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Determination of rat oral bioavailability of soy-derived phytoestrogens using an automated on-column extraction procedure and electrospray tandem mass spectrometry

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Abstract

In recent years, consumption of herbal supplements as an alternative to pharmaceutical drug therapy has increased. For example, with the health claims labeling which describes the link between soy-protein and a reduced risk of coronary heart disease (CHD), the consumption of soy and soy-derived phytoestrogens has increased dramatically. That being said, the oral bioavailability of only a few soy phytoestrogens such as Daidzein and Genestein have been previously estimated. In this paper, we present the calculated percent of rat oral bioavailability of five soy-derived phytoestrogens (Genistein, Daidzein, Biochanin A, Coumestrol, and Zearalenone) in male Sprague–Dawley rats. The plasma quantitation required for the bioavailability calculation is performed by using a rapid on-line plasma extraction procedure for the quantitative analysis. To further speed up the analysis the rats were dosed using the 'n-in-one' (cassette) protocol. The rapid on-line extraction/quantitation methodology coupled to the cassette dosing analysis of phytoestrogens is the key point of this paper. The limit of quantitation (LOQ) for each compound was 1–1000 ng/ml with each plasma sample analysis taking less than 2 min. In general the percent oral bioavailability was determined to be between 11 and 28%.

Keywords: Phytoestrogens; Plasma quantitation; Oral bioavailability

1. Introduction

1.1. Soy phytoestrogen research

In recent years, consumption of herbal supplements as an alternative to traditional drug therapy [1] hormone replacement [2–8] or diet aid (e.g. The Soy Zone, Chapter 10 and 45 references therein [9]) has

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increased dramatically. On 26 October 1999, the FDA approved health claims labeling which describes the link between soy-protein and a reduced risk of coronary heart disease (CHD) [10]. This assumes that one is able to consume a minimum of 25 g of soy-protein per day. Since that date one cannot go to a drug or food store without seeing aisles of soy-based or soy-derived products with claims about all aspects of ailments associated with the aging process including Type II Diabetes [11] and Alzheimers Disease [12]. One reason for these claims is the presence, in these products, of natural estrogenic compounds known as isoflavanoids. These so-called "phytoestrogens" are naturally occurring and can have similar bioactivity to estrogen compounds. Much research has been reported describing the possible effects of the consumption of phytoestrogens [13–15]. Other examples of the effects of phytoestrogen consumption include the areas of cardiovascular disease [16] hormone replacement therapy [17,18], memory loss [19], osteoporosis [20,21], the stimulation or reduction of hormone dependent cancers (e.g. breast and prostate) [22–25], chronic renal disease [26], and brain structure [27] and function [28] (including pain management) [29]. There have even been unsubstantiated reports that hop-based phytoestrogens [30,31] can be used as a means to increase breast size [32]. All of these effects attributed to phytoestrogens are interesting considering that these compounds have been estimated to have a binding affinity to the estrogen receptor alpha up to 30,000-fold lower (e.g. Biochanin A) as compared to the endogenous estrogen estradiol [33,34].

Postmenopausal women have an increased risk of coronary heart disease. Oxidation of low-density lipoprotein (LDL) has been implicated in atherogenesis, and the presence of modified LDL (LDL (−)) in plasma appears to represent LDL oxidation in vivo [35]. Previous studies have also demonstrated a strong antiatherogenic effect of estrogen due to its antioxidant activity and similar antioxidant activity has been observed for specific isoflavones. Thus, the antioxidant activity of phytoestrogen extracts derived from soy and alfalfa was also studied and the protective effect of these extracts was attributed to the presence of flavonoids [36]. In addition, Setchell published a supplement describing the dozens of positive effects of phytoestrogens since it is accepted that many of the major diseases of Western populations are hormone dependent [37].

Consumption of soy milk in the US alone has increased from US$ 2 million in 1980 to over US$ 300 million by 2000 [38]. Nevertheless, there are now many reports suggesting that the effects of a high soy-protein diet, rich in phytoestrogens, may have little or no positive health benefit whatsoever [39,40]. In fact, a recent review implicates soy and soy-based products as the major cause of many of the worlds most significant health problems [41,42]. For example, there are reports which link potential reproductive problems to the consumption of soy-based infant formula early in life [43–45]. A similar report indicates that phytoestrogens circulate at concentrations that are 13,000–22,000 times higher than plasma estrogen concentrations in soy-formula fed infants which typically range from 40 to 80 pg/ml in early life [46]. With this being said, there have been few reports of toxicity due to the high intake of phytoestrogen compounds. One such report suggests a soy diet containing Genistein may stimulate the growth of estrogen dependent tumors in a dose dependent manner [47]. Another report published in JAMA indicated that in a study of over 800 men and women, no statistically significant differences were observed between groups of men or women, for over 30 outcomes followed. Women who were fed soy-formula reported slightly longer duration of menstrual bleeding (adjusted mean difference was 0.37 days) [48]. There are reports indicate the bioavailability of soy isoflavones, especially Genestein and Daidzein, as well as a tabulation of total isoflavones for a series of 33 different commercially available soy supplements [49]. With the constant emergence of negative or inconclusive scientific evidence [50,51] we felt that an investigation of the oral bioavailability of phytoestrogen compounds would be an interesting and important scientific endeavor. In this report, we determine the percent oral bioavailability of five soy-derived phytoestrogen aglycones [52].

1.2. Pharmaceutical properties

One important aspect of this manuscript is the ability to rapidly evaluate the pharmacokinetic properties of any new chemical entity (NCE) prior to advancement into development track. Traditionally, the investigation of pharmaceutical properties, such

as permeability, solubility, metabolic stability, etc. has occurred during the development phase of a potential NCE. Over the years, however, more than 40% of drug candidates have failed in development due to poor biopharmaceutical properties [53,54]. Thus many Discovery programs have unkertaken the task of evaluating the pharmaceutical properties of potential NCE's so as to increase the understanding of a series of compounds. In an effort to increase the likelihood of selecting a compound for development that will make it through the pipeline, a number of recent reviews have evaluated this new pre-clinical paradigm [55,56]. The primary issues discussed recently have been in the areas of ADME/Tox [57], more specifically the areas of metabolic stability [58–61], cytochrome P450 inhibition (and induction) [62] and absorption [63]. With all of these new assays to assess the pharmaceutical properties of potential NCE's comes the bottleneck of rapid quantitative analysis of a large number of new samples [64,65]. Recently, Kerns reviewed several assays currently used by the pharmaceutical industry to evaluate the pharmaceutical properties of potential NCE's [66]. In addition, Kerns has published on the use of multivariate analysis to evaluate structure activity (SAR) data along with structure property (SPR) data to best drive the synthetic optimization efforts of drug discovery [67].

