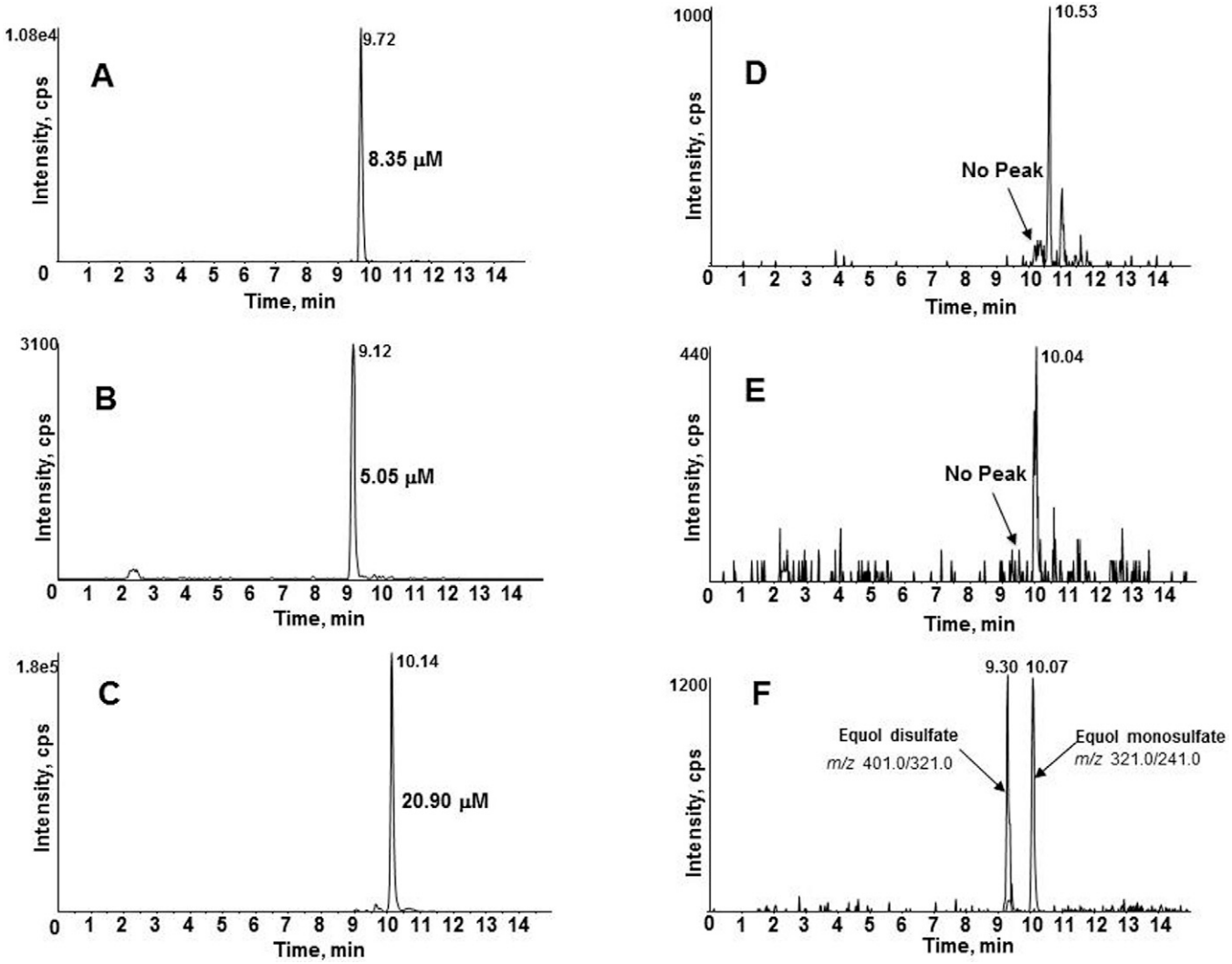

Figure 3. Individual multiple reaction ion-monitoring channels for plasma isoflavone sulfate conjugates from a cat fed a soy extract tablet bolus. (A) Daidzein monosulfate, (B) daidzein disulfate, (C) genistein monosulfate, (D) equol monosulfate, and (E) equol disulfate. There were no peaks that corresponded to the standards (0.1 *M* solution) for (D) and (E) as illustrated in (F). The concentrations (µmol/L) of each isoflavone sulfate are given on the left side of each peak. cps = counts per second; *m*/*z* = mass-to-charge ratio.

