Radioimmunoassay Determination of Formononetin in Murine Plasma and Mammary Glandular Tissue[1] (44233)

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Abstract. **Formononetin is an isoflavonoid phytoestrogen found in animal fodder and in certain human foodstuffs. Since high consumption of formononetin has been implicated in infertility among ruminants and may relate to human breast cancer, we developed a radioimmunoassay method and applied it to quantitate formononetin in murine plasma and mammary glandular tissue for animal model studies.**

The radioimmunoassay utilized an antiserum raised in rabbits following immunization with formononetin-7-*O*-(carboxymethyl)ether coupled to bovine serum albumin. The tracer was an [^{3}H]leucine derivative of formononetin synthesized by mixed anhydride reaction between formononetin-7-*O*-(carboxymethyl)ether and [^{3}H]leucine. The bound and free forms of formononetin were separated by adding dextran-coated charcoal. This radioimmunoassay procedure enabled the quantification of 4 ng/ml of plasma or 50 pg/mg of mammary tissue, and the antiserum showed no marked cross-reaction with the reactants tested. The reliability and reproducibility of the assay were demonstrated by intra- and inter-assay variation that was 6.5% and 11.9%, respectively. This radioimmunoassay was compared with a high-performance liquid chromatographic method by determining concentrations of formononetin in ethanol extracts of red clover. Good correlation existed between the radioimmunoassay and high-performance liquid chromatographic method (r = 0.980), but the radioimmunoassay values were on the average 5% higher than high-performance liquid chromatographic values. Furthermore, we used the radioimmunoassay to assess the pharmacokinetics of formononetin in castrated female BALB/c mice after a single subcutaneous injection at 40 mg/kg dose. The sensitivity of the radioimmunoassay permitted the detection of a prolonged elimination phase for formononetin in both plasma and mammary glandular tissues, with mean elimination half-lives of 2 and 2.5 hr, respectively.

A specific, sensitive, and fast radioimmunoassay procedure has been developed for the determination of formononetin in murine plasma and mammary glandular tissue. We conclude that the presented radioimmunoassay is a valid alternative method to the quantification of formononetin in biological fluids and/or plants.

[P.S.E.B.M. 1998, Vol 217]

Phytoestrogens are natural plant substances that, by virtue of structural similarity to gonadal sex steroids, may exert estrogenic or antiestrogenic effects when introduced into mammalian systems (1–3). The most potent of these natural phytoestrogens are the isoflavonoids that are abundant in the bean subfamily of the *Leguminosidae,* including many plants commonly consumed by humans and livestock (4). The biological response to isoflavonoids was firstly observed in Australian sheep that had grazed on isoflavonoid-rich pasture and become sterile (5). Later, many other biological activities of isoflavonoids in mammals such as antiestrogenic (6), antioxidant (7), and antiproliferative effects (8) have been reported. In particular, the dietary isoflavonoids are currently receiving increasing attention because of their possible protective role against human cancer and/or other diseases (9–10). Therefore, the efforts in analyzing dietary isoflavonoids in both diet and biological

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These studies presented at the *Third International Conference on Phytoestrogens,* held December 3–6, 1995, in Little Rock, Arkansas, were partially supported by grants from the National Cancer Institute CA-01686, the American Heart Association HIFW-1595, and the Hawaii Community Foundation 955464.

0037-9727/98/2173-0281$10.50/0

fluids appear crucial to explore the possible mechanism underlying between isoflavonoid consumption and cancer risk.

Formononetin (7-hydroxy-4′-methoxyisoflavone) is a major dietary isoflavonoid responsible for reproductive dysfunction in ruminants (11). Formononetin content in animal fodder and in human diet may be as high as 1.3% (12) and 0.2% of dry weight (1), respectively. High consumption of formononetin has been implicated in bovine and ovine infertility, which was attributed to an estrogenic function of formononetin and/or its major metabolite, equol (13). In addition, formononetin was recently used in investigating the hormonal function as a possible mechanism of influence on breast cancer risk (14).

Precise methodologies for monitoring formononetin levels in either pasture or animal fluids are established involving the thin-layer chromatography (TLC) (15), the liquid chromatography (LC) (16), the high-performance liquid chromatography (HPLC) (17), and gas chromatography–mass spectrometry method (GC-MS) (18). This present study described the development of a radioimmunoassay method (RIA) as an alternative measurement for formononetin quantification in biological fluids. The application of this method for the pharmacokinetic properties of formononetin in a murine model study was also presented.