1.3. Plasma quantitation methods

Another significant challenge to most NCE's is in the area of oral bioavailability. It is for this reason that more and more in vivo exposure animal studies are being performed in discovery, thus, improving the likelihood that a candidate with good ADME properties will be selected for advancement into development [68,69]. In addition to the increase in number of compounds for investigation, has come the need to reduce the number of animals used in pharmaceutical research. This need has recently been addressed through the novel approach of cassette or so-called "n-in-one" dosing [70]. One obvious limitation of the "n-in-one approach" for wider application, such as definitive ADME studies, is the inability to determine which compound may have produced a metabolite. In a Drug Discovery environment, however, as many as 10 compounds can be simultaneously dosed in a single animal, decreasing the number of animals used, while increasing the number of compounds investigated. In this paper we demonstrate the use of cassette dosing to investigate the percent rat oral bioavailability of the phytoestrogens discussed above.

One issue related to the rapid evaluation of multiple compounds is the mounting number of plasma samples for quantitation. A wide variety of research on the topic of rapid or high-throughput quantitation has produced a variety of methods. Examples range from automated liquid–liquid [71,72], solid-phase [73,74], direct injection (so-called "dilute and shoot") [75], and on-line extraction using a restricted access media column [76]. In fact, several recent reviews have been written which discuss the relative merits of these on-line/off-line high throughput extraction and mass spectrometry techniques as well as their impact on pharmacokinetic (PK) studies performed in drug discovery [77–80]. One very simple but elegant method employs plasma pooling as a means to increase the throughput of in vivo samples [81]. The area under the curve (AUC) observed using this method differed up to 15% from those calculated when sample pooling was not performed, making this a very useful and viable option. Another alternative is to transfer traditional solid-phase extraction (SPE) cartridge techniques to 96 or 384-well solid-phase extraction plates [82,83]. Still another is the use of repetitive injection packed column supercritical fluid chromatography MS/MS analysis. With this technique a 96-well plate has reportedly been analyzed in as little as 10 min [84]. In all cases, there is still likely the need for a lengthy clean-up, drying and reconstitution of the sample plate (or sample). In most cases the dynamic range of quantitation was approximately two orders of magnitude with a limit of quantitation (LOQ) of 5 ng/ml [85].

More recently, other rapid methods for the on-line quantitation of plasma have been published. One published on-line procedure uses turbulent flow chromatography to perform the extraction of pharmaceutical compounds from plasma. This was accomplished using high flow rates and large particle size stationary phases. A similar approach to the turbulent flow system was published using a dual HPLC, multi-valve system [86,87]. Several papers describing the use of similar setups have also been published for single component analysis [88,89]. In one such paper, a 100% recovery was reported versus a 75.1% recovery

when acetonitrile protein precipitation was used for sample extraction [90].

Herein we report a rapid method for the quantitation of a series of phytoestrogens from blood plasma after oral or subcutaneous (SC) administration in male Sprague–Dawley rats. This automated method is based upon the use of rapid on-column solid-phase extraction coupled with traditional reverse phase chromatography previously described by Jemal et al. [86,87]. In general, as little as 20 μl of plasma is combined with an equal volume of internal standard dissolved in an appropriate solvent (usually water). This sample is then placed in the HPLC autosampler, where approximately 10 μl is injected. In this paper, we show the ability to quantitate five different phytoestrogens after cassette dosing of male Sprague–Dawley rats at 3 mg/kg per compound. The range of quantitation is 1–1000 ng/ml with a detection limit of approximately 100 pg/ml. In addition to the quantitative analysis, all pertinent pharmacokinetic parameters are determined, including (but not limited to) the percent oral bioavailability (in rats) of each of the soy phytoestrogens investigated.

2. Experimental

In general, the animals were dosed either SC or orally (PO), blood was drawn at particular time points, the plasma was spun down and analyzed using an on-line extraction/quantitation procedure. A description of the instrumentation and chemicals used is presented below. The details of the extraction procedure are discusssed in Section 3.

2.1. Chemicals

All HPLC solvents (acetonitrile, triethylamine, and dimethylsulfoxide) were purchased through VWR Scientific Products Inc. The 18.2 MΩ HPLC water was produced using a Milli-Q water polishing system from Millipore Inc. The phytoestrogens Daidzein (D7802), Zearalenone (Z2125), and Genistein (G6776), were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification (see Fig. 1 for structure of evaluated compounds). Biochanin A (14,563–14,567) was purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. Coumestrol (27885) was purchased from Fluka Chemie (a division of Sigma–Aldrich; Steinheim, Germany) and used without further purification. The internal standard used in this study (*n*-(3-chloro-4-fluoro-phenyl)-2-morpholin-4-yl-kacetamide) was purchased from the Sigma–Aldrich Library of Rare Chemicals (R256625; Milwaukee, WI) and used without further purification. This internal standard was chosen primarily due to experience with this compound in previous discovery programs. While it bears only some resemblance to the compounds of interest, it is well behaved and a good internal standard for the determination of extraction efficiency and instrument performance. The use of deuterated internal standards is of course preferred, but usually impractical in our discovery environment.

For animal dosing, each compound was dissolved in 2% Tween-80, 0.5% methyl cellulose and administered to each animal at a final concentration of 3 mg/kg per compound (total concentration of 15 mg/kg).

2.2. Animal dosing

Male Sprague–Dawley rats (ca. 200 g) were used for this study. The animals were dosed either subcutaneously or orally to determine the percent oral bioavailability for each of the five phytoestrogens. After the animals were dosed, blood was drawn from three rats at each time point. Blood was collected via cardiac puncture after CO_2 asphyxiation. All animal procedures were performed in accordance with Institutional Animal Care and Use Committee guidelines and followed approved protocols. The SC route of administration was initially chosen since it was easily accomplished with the rats that were available to our laboratory. Also, with the compounds investigated, SC typically showed >90% of the quantitative area under the curve of an IV administration.[1] The time points were 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h, using a total of 24 rats per administration. In comparison, one would need to use 120 rats if each phytoestrogen was evaluated separately (i.e. five compounds times 24 rats). Thus 48 rats were used to accomplish our

[1] The comparison between IV and SC dosing occurred internally with proprietary Wyeth compounds and therefore is not presented herein.