Table 2. Mean plasma appearance variables in adult cats ($n = 6$) administered with 2.22 mg genistein and 2.86 mg daidzein/kg BW via a soy extract tablet[1,2]

Metabolite	T_{max}, h	C_{max}, μmol/L
Unconjugated genistein	2.40 ± 0.09	0.09 ± 0.04
Unconjugated daidzein	2.40 ± 0.69	0.10 ± 0.10
Genistein monosulfate	3.00 ± 0.68	14.43 ± 4.02
Daidzein monosulfate	4.33 ± 1.58	6.66 ± 1.67
Daidzein disulfate	4.67 ± 1.52	3.27 ± 0.73

[1]Phytolife1 One a Day (Healthy Living, Palmerston North, New Zealand).

[2]C_{max} = the maximum plasma concentration; T_{max} = time to achieving C_{max}.

cats ingesting the soy extract tablet (Exp. 1) and the cats consuming an isoflavone-containing diet (Exp. 2). The incidence of unconjugated genistein and daidzein were both greater in Exp. 1 than Exp. 2 whereas the incidence of mono- or disulfates of either of these isoflavones was similar between experiments. Equol mono- and disulfates as well as dihydrodaidzein and *O*-DMA were only detected in Exp. 2. The proportion of total genistein or daidzein metabolites detected at 12 h as a conjugated form was similar between dietary forms.

DISCUSSION

A key element in the present study was the development of a rapid and sensitive LC-ESI-MS/MS assay to simultaneously quantify isoflavone aglycones and sulfates in plasma to enable the identification and quantification of isoflavone metabolites in domestic cat plasma. The methods developed in the present study will be of value in supporting future pharmacokinetic studies in cats. We optimized chromatographic conditions to provide shorter retention times, adequate peak shape as a modification of the LC-MS/MS method for quantification of intact sulfate, and glucuronide isoflavones in human urine reported by Clarke et al. (2002).

Table 3. Mean plasma appearance variables in cats ($n = 3$) and 1 outlier cat fed a diet containing 151 μg genistein and 198 μg daidzein/g DM[1,2]

	T_{max}, h		C_{max}, μmol/L	
Metabolite	Mean, $n = 3$	$n = 1$	Mean, $n = 3$	$n = 1$
Unconjugated genistein	ND[3]	1.50	ND	0.26
Unconjugated daidzein	ND	4.00	ND	0.19
Genistein monosulfate	1.83 ± 0.29	1.50	1.78 ± 0.33	0.19
Daidzein monosulfate	1.83 ± 0.29	1.50	1.33 ± 0.28	0.05
Daidzein disulfate	2.50 ± 0	ND	1.46 ± 0.16	ND[3]
Equol monosulfate	7.00 ± 1.15	ND	1.43 ± 0.71	ND[3]
Equol disulfate	8.25 ± 3.75	ND	0.64 ± 0.20	ND[3]

[1]Average intake of isoflavones was 0.62 mg genistein and 0.82 mg daidzein/kg BW.

[2]C_{max} = the maximum plasma concentration; T_{max} = time to achieving C_{max}.

[3]ND = not detected.