Materials and Methods

Chemicals and Reagents. Formononetin, biochanin A, daidzein, genistein, quercetin, coumestrol, and estradiol-17β were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Equol was a gift from Dr. Herman Adlercreutz, Department of Clinical Chemistry, University of Helsinki, Helsinki, Finland. The 2′-methoxyformononetin and matheucinol were kindly provided by Professor Gongyu Han, Department of Plant Chemistry, The Second Army Medical University in Shanghai, China. L-[4, 5-^{3}H(N)] Leucine (40 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA). The radiochemical purity was checked by TLC prior to use and estimated to be > 97%. All other reagents, unless indicated otherwise, were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Immunogen. The immunogen was synthesized by following two steps as shown in Figure 1.

Synthesis of formononetin ether hapten. The ether of formononetin was prepared by refluxing 500 mg (1.8 mmol) of formononetin and 430 mg (3.6 mmol) of sodium chloroacetate for 6 hr in 20 ml of absolute ethanol and 20 ml of dry pyridine. The solution was subsequently dried repeatedly *in vacuo* to remove the pyridine. The yellow oily residue was dissolved in 1 ml of warm water, and the solution was allowed to crystallize by keeping at 4°C for 72 hr. The crystallized product was then collected by filtration, washed with cold water, and dried over P_2O_5. A total of 247 mg (0.8 mmol) of formononetin-7-*O*-(carboxymethyl) ether (I) was recovered.

Synthesis of formononetin ether immunogen. Forty mg (150 μmol) of the formononetin ether (I) was dissolved

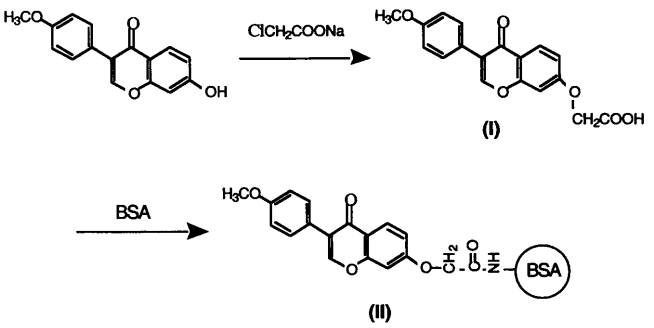

Figure 1. Outline of the synthetic scheme and structure of the formononetin-7-*O*-(carboxymethyl) ether hapten (I) and the formononetin-7-*O*-(carboxymethyl) ether-bovine serum albumin conjugate (II) used as immunogen for antibody production. Formononetin reacted with sodium chloroacetate in absolute ethanol produced to the formononetin-7-*O*-(carboxymethyl) ether. Then the formononetin-7-*O*-(carboxymethyl) ether was coupled to bovine serum albumin by the mixed anhydride reaction (see Materials and Methods).

in 2 ml of dimethylformamide (DMF). To the solution were added 30 μl (120 μmol) of tri-n-butylamine and 30 μl (220 μmol) of isobutyl chlorocarbonate followed by cooling on ice. After 20 min, this solution was added to a rapidly stirring mixture of 120 mg of bovine serum albumin (BSA), 3 ml of water, 3 ml of DMF, and 0.1 ml of 1 *M* NaOH. After 1 hr, 0.1 ml of 1 *M* NaOH was added. After an additional 5 hr, the solution was dialysed against 5 l of water for 48 hr twice and then lyophilized to give the formononetin ether conjugate (II) as a fluffy powder. This immunogen was stored at −20°C without further purification. From spectrophotometric analysis, a coupling ratio of 15 mol of formononetin ether per mol of BSA wascalculated.

Preparation of Antiformononetin Antisera. Four randomly bred rabbits (New Zealand white, approximately 2 kg) received intradermal injection of an emulsified mixture of Freund's complete adjuvant and 1 mg of formononetin ether immunogen. This preimmunization was followed by a monthly intradermal boost with 0.5 mg of the formononetin conjugate emulsified in Freund's noncomplete adjuvant for an additional 5 months. Two weeks after the last booster injection, blood was collected from two rabbits having high antibody titer. After standing at room temperature for a few hours, the blood was centrifuged at 2000 rpm for 10 min. The supernatant was stored at −80°C as antiformononetin antisera.