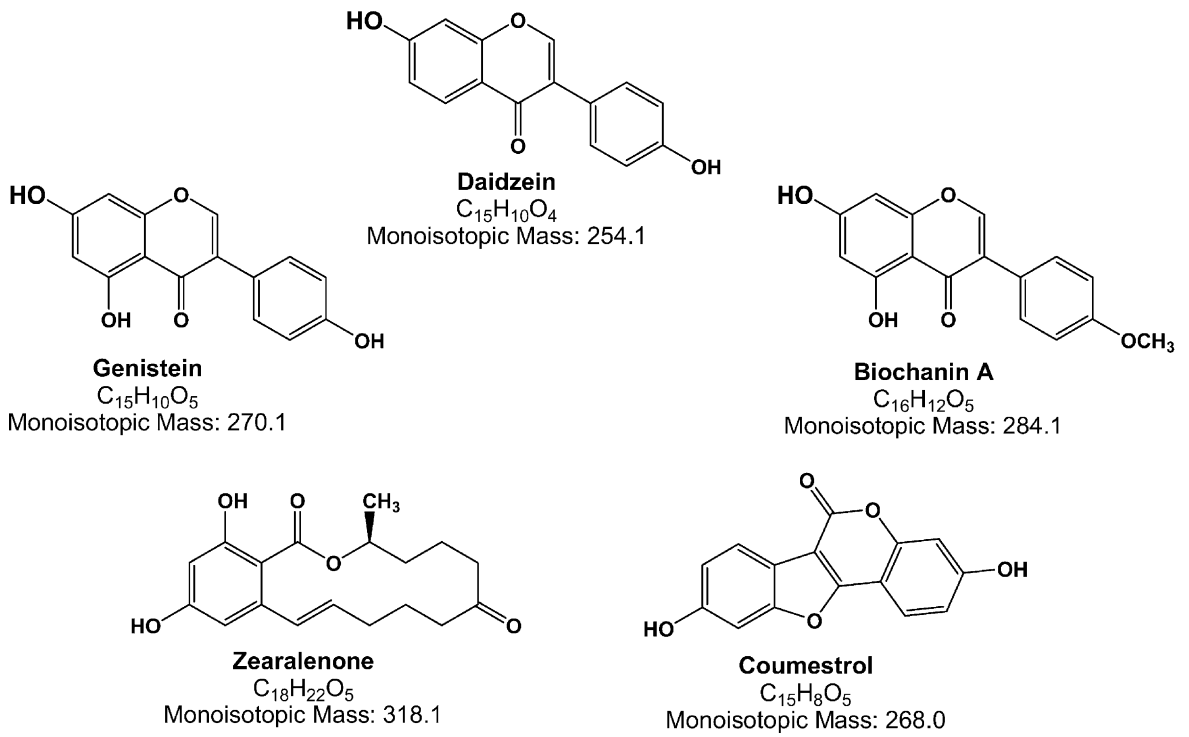

Fig. 1. Structure, formula, and monoisotopic mass for the phytoestrogens investigated within this manuscript.

investigation in comparison to 240 if evaluated as individual NCE's, representing a savings of 192 animals.

Once the blood was drawn (ca. 1 ml), 100 μl of 0.5 mM EDTA was added to the blood, the blood centrifuged (at 1000 × *g*) for 15 min, and the supernatant plasma was collected. The plasma was then frozen at −80 °C until thawed for analysis. Prior to analysis using mass spectrometry, 100 μl of plasma was combined with 100 μl of the internal standard (see mass spectrometry quantitation for more details). The internal standard, described above, was dissolved in 18 MΩ water to a final concentration of 200 ng/ml.

2.3. Mass spectrometry quantitation

All plasma samples were analyzed using a Waters (Micromass) Quattro Ultima tandem quadrupole mass spectrometer equipped with a Z-spray electrospray interface. To perform the quantitation, MS/MS transitions were determined to maximize the sensitivity and selectivity for each compound. The instrument was run in the negative ion mode using a technique called Multiple Reaction Monitoring (MRM). With MRM all five compounds can be quantitatively evaluated simultaneously. To correct for any potential changes in the extraction procedure or mass spectrometer performance, an internal standard was used (see Section 2.2). The transitions used for the MRM quantitative analysis are described in Section 3 and are presented in Table 1. In general, the conditions used for all analyses were the same and are summarized here. The

Table 1
List of the phytoestrogens analyzed, the precursor and product ions used for MRM quantitation, along with the optimum cone and collision energies for each compound

Compound	Precursor ion	Product ion	Cone voltage	Collision energy
Genistein	269	133	40	35
Zearalenone	317	131	70	30
Coumestrol	267	91	40	48
Biochanin A	283	211	30	35
Daidzein	253	91	40	40
Internal standard	271	144	30	25

electrospray capillary voltage was set to 2750 V, while the source block and desolvation temperatures were set to 120 and 350 °C, respectively. The desolvation and cone gas flows were set to 700 and 60 l/h, respectively. The LM and HM mass resolution was set to 10.0 and 10.0 for the first quadrupole, and 12.0 and 12.0 for third quadrupole, respectively. The LM and HM values are lower to allow for greater sensitivity for each MRM transition. Although it is possible that interferences can be detected due to such low resolution value settings, each phytoestrogen was analyzed separately to determine whether carry-over would be observed in the other MRM channels. In general, no significant carry-over was observed for any of the phytoestrogens examined. The ion energy was set to 1.0 V for the first quadrupole and 2.0 V for the third quadrupole; the multiplier was set to 650 V. The MRM dwell time was set to 0.08 s per transition with a 0.02 s interscan delay for a total cycle time of 0.60 s per compound. This enabled the acquisition of at least 10 data points per compound for peaks that are at least 0.1 min wide. All samples were analyzed in the negative ion mode. Since the cone and collision energy were set to optimize sensitivity, those settings are also presented in Table 1. The software used for these methods was Masslynx^{TM} version 3.4 equipped with Quanlynx^{TM} for quantitation.

Two HPLC were used for the on-line plasma extraction procedure. A Waters Alliance 2790 HPLC equipped with a 4-position multi-well plate carousel was used to inject and wash all plasma samples. In general, samples were placed into standard 2 ml HPLC vials and the autosampler carousel was set to utilize the standard 48-position 2 ml vial configuration. A mobile phase consisting of 0.02% triethylamine in 18.2 MΩ water running at a flow rate of 4 ml/min was used for component washing/trapping. The second HPLC, a Waters Alliance 2690, was used for the gradient elution of the trapped components from the Oasis cartridges. The mobile phases used for this system were 0.02% triethylamine dissolved in 18.2 MΩ water (A) and 0.02% triethylamine dissolved in HPLC grade acetonitrile (B). Both systems were connected via 0.010 ID (blue) PEEK (polyetheretherketone) polymer tubing (Upchurch Scientific, Oak Harbor, WA) to a 10-port valve which was timed to properly wash, trap, and elute the compounds of interest from the Oasis trapping column (Waters Corporation, Milford, MA; 2.1 × 20; 25 μm particle size; ambient temperature). An XTerra 3 mm × 30 mm (Waters Corporation, Milford, MA; 3.5 μm; 40 °C) column was also utilized to improve peak shape and chromatographic resolution. System extraction details are presented in Section 3.

A Rheodyne Lab-Pro 10-port, 2-column switching valve was used to switch between the two trapping columns. In general, the system was switched in a manner such that while one cartridge was being used for the extraction and quantitative analysis the second cartridge was being properly washed and equilibrated for the next run.