The first indication that dietary isoflavones may be absorbed by a felid species was provided by Setchell et al. (1987). Cheetahs (*Acinonyx jubatus*) consuming a diet containing soy protein produced urine with a number of unidentified phenol-like compounds in the unconjugated steroid fraction (Setchell et al., 1987). These compounds were thought to represent metabolites of genistein and daidzein, which were found in the diet (Setchell et al., 1987), but the urinary compounds were not positively identified. Likewise, Brown et al. (1994) provided indirect evidence that domestic cats may be capable of converting daidzein into a more biologically potent form, equol, through their incidental finding of this metabolite in domestic cat feces. More recently, quantification of the relative proportions of unconjugated and conjugated genistein in cat plasma showed that, contrary to previous hypotheses, cats do indeed have capacity for the conjugation of genistein although the specific metabolites were not identified (Bell et al., 2006a; Cave et al., 2007a).

In agreement with the findings of Cave et al. (2007a), both genistein and daidzein appeared in detectable concentrations in the plasma of adult domestic cats after ingestion of an isoflavone-containing tablet. Furthermore, the current study has also confirmed the ability of dietary isoflavones to cross the intestinal barrier of the cat when ingested as part of a commercially available feline diet.

Table 4. Comparative incidence of the detection of isoflavone metabolites in the plasma of cats fed either a soy extract tablet (Exp. 1) or a diet containing isoflavones (Exp. 2)[1]

Metabolite	Exp. 1 (soy extract, $n = 6$)	Exp. 2 (dietary matrix, $n = 4$)
Unconjugated genistein	83.3	25[2]
Unconjugated daidzein	83.3	25[2]
Glucuronide from genistein or daidzein	0	0
Genistein monosulfate	100	100
Genistein disulfate	0	0
Daidzein monosulfate	100	100
Daidzein disulfate	100	75
Unconjugated equol	0	0
Equol monosulfate	0	100
Equol disulfate	0	75
Dihydrodaidzein	0	25[2]
O-desmethylangolensin	0	25[2]
Mean proportion of plasma genistein metabolites conjugated at 12 h	99.9	100
Mean proportion of plasma daidzein metabolites conjugated at 12 h	99.9	100

[1]Values are expressed as the percentage of cats detected with each metabolite present in their plasma at 1 or more time points where the number detected vs. the total number of cats is shown in parentheses.

[2]One cat exhibited a divergent metabolite profile to other cats in Exp. 2; therefore, these results should be interpreted with caution.

After hydrolysis in the gastrointestinal tract and deconjugation of dietary isoflavone glycosides, isoflavones are absorbed in their unconjugated state (Setchell et al., 2002). In humans and rodents, isoflavone metabolism typically involves conjugation (Coldham et al., 1999; Setchell, 2000). This phase II type of metabolism has been shown to occur in both the enterocyte and hepatocyte, with β-glucuronides being the predominant conjugate species (Setchell, 2000). However, isoflavone sulfates are also formed in male rats (Coldham et al., 1999). In common with humans and rodents (Joannou et al., 1995; Cimino et al., 1999; Heinonen et al., 1999), the daidzein metabolites, dihydrodaidzein, equol, and *O*-DMA, were detected in feline plasma along with sulfoconjugates, unconjugated genistein, and daidzein. The presence of other potential isoflavone metabolites, such as dihydrogenistein, 6′-hydroxy-*O*-DMA, 4-hydroxyphenyl-2-propionic acid, *p*-ethyl-phenol, tetrahydrodaidzein, 4-OH-equol, and 2-dehydro-*O*-DMA, was not investigated.