Measurement of the Titer of Antiformononetin Antisera. The titer of antiformononetin antisera was measured as follows: a mixture of 0.1 ml of stepwise diluted antiformononetin antisera and 0.5 ml of 0.1% gelatin in 10 m*M* phosphate-buffered saline (PBS, pH 7.0) was incubated with 0.1 ml of [^{3}H]formononetin derivative (~2.1 pmol of [^{3}H]formononetin derivative, ca. 10,000 cpm at 14% counting efficiency) in a plastic test tube at 4°C for 48 hr. Then the bound from free formononetin was separated as described in the assay procedure below. The titer was deter-

mined as the dilution required to obtain 50% binding of radioactivity. Two of the four rabbits immunized produced sera that bound 10,000 cpm (ca. 2.1 pmol) of [^{3}H]formononetin derivative at a final dilution of 1:10,000. From a Scatchard plot of binding data, a maximum affinity constant of $K_a = 6.8 \times 10^9\ M^{-1}$ was calculated.

Synthesis of [^{3}H]Formononetin Derivative. The [^{3}H]leucine derivative of formononetin was also prepared by the mixed anhydride reaction as shown in Figure 2. Fifteen mg (46 μmol) of formononetin-7-*O*-(carboxymethyl)ether (I) was first dissolved in 0.4 ml of DMF. After 12 μl (48 μmol) of tri-n-butylamine and 6 μl (44 μmol) of isobutyl chlorocarbonate were added, the solution was cooled to 0°C for 30 min. The activated solution was then mixed with 200 mCi (5 μmol) of [^{3}H]leucine. The coupling of the carboxyl and amino groups was allowed to proceed at 4°C for 24 hr, and the major product was isolated by chromatography on silica gel plates (Merck, Rahway, NJ), developed in methanol-chloroform (11:89, MC) [Rf of formononetin-7-*O*-(carboxymethyl)-ether = 0.40; Rf of major radioactive product = 0.49]. The product of [^{3}H] formononetin derivative was eluted from the plates with methanol and dried thoroughly *in vacuo* over P_2O_5. A total of about 22% of radioactivity was recovered. The specific activity of the product was estimated at 15 Ci/mmol by the self-displacement method (19). This [^{3}H]formononetin derivative product (III) was stored at 4°C until use, but never longer than 2 months.

Assay Procedure. Each assay tube consisted of 0.5 ml of either 0.1% gelatin PBS or sample, 0.1 ml of [^{3}H]formononetin derivative, and 0.1 ml of either antiserum or PBS (for unspecific binding determination). For the preparation of standard curves, standard formononetin solution was used instead of samples. All samples were assayed in triplicate. The tubes were incubated at 4°C for 48 hr, and the bound from free formononetin was separated by adding 0.4 ml of dextran-coated charcoal (1 g of charcoal was suspended with 100 ml of 0.1% dextran in PBS solution). Tubes were vortexed and incubated for 30 min at 4°C and centrifuged at 2000 rpm for 10 min at 4°C. All of the supernatant was carefully withdrawn into a counting vial and mixed with 10 ml of scintillation cocktail. The tritium radioactivity bound to the antibody was calculated by liquid scintillation counting (Beckman LS 9800, Fullerton, CA) after correction for the unspecific binding values.

RIA Validation. The sensitivity of the RIA was calculated statistically by the method of Rodbard (22). The intra- and interassay precision and accuracy were conducted by measuring formononetin concentration in control plasma on the same day or on different days, respectively. The control plasma was prepared by adding reference formononetin to formononetin-free human plasma at concentrations of 0.5, 5, and 50 ng/ml. Plasma controls were stored frozen in aliquots at –20°C. Recovery of formononetin was determined using control plasma and the tissue homogenate samples to which a known amount of formononetin had been added. The specificity of the RIA was investigated by comparing the cross-reactivity of formononetin with a number of structurally related compounds. The relative amounts required to reduce the initial binding of [^{3}H]formononetin derivative by half, where the mass of unlabeled formononetin was arbitrarily taken as 100%, were calculated from the standard curve. This RIA procedure was compared with a reversed-phase HPLC method (23) by determining concentrations of formononetin in the ethanol extracts of 12 red clover pastures.