2.4. Standard curve sample preparation

The samples used to create the standard curve for each phytoestrogen were made using rat plasma purchased from Pel-Freez Biologicals Inc. (Rogers, AR). In general, the phytoestrogens were dissolved in DMSO at an initial concentration of 1 mg/ml and then further diluted to 10 μg/ml in rat plasma. Subsequently, the samples were diluted into rat plasma to an initial total concentration of 1000 ng per compound/ml. Since the dosing was performed using cassette dosing, the standard curve samples were prepared in a similar fashion to contain the same mixture of compounds. From the 1000 ng/ml solution, standards were created at 500, 200, 100, 50, 25, 10, 5, 2, and 1 ng/ml per compound and analyzed correspondingly using the LC/MS/MS technique described above.

3. Results and discussion

There are a few key points to this study. The first key point is the wider range of quantitation (three orders of magnitude) for each of the phytoestrogens evaluated than previously published. A second is the extension of this rapid on-column plasma clean-up and extraction procedure to the analysis of compounds evaluated through cassette dosing administration (i.e. six compounds extracted at once). The third key point of this study is the determination of the percent oral bioavailability (in Sprague–Dawley rats) of the five phytoestrogens presented in this manuscript. Therefore, the paper will be broken down into these parts.

3.1. LC/MS/MS extraction and quantitation procedure

As described in Section 2, two HPLC systems were used to perform the extraction and elution of the components of interest. Fig. 2A shows the first step of the quantitation procedure. In our system, the Waters Alliance 2790 HPLC was used to inject 10 μl of the prepared plasma sample. The compounds of interest were trapped onto the Oasis cartridge column and washed for 0.3 min at 4 ml/min, with a mobile phase consisting of 0.02% triethylamine in water to remove salts, proteins, and etc. from the plasma sample. The Rheodyne Lab-Pro 10-port valve was then switched to position "2" enabling the second HPLC (Waters Alliance 2690) to rapidly elute the compounds of interest from the Oasis cartridge. This reverse flow through the Oasis cartridge trapping column pushes the trapped compounds of interest onto the Waters XTerra column for chromatographic resolution and MRM quantitation using the Micromass Quattro Ultima tandem quadrupole mass spectrometer (see Fig. 2B). The elution of the trapped phytoestrogens through the Oasis cartridge and ultimately through the XTerra column was performed using the fast gradient described in Table 3.

The initial conditions of the gradient were 50% A and 50% B held for 0.2 min after which the gradient was ramped to 100% B in 1 min and held at 100% B for a total of 0.3 min (total run time of 1.5 min plus the initial 0.3 min wash cycle). During this stage of the procedure, the 2790 HPLC was constantly pumping through the second Oasis cartridge, switched to 100% B at 0.4 min, held at 100% B for 0.9 min, and returned to 100% A for equilibration of Oasis cartridge 2 for the next injection (all part of Fig. 2B; see also Table 2 for the 2790 HPLC gradient table). This is performed to properly wash and equilibrate the extraction cartridge prior to the next injection.

Table 2
Gradient used for the Waters 2790 HPLC and autosampler. This system is used to wash all plasma samples onto the Oasis cartridges

Time (min)	A (%)	B (%)	Curve	Flow rate
0	100	0	1	4.0
0.30	100	0	1	4.0
0.40	0	100	11	4.0
1.20	0	100	11	4.0
1.30	100	0	11	4.0
1.60	100	0	11	4.0
2.00	100	0	11	4.0

Table 3
Gradient used for the Waters 2690 HPLC. This system is used to elute all extracted components from the Oasis cartridges

Time (min)	A (%)	B (%)	Curve	Flow rate
0	50	50	1	1.0
0.20	50	50	1	1.0
1.20	0	100	6	1.0
1.5	50	50	11	1.0

The second sample was analyzed in a similar fashion with the plasma sample being injected onto the second Oasis cartridge and washed at 4 ml/min for 0.3 min (Fig. 2C). At that time, the 10-port valve was switched back to position "1" which allowed the 2690 HPLC to elute the trapped compound of interest. The reverse flow through the Oasis cartridge trapping column was eluted onto the Waters XTerra column for chromatographic resolution and MRM quantitation using the Micromass Quattro Ultima tandem quadrupole mass spectrometer (see Fig. 2D). The elution of the components of interest, through the Oasis cartridge and ultimately through the XTerra column, was performed using the same fast gradient which is described in Table 3. A chromatogram displaying the peaks observed for the cassette standard at 100 ng/ml of the five phytoestrogens, plus the internal standard, is shown in Fig. 3.

With this dual column setup, it is very important to use a reliable internal standard since the trapping efficiencies of the two Oasis cartridge columns could be different. Thus, the area response of the internal standard from the 72 injections performed during this analysis was plotted versus injection number (see Fig. 4). Only a 5% variability was observed from injection to injection. It is important to note that this variability is more than adequate when analyzing plasma samples in our rapid, high throughput, drug discovery environment. In addition, each of the five references standards were injected 10 times at a concentration of 100 ng/ml in plasma to evaluate the precision of the method (see Table 4 for details).

3.2. Percent oral bioavailability of five phytoestrogens

The concept of n-in-one (cassette) dosing was used to attempt to limit the number of animals necessary for