The lack of detectable β-glucuronide conjugates in the plasma of domestic cats in the present 2 experiments is in agreement with previous findings of a poor glucuronidation capacity (Robinson and Williams, 1958). Indeed, conjugation to form β-glucuronides is known to be nonfunctional in the cat against a large number of substrates, including phenols (Capel et al., 1972, 1974; Court and Greenblatt, 2000). However, before the current study, no information was available regarding the glucuronidation activity toward isoflavones in the cat. Hence, a number of authors (Setchell et al., 1987; Court and Freeman, 2002) have speculated that the reduced capacity of cats to form β-glucuronides may render them unable to conjugate isoflavones. This, in turn, has been hypothesized to translate into a greater susceptibility of felids to the biological effects

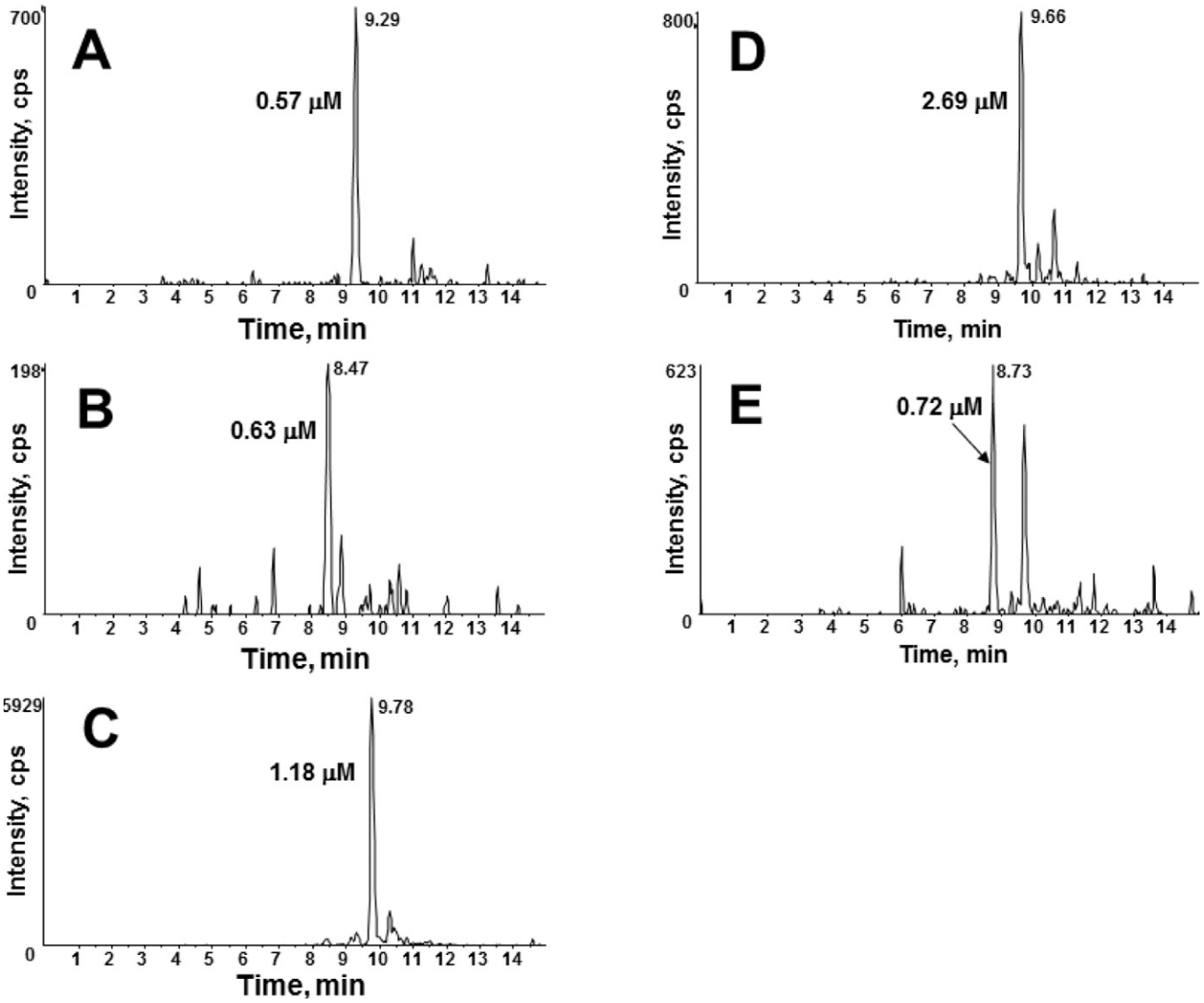

Figure 5. Individual multiple reaction ion-monitoring channels for plasma isoflavone sulfate conjugates in a cat fed a commercial isoflavone-containing feline diet. (A) Daidzein monosulfate, (B) daidzein disulfate, (C) genistein monosulfate, (D) equol monosulfate, and (E) equol disulfate. The concentrations (µmol/L) of each isoflavone sulfate is given on the left side of each peak. cps = counts per second.