Application to Pharmacokinetic Study. The pharmacokinetics of formononetin after a single administration was investigated in ovariectomized BALB/c mice that were housed under standard conditions (12:12-hr light-dark cycle, humidity 50 ± 15%, and temperature 22 ± 2°C). Food and water were provided *ad libitum.* Virgin mice were ovariectomized at 60 days of age and were used 1–2 weeks after ovariectomy.

Formononetin as a suspension in 1% ethanol in saline was injected subcutaneously (sc) at the dose of 40 mg/kg. On the study day, a group of five animals was sacrificed prior to the administration. Then each five-animal group was sacrificed at 2, 4, 6, 10, 16, and 24 hr, respectively, after dosing. Both blood and the inguinal mammary gland samples were immediately obtained from each sacrificed mouse. Plasma samples were obtained and stored frozen at –20°C until later analyzed for formononetin. The lymph node–devoid inguinal mammary glands were isolated by sharp dissection according to the criteria of DeOmer and co-workers (21). Then the glandular tissue was washed twice with cold PBS and homogenized at 0–4°C using a Polytron Homogenizer (Brinkman Instruments, Inc., Westbury, NY) in 10 m*M* Tris buffer and 1 m*M* EDTA (1:3 wt/vol). Cytosol fractions were obtained by centrifugation of the homogenate at 12,000 rpm for 10 min and kept frozen at –20°C prior to analysis by RIA.

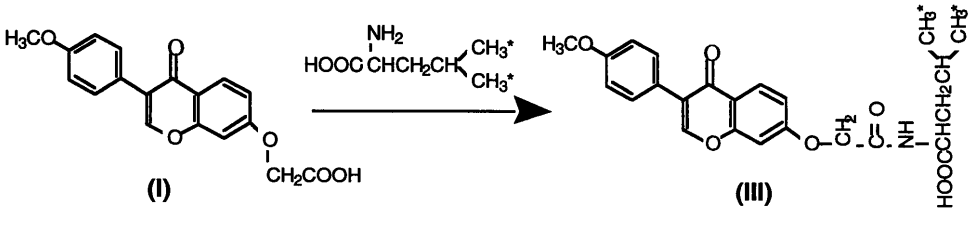

Figure 2. Synthesis of the [^{3}H] formononetin derivative (III) used as radioactive tracer. The [^{3}H]leucine derivative of formononetin was prepared by the mixed anhydride reaction between the formononetin-7-*O*-(carboxymethyl) ether and the [^{3}H]leucine. The asterisks denote possible sites of tritium (see Materials and Methods).

Results

RIA Validation. The binding of tritiated tracer to the antibodies was competitively inhibited by the addition of increasing amounts of formononetin standard. A typical standard curve is shown in Figure 3. Although the sensitivity of the RIA was estimated to be 0.05 ng/assay, we assigned 0.1 ng/assay as a lower limit of quantification to decrease the potential for false-positive results resulting from differences in the sample matrix. The optimal working range of the RIA was 0.4–40 ng/assay as determined from the logit linear transformation of the standard curve data. The sensitivity of this assay (at the 99.5% confidence limit) is 4 ng/ml in plasma sample and 50 pg/mg tissue in mammary glandular tissue, respectively.

The specificity of the formononetin antibodies was detected by measuring the inhibition of tracer binding in the presence of increasing amounts of potential cross-reacting compounds, and the results were shown in Table I. The percent cross-reactivities was calculated from the ratio of the 50% effective dose (ED_{50}) of formononetin to the ED_{50} of each compound. All of the compounds tested except biochanin A showed no measurable (<1%) cross-reaction with the antiserum. The observation that the antiserum cross-reacted with biochanin A to the extent of 6% was not unexpected, since the immunogen was prepared from a hapten that was the 9-dehydroxyl derivative of this compound.

A further assessment of the specificity of the antiserum was carried out by comparing the assay values obtained by the RIA with those by HPLC (23) in the ethanol extracts of 12 red clover pastures. The values from both methods showed good correlation (r = 0.980). However, the values of formononetin content in the red clover pasture by RIA were about 5% higher than those by HPLC. It is unknown why RIA values were on the average 5% higher than HPLC values, although this may be due to the cross-reactivity of biochanin A to the antisera. However, this difference was randomly distributed and did not increase with increasing sample concentration, which may suggest that the difference was independent of the concentration range studied and was probably not due to cross-reactions from biochanin A and/or other untested compounds.