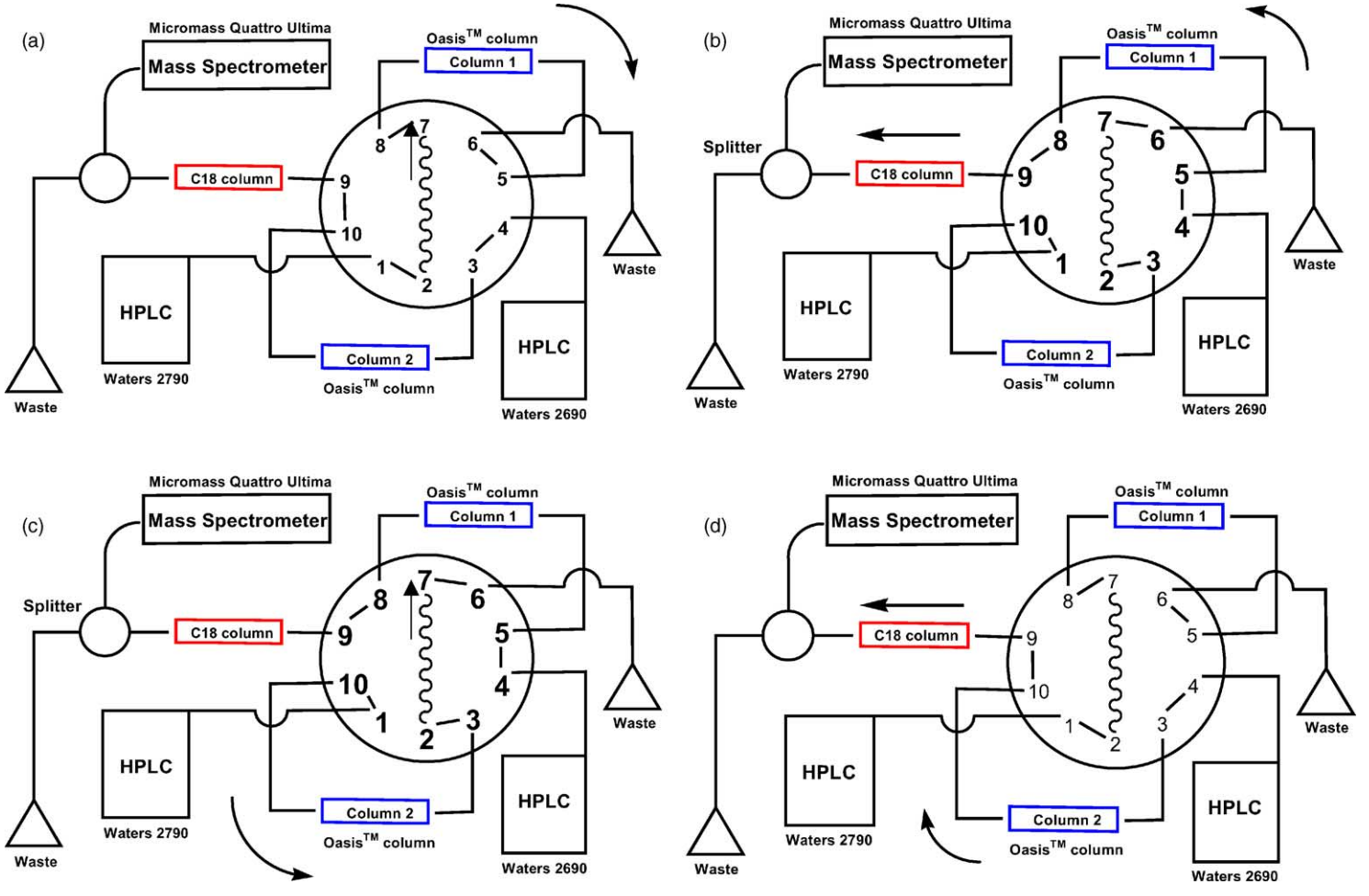

Fig. 2. Graphical schematic which describes the flow path for the on-line extraction/quantitation procedure. (A) The flow path of the first injection wherein the plasma sample is injected onto the first Oasis extraction cartridge. (B) The 10-port valve is switched, the flow path is reversed such that the compound of interest is back-flushed onto a high resolution C18 HPLC column, and the compound sent to the triple quadrupole mass spectrometer for quantitation. (C) The second injection onto the second Oasis cartridge begins while the first cartridge is being cleaned. (D) The 10-port valve is switched again, reversing the flow path on the second cartridge, backflushing the compound of interest onto the HPLC column, and the compound is again sent to the triple quadrupole for quantitation. The extraction, elution, and quantitation procedure for each sample takes approximately 2 min.

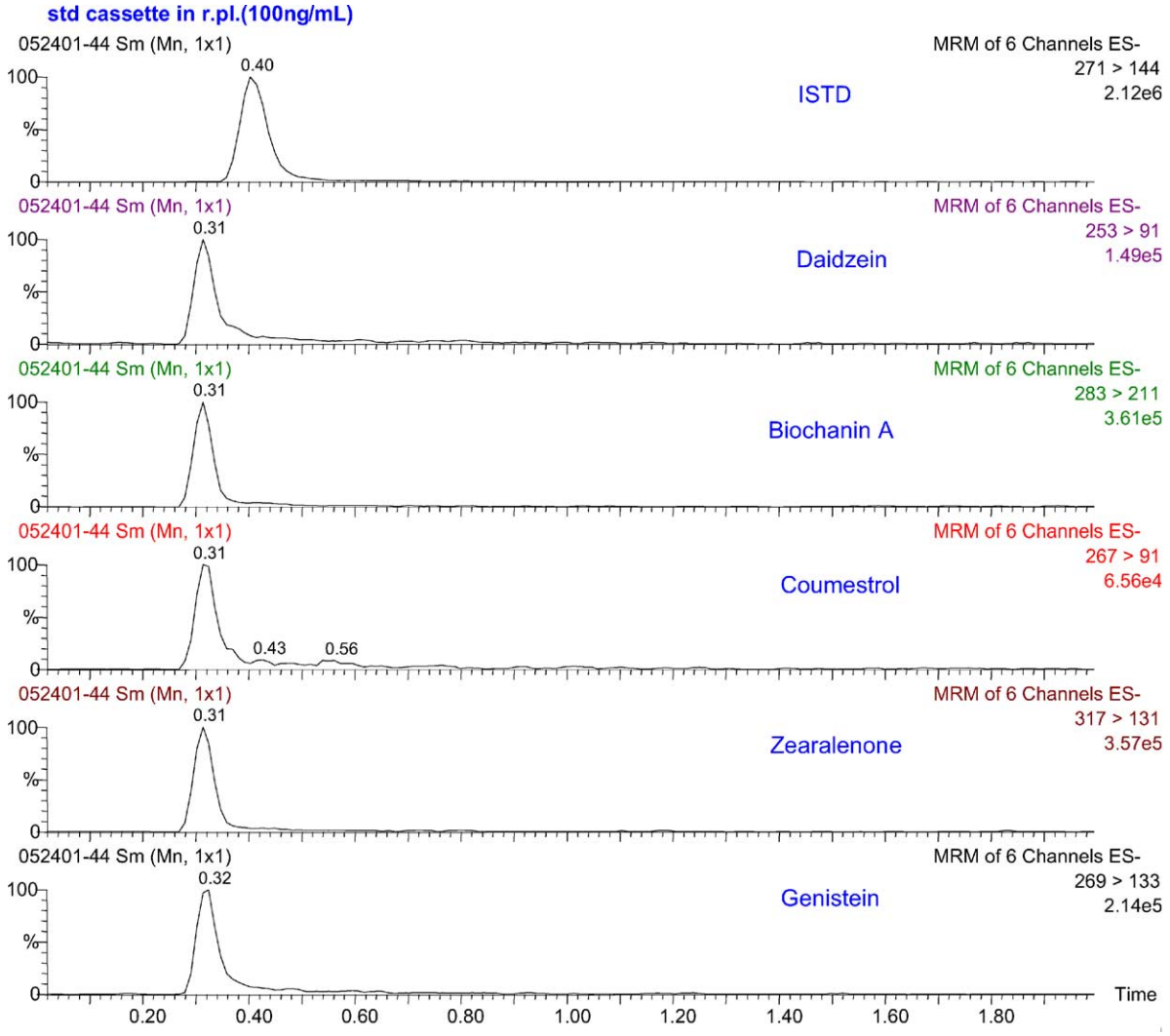

Fig. 3. Example of the LC/MS/MS chromatograms for the standard set at 100 ng/ml of each phytoestrogen in rat plasma in a cassette style sample.

