Radioimmunoassay for Quantitative Analysis of Formononetin in Blood Plasma and Rumen Fluid of Wethers Fed Red Clover†

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Formononetin concentration was determined by radioimmunoassay (RIA) in plasma and rumen fluid of wethers fed on red clover. The RIA utilized antisera raised against a formononetin-7-*O*-(carboxymethyl) ether hapten. The tracer used was a ^{3}H-labeled derivative of formononetin, and the detection limit of the assay was 0.7 pmol/assay of formononetin. The reliability and reproducibility of the assay were demonstrated by intra- and inter-assay variations, which were 6.5% and 11.9%, respectively. With this assay, the concentration of formononetin was determined in blood plasma and rumen fluid from wethers at different times after red clover feeding. The maximum levels in both plasma and rumen fluid were within 4 h after red clover intake. At this time, the rumen fluid level was about 3 times higher than the plasma level. These results show that (a) a powerful RIA has been developed for formononetin quantification and (b) the biological half-lives of formononetin concentration in blood plasma and rumen fluid of wethers after red clover feeding were 8 and 2 h, respectively, which suggests the potential activity of formononetin besides its metabolites.

Keywords: *Formononetin, radioimmunoassay, wether, red clover*

INTRODUCTION

Some economically important pasture or forage plants of the Leguminosae family, e.g., red clover, contain high levels of phytoestrogens. Phytoestrogens have estrogen-like activity and may interfere with normal reproduction in ruminants [see reviews: Adlercreutz (1990), Kaldas and Hughes (1989), and Shutt (1976)]. The main phytoestrogen in red clover is formononetin (7-hydroxy-4′-methoxyisoflavone), which plays a major role in reproductive dysfunction in sheep (Millington et al., 1964). It has been suggested by Shackell and Kelly (1984) that the ingestion of clover pastures containing over 0.68% formononetin would cause infertility in ewes. On the other hand, it has been found that formononetin is metabolized by microorganisms in the rumen to daidzein and further to equol (Dickinson et al., 1988; Nilsson et al., 1967). Therefore, precise methodologies for monitoring formononetin levels in both pasture and blood are needed. Methods involving thin-layer chromatography (Wang and Han, 1989; Francis and Millington, 1965), gas chromatography–mass spectrometry (Adlercreutz et al., 1991), and high-pressure liquid chromatography (Wang, G., et al., 1990; Setchell et al., 1987; Pettersson and Kiessling, 1984) have been used for the determination of phytoestrogens. However, little is known about the dynamic changes of formononetin concentration both in rumen fluid and in blood.

The objective of this research was (1) to develop a radioimmunoassay (RIA) for formononetin that uses a tritiated tracer and is characterized by great selectivity and sensitivity and (2) to determine the in vivo clearance of formononetin both in rumen fluid and in blood plasma of wethers after red clover feeding.

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MATERIALS AND METHODS

Animals. Two 2-year-old ruminally fistulated wethers (mean live weight about 30 kg) were pen-fed twice a day with fresh grasses for 14 days prior to blood sampling. In the control period, the wethers were fed in a similar way as during the prefeeding period and given their feed ration of 5 kg of fresh grasses at 8 a.m. and another 5 kg at 5 p.m. In the experimental period, the wethers were fed the same rations except that the first daily feed ration at 8 a.m. was replaced by 5 kg of red clover.

Sample Collection. Both blood and rumen fluid samples were collected about 10 min before the first feed was offered and at 2, 4, 7, 10, and 13 h after the feeding during both the control and experimental periods.

Jugular blood samples were collected in 10-mL heparinized tubes to obtain plasma and were kept frozen at −20 °C until analyzed for formononetin by RIA.

Rumen fluid samples were strained through four layers of cheesecloth from the rumen fistula and kept at −20 °C after centrifugation at 4000 rpm for 10 min. Before analysis, 2 mL of defrosted fluid sample was extracted twice with a total volume of 5 mL of ether. Three milliliters of ether extracts was evaporated to dryness under air flow and were reconstituted in 0.5 mL of 0.1% gelatin in 10 mM phosphate-buffered saline (PBS, pH 7.0). The extracts were then analyzed for formononetin by RIA.