Table I. Cross-Reactivities of Structurally Related Compounds in the Radioimmunoassay for Formononetin[a]

Compounds	ED_{50} (n*M*)	% Relative potency
Formononetin	9.3	100
Biochanin A	164.6	5.7
Equol	650.0	1.4
2′-Methoxyformononetin	852.8	1.1
Daidzein	1104.5	0.8
Genistein	1300.0	0.7
Quercetin	>9300	<0.1
Matheucinol	>9300	<0.1
Coumestrol	>>9300	<<0.1
Estradiol-17β	>>9300	<<0.1

[a] Standard curves of formononetin and the test compounds were analyzed in triplicate by the radioimmunoassay method described in the Materials and Methods. ED_{50} was estimated as the concentration of analyte necessary to displace 50% of the bound radiolabeled formononetin. The percentage relative potency was then calculated from the ratio of the ED_{50} for formononetin to the ED_{50} for each test compound x 100.

Various amounts of formononetin were added to control plasma to determine the precision and reproducibility of this assay, which were characterized by intra- and interassay coefficient of variation as shown in Table II. For the plasma controls, the intraassay coefficients of variation were 6.7% (0.5 ng/ml), 6.1% (25 ng/ml), and 6.8% (50 ng/ml), respec-

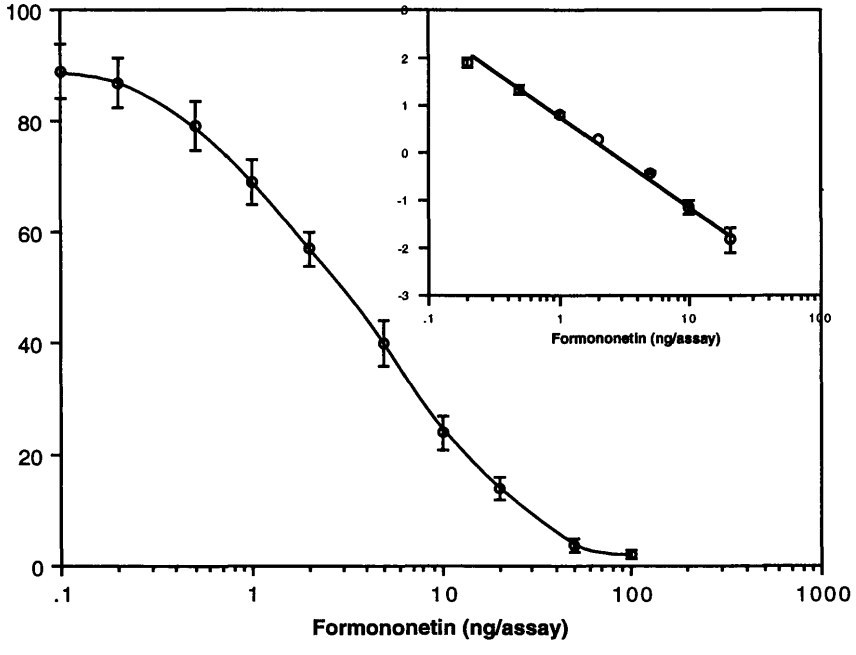

Figure 3. Standard curve for formononetin radioimmunoassay using the [^{3}H]-formononetin derivative as the tracer. Bars indicate standard deviations for triplicate samples. B = binding of the [^{3}H]-formononetin derivative to antibodies in the presence of unlabeled formononetin; Bo = binding in the absence of unlabeled formononetin. Insert shows the linear transformation of the standard curve using the logit plot [logit (B/Bo)] = 1n [(B/Bo)/(100–B/Bo)]. (Reprinted from Ref. 20, by permission of the American Chemical Society.)

Table II. Intraassay and Interassay Variances of the Radioimmunoassay for Formononetin

	Control plasma concentration of formononetin (ng/ml)		
	50	25	5
Intraassay variance[a]			
Mean ± SD	48.7 ± 3.3	24.6 ± 1.5	5.1 ± 0.3
CV (%)	6.8	6.1	6.7
Interassay variance[b]			
Mean ± SD	49.2 ± 4.5	25.3 ± 3.3	5.3 ± 0.7
CV (%)	9.1	13.0	13.6

[a] Intraassay variance was calculated from three assay readings of control plasma obtained on a single day of analysis.
[b] Interassay variance was calculated from assay readings of same control plasma on 3 different days of analysis.

tively, with the mean coefficient of variation of 6.5%. However, the mean interassay coefficient of variation was 11.9%, which ranged from 9.1%–13.6% (Table II). The accuracy of this RIA was tested by measuring formononetin both in plasma controls and tissue homogenates spiked with formononetin. The mean recovery of formononetin addition in the plasma was about 87%. Formononetin added to mammary tissue homogenates was recovered at approximately 98%.