of isoflavones because the unconjugated forms are considered to have greater biological potency (Kinjo et al., 2004). Furthermore, the excretion or enterohepatic recirculation of isoflavones, or both, may depend, to some extent, on the manner in which isoflavones are metabolized (Sfakianos et al., 1997).

However, the findings of this study provide the first evidence that domestic cats are capable of considerable metabolism of ingested isoflavones. Indeed, the bulk of the detected isoflavones in feline plasma were present as conjugates, with only minimal proportions found in an unconjugated form. Over 99% of the isoflavones detected were conjugated at all time points, indicating that the conjugation process is sufficient to keep up with the rate of absorption achieved in the present study. Hence, assuming that absorption rate in cats is similar to that of other species, felids appear to have a conjugation capacity comparable to other species, at least when exposed to an acute dose of isoflavones. It can, therefore, be expected that cats have a susceptibility similar to the physiological changes observed after acute isoflavone exposure in other species although the metabolic capacity after chronic exposure was not investigated and no changes in physiological variables were investigated in the current study.

The metabolite profile of feline plasma reported in the present study indicates that phase II metabolism of genistein and daidzein involves conjugation with only sulfate rather than the typically glucuronide-dominant pathways observed in other species. The family of enzymes responsible for uridine diphosphate-glucuronosyltransferase (**UGT**) activity is nonetheless expressed in the cat liver (Court and Greenblatt, 1997, 2000). However, the range of isoforms is considerably limited, and UGT activity is considerably less in the cat than other mammalian species (Hietanen and Vainoo, 1973; Court and Greenblatt, 1997, 2000). Our findings support the notion that isoflavones are conjugated to a sulfate moiety and subsequently excreted in this form.

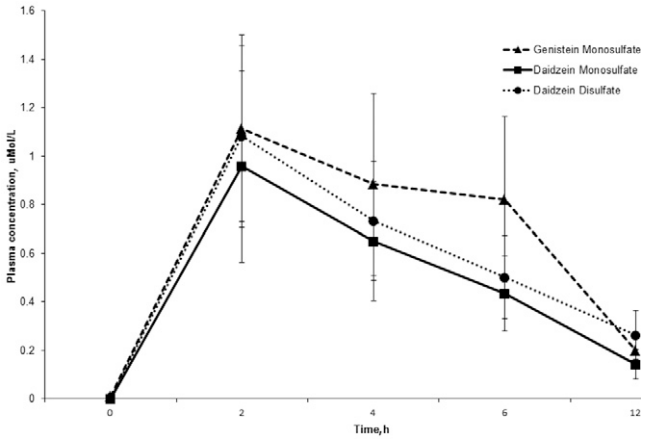

Figure 6. Mean plasma concentration of 3 major metabolites identified in domestic cats ($n = 4$) after exposure to a commercially prepared feline diet containing 151 µg genistein and 198 µg daidzein/g DM. Average intake was 0.62 mg genistein and 0.82 mg daidzein/kg BW.

Sulfotransferases (**SULT**) are ubiquitous in mammalian cells but highly concentrated in the liver where they are responsible for conjugation of hydrophobic molecules (Shangari et al., 2005). In other species, it is thought that SULT work in a complementary manner with UGT, providing an efficient "back-up" for conjugating molecules when glucuronidating enzyme activity is reduced or saturated (Goon and Klaasson, 1990; Shangari et al., 2005). Dose–response differences in metabolite profiles were not investigated but may be of interest for future research.