this exposure determination. Each animal was dosed at 3 mg/kg per compound, either subcutaneous or orally depending on which arm of the study the animal was placed. As described above, the standards were made up in rat plasma and quantified in alternating scans identically as for the rat plasma samples. The quantitation curves for the five phytoestrogens investigated are shown in Fig. 5. The curves are plotted as the ratio of the area counts for the compound of interest to the area counts for the internal standard versus the proposed concentration of each reference standard. All five of these curves are represented as fairly linear relationships from 1 to 1000 ng/ml of plasma. The back calculated concentrations for all reference standards were off by less than 20% in all cases (criteria for this experiment). It is important that a standard curve be determined for each compound separately, however, each compound must be quantified in the presence of the other four (plus internal standard) in order to eliminate the possibility of ion suppression becoming

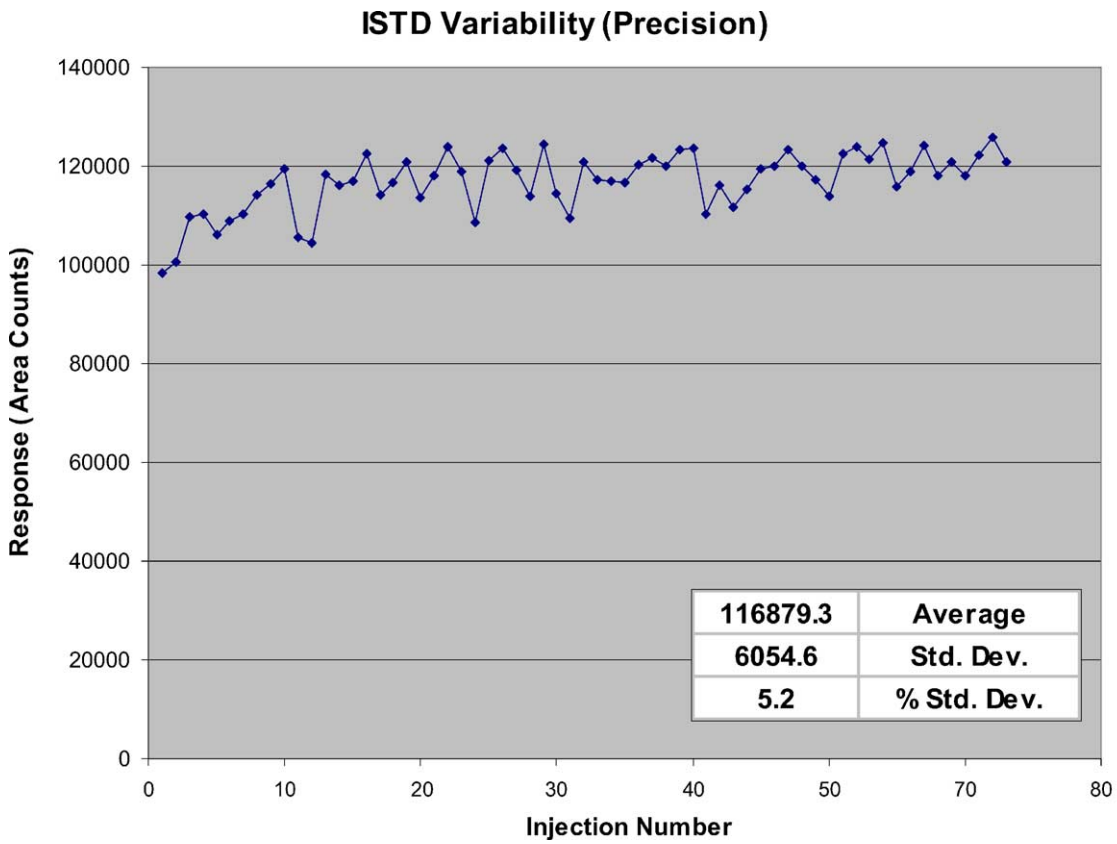

Fig. 4. Graph of area counts versus injection number for the internal standard employed during this study. Note that even though two separate extraction cartridges are used, the standard deviation is only ca. 5%.

Table 4
Area counts of each phytoestrogen standard for ten repeat injections. The standard deviation ranges from 4.6 to 10.3 percent even though two separate extraction cartridges are used. Correcting with the internal standard area counts, the standard deviation of the ratio of standard to internal standard area counts drops to ca. 5 percent

Injection no.	Daidzein	Biochanin A	Coumestrol	Zearalenone	Genistein
1	5330	11346	1804	11797	8133
2	5667	12436	2523	13107	7699
3	5497	11931	2165	13917	7047
4	6008	13313	2471	15139	7626
5	6307	12966	2099	13763	7980
6	5898	12720	2174	15165	7964
7	5883	12320	2570	13459	7217
8	6187	12991	2228	14343	9352
9	5894	12198	2106	13963	7595
10	6430	12760	2318	14392	8193
Average	5910	12498	2246	13905	7881
Standard deviation	347	579	232	991	638
Standard deviation (%)	5.9	4.6	10.3	7.1	8.1