Application to Pharmacokinetics Study. An applied experiment utilizing a single sc injection of formononetin (40 mg/kg) in castrated female BALB/c mice was conducted to determine formononetin pharmacokinetics. Persistent levels of formononetin were quantifiable both in plasma and mammary tissue up to 24 hr after injection as shown in Figure 4. The formononetin concentrations in plasma reached a peak value of 2.5 ± 0.8 μg/ml at 2 hr then gradually decreased over the following 22 hr. The formononetin concentrations in mammary tissue reached a peak of 2.0 ± 0.2 ng/mg tissue at 4 hr, then gradually decreased until near undetectable levels by 24 hr after administration. The biological elimination half-lives of formononetin in plasma and in mammary tissue were 2 hr and 2.5 hr, respectively.

Discussion

In this report we described the development of a sensitive and specific RIA for the quantification of formononetin, an isoflavonoid phytoestrogen. This RIA utilized the formononetin derivatives for the production of antiserum and tracer. The position on the formononetin molecule chosen for derivatization was the hydroxyl group at C7. The synthesis of a 7-*O*-(carboxymethyl)ether derivative introduced a carboxyl group that could be coupled to amino residues on protein as well as the α-amino group of leucine. The latter derivative can serve as a tracer when leucine is labeled with tritium (Figs. 1 and 2).

Immunization of rabbits with this 7-*O*-(carboxymethyl) ether of formononetin coupled to BSA resulted in the production of a specific antiformononetin antiserum. We systematically investigated the specificity of the antiserum by evaluating the cross-reactivity of a number of structurally related compounds in competitive-binding experiments (Table I). All the test compounds except biochanin A had a negligible level of cross-reactivity in the RIA. Even though biochanin A had a 6% cross-reactivity, it was nearly 20-fold less potent than formononetin. Noteworthy is that equol and daidzein, the major metabolites of formononetin in the gastrointestinal tracts (24), competed with this antiserum only 1% as well as formononetin. Furthermore, the RIA assay shows good correlation with a HPLC reference method, indicating again that no significant cross-reactions were occurring. Thus, we concluded that the antiserum is highly specific for formononetin.

With this antiserum and the synthesized tracer, we developed an RIA procedure for formononetin determination. A typical standard curve is shown in Figure 3. Useful dose-response curves could be carried in the range of 0.4–40 ng/assay. The quantitative limits of formononetin in plasma and tissue homogenates were approximately 4 ng/ml plasma and 50 pg/mg tissue, respectively. For the measurement of the formononetin concentration in plasma and in mammary