Sulfation is generally more easily saturated than glucuronidation (Goon and Klaasson, 1990), and increased isoflavone intake by cats may result in disproportionately increased unconjugated isoflavones, which has implications for the potential biological activity of these compounds in the cat. Furthermore, unlike other species, in which glucuronidation and sulfation would both need to be impaired, changes in sulfation capacity (e.g., via polymorphisms or dysfunctional metabolism) in individual cats is likely to infer greater physiological consequence than would be predicted in other species.

Some differences in metabolite profiles were observed after ingestion of 2 divergent forms of isoflavones. In general, the efficiency of isoflavone conjugation was nearly identical in Exp. 1 and 2 when the proportion of conjugated metabolites at the 12 h sampling point was compared. However, a much greater number of cats ingesting soy extract were shown to have detectable concentrations of unconjugated genistein and daidzein at any time point compared with cats consuming an isoflavone-containing diet. This indicates that there may be differences in bioavailability or enzyme adaptation, or both, depending on the type of isoflavone ingested, or the duration of exposure.

Alternatively, provision of isoflavones as a soy extract tablet may have resulted in a more rapid absorption (which was not detectable within our sampling regimen) of isoflavones, thus resulting in an earlier saturation of conjugating pathways. However, the absolute values reported after soy extract supplementation should be interpreted with caution because mixing of the tablet may not have been complete, and variation in esophageal transit of the tablets may have resulted in the isoflavones entering the stomach or intestines before the bulk of the meal. However, variable esophageal transit is unlikely to have had an effect, and the limited sampling regime was not appropriate for the estimation of true pharmacokinetic variables or bioavailability.

Given the restricted sampling regime, differences in dose received via tablet and dietary administration, and the detection of metabolites close to the detection limit

of the assay, the observed differences between Exp. 1 and Exp. 2 should be interpreted with caution. Studies with extended and more frequent sampling regimens to enable comparison of concentrations rather than presence or absence are warranted.

A crucial role for intestinal microbiota has previously been demonstrated for the production of equol and *O*-DMA (Atkinson et al., 2005; Cassidy, 2005), and equol is thought to be produced in the distal intestine, most likely the colon (Setchell et al., 2003). The production of equol was only apparent in cats consuming isoflavones as part of a dietary matrix whereas this metabolite was not detected in cats exposed to soy extract tablets. Equol is known to possess greater affinity for estrogen receptor binding compared with daidzein in other species (Sathyamoorthy and Wang, 1997; Lehmann et al., 2005). Equol may, therefore, subsequently exert greater physiological influence than daidzein, so the finding that all cats (4) exposed to a daidzein-containing diet were capable of producing equol may have ramifications for the long-term reproductive physiology of domestic cats fed diets containing daidzein. However, there is a discordance between the estrogenic effects of equol in vitro or cell culture vs. its lack of estrogenic effect in vivo on the growth of human estrogen receptor-positive Michigan Cancer Foundation – 7 (**MCF-7**) cells in the ovariectomized nude mouse model (Ju et al., 2006). In this mouse model, expected extensive β-glucuronidation of equol may have substantially attenuated its estrogenic effects in vivo. It remains to be determined in felids whether sulfoconjugates of isoflavones and their metabolites retain estrogenic activity.

The 12-h sampling period used here was predicted to provide sufficient time for the detection of equol, but none of the 6 cats ingesting soy extract exhibited detectable concentrations of equol within this sampling regimen. This is in contrast to the 100% of cats (4 out of 4) consuming the isoflavone-containing diet that produced detectable equol by 12 h. Because equol was present in baseline samples from noncoprophagic cats adapted to an isoflavone-containing diet, some unforeseen overlap in plasma isoflavone content from previous meals is thought to have occurred, despite the 18-h food-withholding period before the trial.