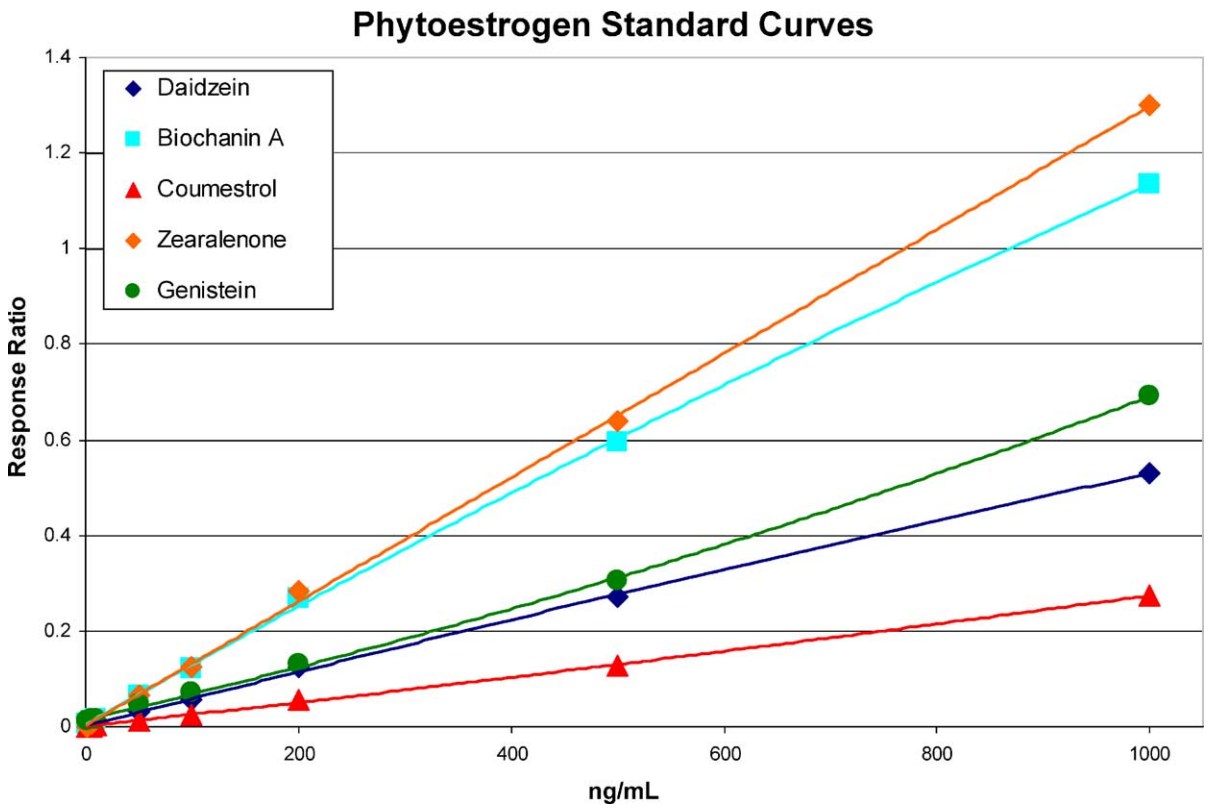

Fig. 5. Standard quantitation curves for the five phytoestrogens of interest. In each case the compounds were analyzed from 1 to 1000 ng/ml of plasma and the curves fit to either a linear, or second order polynomial curve with $1/X^2$ weighting. The R^2 values for each standard curve were determined to be 0.999 or greater.

a factor in the quantitation of the plasma samples. It is interesting to note that the ionization efficiency of each compound appears to vary and can be characterized as: Daidzein > Biochanin A ≫ Genistein > Zearalenone > Coumestrol. This is interpreted largely by the vast differences in the slope of each quantitation curve. Given the structural similarities, the authors would not have predicted that the ionization efficiency of these compounds would be so different. It is possible, however, that differences in ionization efficiency do not play a role but rather protein binding or possibly salt ion formation, thus limiting the production of the $[M - H]^-$ molecular ion of each compound.

Figs. 6 and 7 show the oral and subcutaneous pharmacokinetic curves for each of the five phytoestrogens of interest. In all cases, the curves in Fig. 6 seem to rise with time, fall off, and then rise again before dropping to the limit of quantitation (at around 24 h). This is tentatively described as enterohepatic recirculation [91] and is well known for these compounds. That is to say that isoflavones are known to be rapidly glucuronidated which could account for the apparent initial decrease in overall plasma concentration. These glucuronides are unstable and thus cleaved and released back into the blood compartment where they are eventually metabolized and eliminated. In Fig. 7, this same phenomenon is observed, although a bit more difficult to see due to the rapid appearance and disappearance of the phytoestrogens. For all compounds the C_{max}, T_{max}, $T_{1/2}$ lambda Z (elimination half-life), and AUC's are presented in Table 5. The values were determined using the software program WinNonLin Professional Version 3.1 (Pharsight Corporation, Mountain View, CA) The percent oral bioavailability is also presented in Table 5.

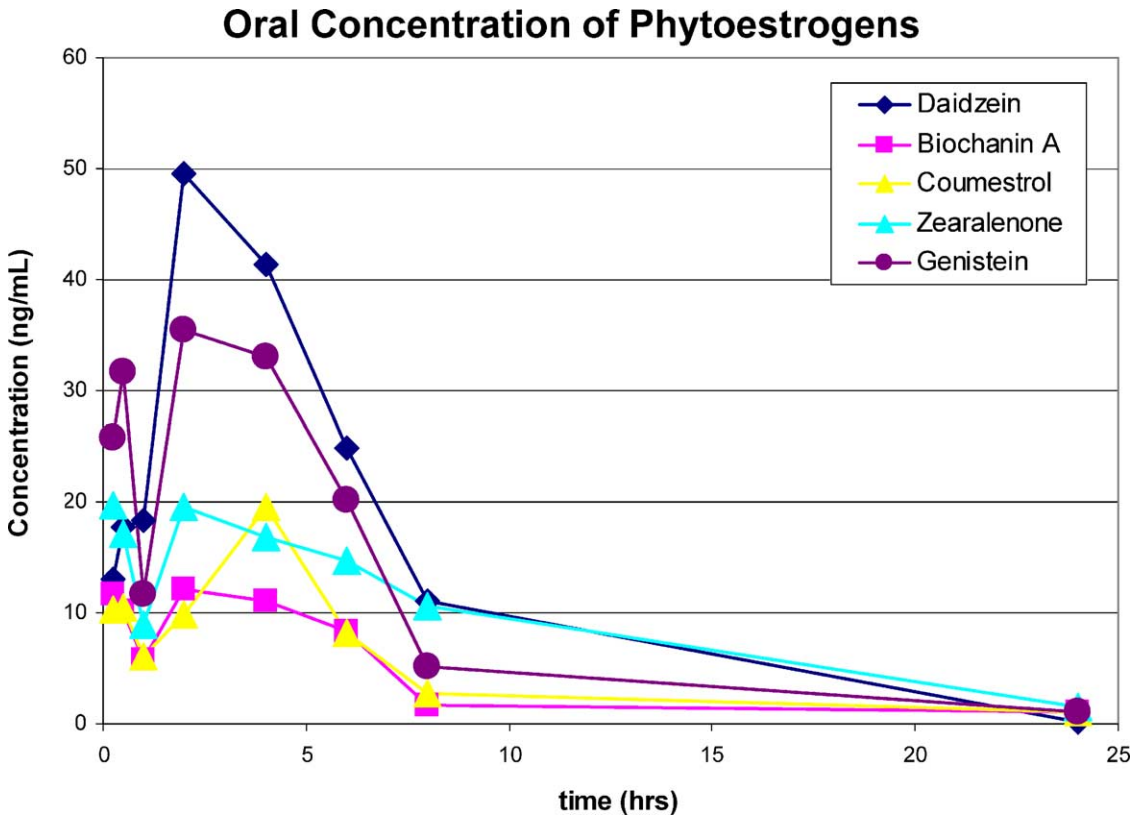

Fig. 6. The pharmacokinetic plots for the five phytoestrogens of interest after oral dosing. Note the dip in each plot suggesting the enterohepatic reuptake phenomenon.

In general, the AUC for the five compounds dosed orally followed the trend of Daidzein > Genistein > Zearalenone ≫ Coumestrol > Biochanin A. It is interesting to note that even though the AUC for Zearalenone is much greater than that for Coumestrol (ca. 80% greater), the C_{max} and elimination half-lives for these two compounds were observed to be identical (ca. 19.7 ng/ml and 5.6 h, respectively). This was primarily due to the rapid onset of the of the C_{max} of Zearalenone (i.e. T_{max} of Zearalenone was 0.25 h versus 4.0 h for Coumestrol).