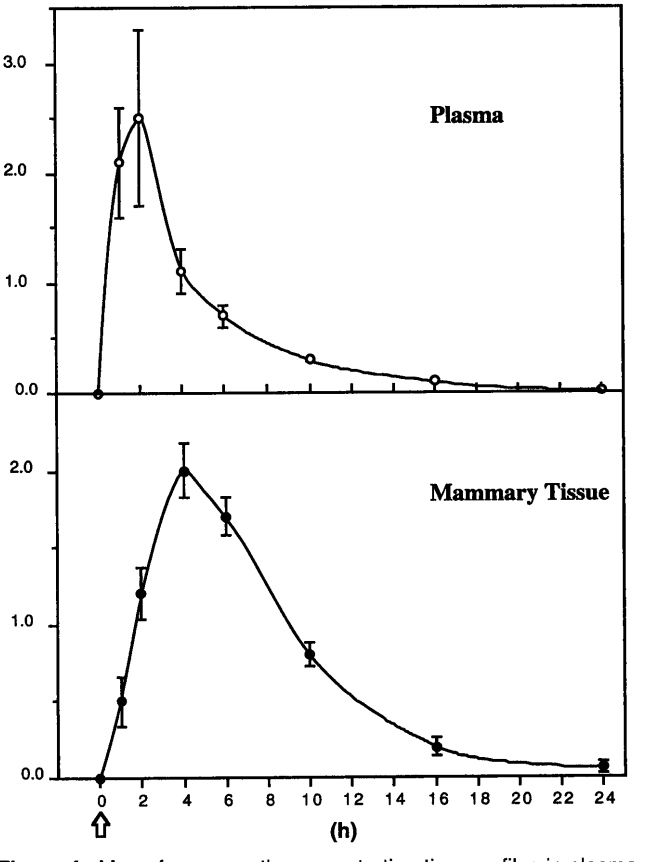

Figure 4. Mean formononetin concentration-time profiles in plasma and mammary gland tissue of ovariectomized BALB/c mice following subcutaneous injection of a single 40 mg/kg dose. Data are plotted as the means ± standard errors of the means for five animals. (Reprinted from Ref. 14, by permission of the Lawrence Erlbaum Associates, Publishers.)

glandular tissues, all samples were analyzed undiluted and following a 1:5 and 1:50 dilution. Moreover, a good precision of the RIA procedure was indicated by low values for the coefficient of variation in both intra- and interassay determinations at all the concentrations examined over the standard curve range (Table II). In practical terms, the assay is efficient since the procedure can readily run 100 samples in a normal working day. Therefore, this RIA, which avoids any sample extraction and preparation, should be suitable for routine monitoring of formononetin in biological fluids.

We used this RIA to assess the pharmacokinetics of formononetin following administration of a single 40-mg/kg sc dose to castrated female BALB/c mice. The RIA permitted the characterization of an elimination phase for formononetin with a mean half-life of 2 hr in plasma (Fig. 4). Meanwhile, formononetin was detected in the mammary glandular tissues with a mean elimination half-life of 2.5 hr.

Considering that the normal route of phytoestrogen intake by mammals is *via* the diet, we also tried to obtain pharmacokinetic information on formononetin given orally. In a preliminary experiment, the ovariectomized BALB/c mice were treated orally by gastric intubation with formononetin as a suspension in 1% ethanol in saline, and then the mice were sacrificed at 2, 4, 6, and 10 hr, respectively, after dosing. Both blood plasma and mammary glandular tissue samples were measured for formononetin by the RIA. However, no detectable amounts of formononetin were found in both plasma and mammary tissue samples, even though the mice were given oral doses up to 100 mg/kg. In fact, we have previously noted that formononetin given orally in the dose range of 10–100 mg/kg did not elicit biological response on vaginal cytology, but a parenteral route administration was deemed most appropriate for murine system (14). Since the formononetin concentration was detectable using the same RIA in blood plasma from sheep at different times after feeding with red clover (20), we hypothesize that formononetin may not be absorbed in the murine gastrointestinal tract or may be already metabolized before it is transported through the peripheral circulation to its target organs, and this may contribute to this species' relative insensitivity to this phytoestrogen (25). Regardless of the mechanism involved, taking this difference into account when exerting the biological effects of phytoestrogen in animal model systems may produce more valid clinical relevance of these dietary phytoestrogens.

In summary, this study demonstrates a sensitive, fast, and specific RIA for formononetin determination. The RIA described herein permits the detection of formononetin at 4 ng/ml in plasma or 50 pg/mg tissue in mammary glandular tissue without any extraction or purification step. With this RIA, it is possible to analyze up to 100 samples/day. The assay shows good correlation with an HPLC method and indicates no significant cross-reactions with tested reactants. The pharmacokinetics of formononetin in plasma and mammary glandular tissue were additionally presented by application of this method after single sc injection in a murine model study. Therefore, the RIA is a valid alternative method for the routine monitoring of formononetin in plants or in biological fluids.