It has been suggested that chronic exposure to daidzein may increase the equol-producing capacity of the gut microbiota of mammals (Hedlund et al., 2005). Certain gastrointestinal bacteria are known to be stimulated by exposure to isoflavones (De Boever et al., 2000; Clavel et al., 2005), such that prolonged intake has been associated with altered isoflavone metabolism or absorption, or both, compared with single bolus doses (Lu and Anderson, 1998; Hedlund et al., 2005). Hence, it is feasible that equol production was increased in Exp. 2 because of the adaptation period, in which the cats consumed the isoflavone diet before sampling. This is supported by the finding that equol was detectable in the baseline sample of the majority of cats in this study. Likewise, overlap from previously ingested meals may explain the earlier peak plasma concentrations observed in the present study (at approximately 8 h) compared with studies in humans, in which equol T_{max} was typically reported to occur after 24 h (Setchell et al., 2003).

Nonetheless, the finding that the greatest concentration of equol sulfates were detected at 8 h after ingestion of the study meal provides evidence that equol production was increased as a result of the study meal. This indicates some form of modulatory role for either the dietary matrix or the duration of daidzein exposure in the equol-producing capacity of domestic cats. However, it must be noted that the sampling regimen used in the present study was not sufficient to accurately predict the true T_{max} and further investigation of the pharmacokinetics of isoflavones in cats is warranted.

Interestingly, 1 cat in Exp. 2 failed to produce detectable concentrations of daidzein disulfate or equol disulfate, but this cat was also the only cat observed to have circulating concentrations of unconjugated daidzein. Moreover, this cat was unique in its production of dihydrodaidzein and *O*-DMA, indicating that daidzein metabolism differed in this individual compared with other cats. It is possible that the presence of daidzein or equol disulfate metabolites in this cat occurred in periods that were not sampled in this study because of the restricted sampling regime. However, the presence of these metabolites at sampled time points in all other cats in Exp. 2, combined with the unusual metabolite profile of this cat, indicates that study design alone cannot explain this finding.

A divergent activity of phase II metabolizing processes may be exhibited in this individual and may be associated with the ability to produce dihydrodaidzein and *O*-DMA. No known variables with the potential to alter gut microbiota in this cat were apparent (e.g., no antibiotics had been administered and coprophagia was not observed or suspected). However, this cat had the greatest intake per kilogram BW, which may indicate a degree of saturation in the phase II metabolizing process that was occurring and could explain the increased presence of unconjugated isoflavones.

In humans, only 30% of subjects challenged with soy isoflavones are equol producers, which has been attributed to the presence of specific gut microorganisms in individuals (Setchell et al., 2003), such that the microbiota of this cat may have differed to others in the study. Alternatively, the presence of a SNP in the SULT of this cat cannot be ruled out. In humans, these

SNP confer functional consequences on the translated proteins (Nowell and Falany, 2006), which may explain the observed metabolic profile in this individual although the confirmation of this was beyond the scope of this study.

In summary, the identification of isoflavone metabolites in the plasma of domestic cats supplemented with soy isoflavone or fed a diet containing isoflavones from soy may have been described for the first time. The potential for conversion of daidzein to equol has also been demonstrated in this species, and an influence of dietary matrix or exposure duration in the production of this metabolite may be indicated. Cats have an adequate capacity to metabolize isoflavones to sulfate conjugates, which is in contrast to previous hypotheses (Setchell et al., 1987; Court and Freeman, 2002). Furthermore, the metabolic capacity for isoflavone detoxification by domestic cats appears to be efficient, with only minimal proportions of the circulating isoflavones detected in their unconjugated forms. Unlike all other species studied to date, cats do not appear to conjugate isoflavones to glucuronide moieties, relying solely on sulfation instead. These findings have implications for the potential for isoflavones to exert physiological activity in the cat. It is unlikely that cats are rendered more susceptible to isoflavone biological activity than other mammals because of their metabolic differences.

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