The trend observed for the compounds of interest when dosed SC was observed to be Daidzein > Genistein > Coumestrol > Zearalenone >

Table 5
Pharmacokinetic parameters calculated for the oral and subcutaneous dosing of the phytoestrogens. The oral bioavailabilities are estimated to range from 11.7 to 28.1 percent

Phytoestrogen	T_{max} (h)		C_{max} (ng/ml)		$T_{1/2}$ lambda Z (h)		$AUC_{(0-24)}$ (ng h/ml)		$AUC_{(0-\infty)}$ (ng h/ml)		Oral bioavailability (%) $AUC_{oral}/AUC_{SC} \times 100$
	Oral	SC	Oral	SC	Oral	SC	Oral	SC	Oral	SC	
Genestein	2.0	0.25	35.5	962.7	4.3	2.9	227.3	1273.9	233.6	1274.3	18.3
Zearalenone	0.25	0.25	19.7	271.2	5.6	4.9	194.8	727.4	207.0	736.5	28.1
Coumestrol	4.0	0.25	19.6	140.8	5.5	3.1	109.5	987.7	117.5	1003.8	11.7
Daidzein	2.0	0.25	49.6	1120.0	2.3	5.3	275.0	1206.5	275.3	1210.4	22.7
Biochainn A	2.0	0.25	12.1	160.4	3.0	3.7	76.8	361.1	77.2	362.1	21.3

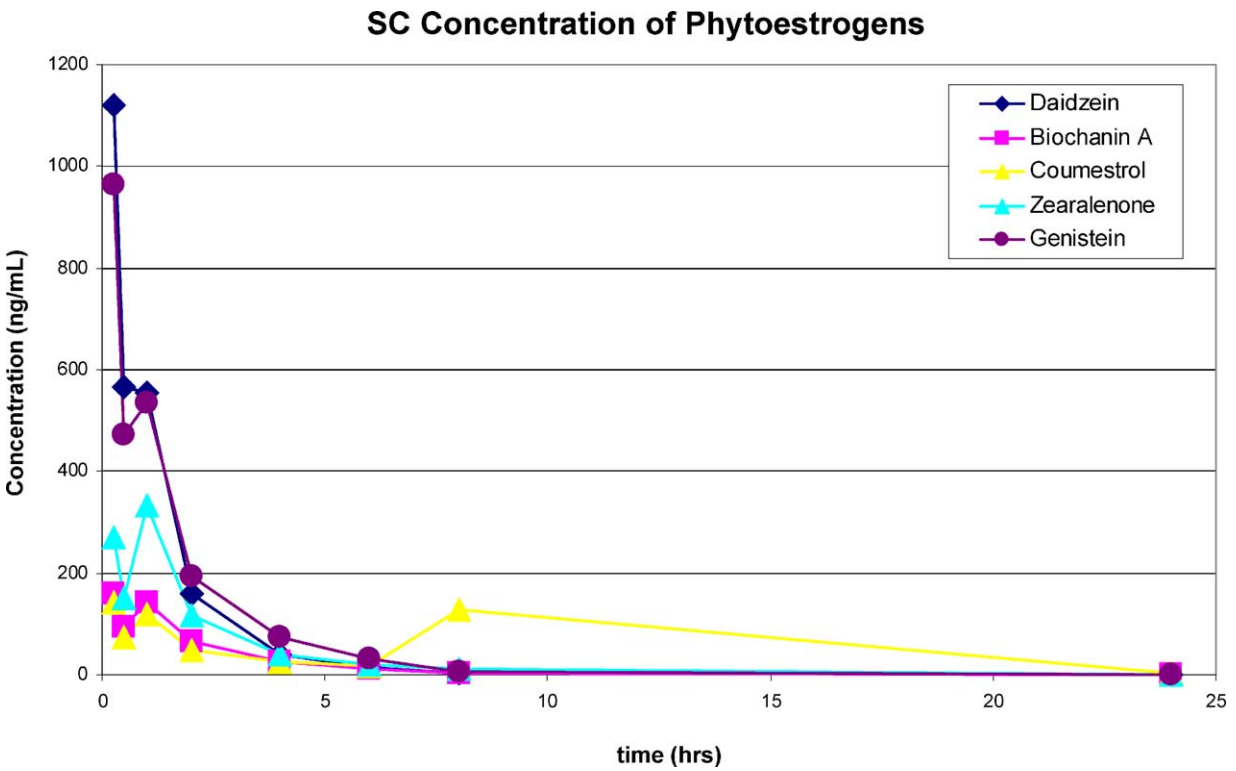

Fig. 7. The pharmacokinetic plots for the five phytoestrogens of interest after sub-cutaneous dosing. Note the dip in each plot also suggesting the enterohepatic reuptake phenomenon.

Biochanin A. Again, comparing Coumestrol with Zearalenone, although the C_{max} for Zearalenone is almost double that of Coumestrol, there appeared to be an increase in Coumestrol plasma level concentration at 8 h leading to the shorter apparent elimination half-life but a much higher AUC. There is no explaination as to why this increase occurred.

The percent oral bioavailability was calculated simply by the ratio of the AUC of the oral over the AUC of the SC dosing multiplied by 100%. This could be done since the rats were dosed at the same concentration in each case. In general the oral bioavailability for these five compounds ranged from ca. 12 to 28%. Specifically the trend observed for the percent oral bioavailability was Zearalenone > Daidzein > Biochanin A > Genistein > Coumestrol. While the value determined for Genestein using this rapid plasma quantitation method compared favorably with the literature data (ranging from 15 to 20% oral bioavailability in rats when dosed at 20 mg/kg/day orally) [92–94], the correlation between rat and human oral bioavailability is generally poor. That is primarily due to the broad range of reported human bioavailability of Genistein (5–37%) [95,96], or Daidzein (16–66%) [97,98]. In these examples, the doses administered ranged from 0.3 to 1.7 mg/kg/day [99].

4. Conclusions

To be able to perform this study, at 8 time points with two different dosing regiments, 48 rats were used. Blood was collected from three rats at each of the 8 time points, thus 24 plasma samples were created for each dosing regimen; 48 plasma samples were prepared for analysis. From the start of sample preparation to the final data analysis, all 48 samples were analyzed and the percent oral bioavailability estimated in 1 day. The quantitation by LC/MS/MS took 2 min per sample to complete this study; including blanks

and standards, 72 injections were made in 2.4 h. Since five compounds were quantified per injection, the per compound analysis time was 0.4 min per injection or 30 min per compound for the 72 injections. Yet with this improvement in speed of sample preparation and compound quantitation, the percent bioavailability determined was equivalent to that previously published [100]. The results of this manuscript suggest that this methodology should enhance the speed with which the pharmacokinetics of new chemical entities can be investigated within a drug discovery environment. In addition, we have shown that the method can be used to evaluate up to five compounds in a cassette dosing mode over a much wider quantitative range than previously reported.

One suggested area of further research is the effect of exposure to these phytoestrogens in early childhood [101]. Phytoestrogens have been reported to circulate in soy-formula fed infants at concentrations that are 13,000–22,000 times higher than plasma estrogen concentrations, which range from 40 to 80 pg/ml in early life [102]. Although the levels of phytoestrogens are an order of magnitude higher than typical plasma concentrations of adults consuming soy foods, there are few reports of toxicity to infants and adults fed soy-based diets. Further studies in this area are therefore extremely necessary.

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