1. Setchell KDR. Naturally occurring nonsteroidal estrogens of dietary origin. In: McLachlan JA, Ed. Estrogens in the Environment II. Influences on Development. New York: Elsevier Press, pp69–83, 1985.
2. Kaldas RS, Hughes CL Jr. Reproductive and general metabolic effects of phytoestrogens in mammals. Reprod Toxicol **3:**81–89, 1989.
3. Whitten PL, Naftolin F. Dietary estrogens: A biologically active background for estrogen action. In: Hochberg R, Naftolin F, Eds. The New Biology of Steroid Hormones. New York: Raven Press, pp155–167, 1991.
4. Harborne JB. Flavonoid pigments. In: Rosenthal GA, Janzen DH, Eds. Herbivores: Their Interaction with Secondary Plant Metabolites. New York: Academic Press, pp619–655, 1979.
5. Bennets HW, Underwood ET, Shier FL. A specific breeding problem of sheep on subterranean clover pastures in western Australia. Aust Vet J **22:**2–12, 1946.
6. Makela SI, Pylkkanen LH, Santti RSS, Adlercreutz H. Dietary soybean may be antiestrogenic in male mice. J Nutr **125:**437–445, 1995.
7. Jha HC, von Recklinghausen G, Zilliken F. Inhibition of *in vitro* microsomal lipid peroxidation by isoflavonoids. Biochem Pharmacol **34:**1367–1369, 1985.
8. Hirano T, Oka K, Akiba M. Antiproliferative effects of synthetic and naturally occurring flavonoids on tumor cells of the human breast carcinoma cell line, ZR-75-1. Res Commun Chem Pathol Pharmacol **64:**69–78, 1989.
9. Adlercreutz H. Western diet and western diseases: Some hormonal and biochemical mechanisms and associations. Scand J Clin Lab Invest **50:**3–23, 1990.
10. Messina MJ, Persky V, Setchell KDR, Barnes S. Soy intake and cancer risk: A review of the *in vitro* and *in vivo* data. Nutr Cancer **21:**113–131, 1994.
11. Millington AJ, Francis CM, McKeown NR. Whether bioassay of annual pasture legumes II. The oestrogenic activity of nine strains of *Trifolium subterraneum* L. Aust J Agric Res **15:**527–536, 1964.
12. Shackell GH, Kelly RW. Residual effects of short-term grazing of red clover pastures on the reproductive performance of ewes. N Z Exp Agric **12:**113–117, 1984.
13. Shutt DA, Braden AWH. The significance of equol in relation to the oestrogenic responses in sheep ingesting clover with a high formononetin content. Aust J Agric Res **19:**545–553, 1968.
14. Wang W, Tanaka Y, Han Z, Higuchi CM. Proliferative response of mammary glandular tissue to formononetin. Nutr Cancer **23:**131–140, 1995.
15. Wang W, Han Z. Extraction and measurement of formononetin content in clover herbage. Pratacultural Sci **6:**20–22, 1989.
16. Lundh TJ, Pettersson H, Kiessling K. Liquid chromatographic determination of the estrogens daidzein, formononetin, coumestrol, and equol in bovine blood plasma and urine. J Assoc Off Anal Chem **71:**938–941, 1988.
17. Franke AA, Custer LJ, Cerna CM, Narala KK. Quantitation of phytoestrogens in legumes by HPLC. J Agric Food Chem **42:**1905–1913, 1994.
18. Juniewicz PE, Morell P, Moser A, Ewing LL. Identification of phytoestrogens in the urine of male dogs. J Steroid Biochem Mol Biol **31:**987–994, 1988.
19. Chervu LR, Murty DRK. In: Freeman LM, Blaufox MD, Eds. Radioimmunoassay. New York: Grune and Stratton, pp33–48, 1975.
20. Wang W, Tanaka Y, Han Z, Cheng J. Radioimmunoassay for quantitative analysis of formononetin in blood plasma and rumen fluid of wethers fed red clover. J Agric Food Chem **42:**1584–1587, 1994.

21. DeOmer KB, Faulkin LJ, Bern HA, Blair PB. Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. Cancer Res **19:**515–520, 1959.
22. Rodbard D. Statistical estimation of the minimal detectable concentration (''sensitivity'') for radioligand assays. Anal Biochem **90:**1–12, 1978.
23. Wang W, Vincent DL, Early RJ, Weems CW. The quantification of phytoestrogens in fresh plant materials by reversed-phase high-performance liquid chromatography. Acta Bot Yunnanica **16:**424–430, 1994.
24. Setchell KDR, Adlercreutz H. Mammalian lignans and phytoestrogens: Recent studies on their formation, metabolism, and biological role in health and disease. In: Rowland IR, Ed. Role of the Gut Flora in Toxicity and Cancer. London: Academic Press, pp315–345, 1988.
25. Fredricks GR, Kincaid RL, Bondioli KR, Wright RW. Ovulation rates and embryo degeneracy in female mice fed the phytoestrogen, coumestrol. Proc Soc Exp Biol Med **167:**237–241, 1981.