Synthesis of Formononetin-7-*O*-(Carboxymethyl) Ether Hapten. The ether of formononetin was prepared by refluxing 500 mg (1.8 mmol) of formononetin [purified and crystallized from the extracts of red clover following the method of Wang and Han (1989)] and 430 mg (3.6 mmol) of sodium chloroacetate for 6 h in 20 mL of absolute EtOH 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 allowed to crystallize by keeping it at 4 °C for 72 h. 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 the hapten was recovered.

Coupling of Formononetin-7-*O*-(Carboxymethyl) Ether to BSA. Forty milligrams (150 μmol) of the formononetin ether was dissolved 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 chlorocarbinate followed by cooling on ice. After 20 min, this solution was added to a rapidly stirring

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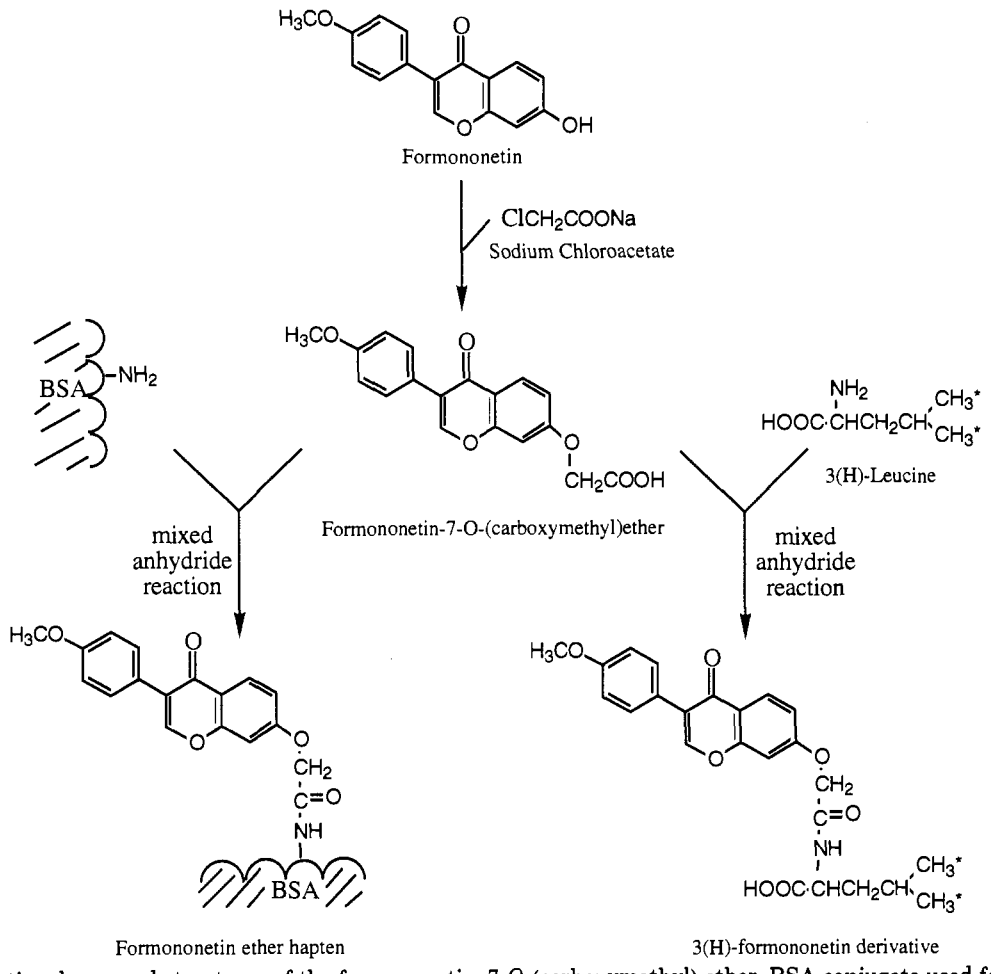

Figure 1. Synthetic scheme and structures of the formononetin-7-*O*-(carboxymethyl) ether–BSA conjugate used for immunization and the (^{3}H)formononetin derivative used as a tracer. The asterisks denote possible sites of tritium (see Materials and Methods).

mixture of 120 mg of bovine serum albumins (BSA), 3 mL of water, 3 mL of DMF, and 0.1 mL of 1 M NaOH. After an hour, 0.1 mL of 1 M NaOH was added. After an additional 5 h, the solution was dialyzed against 5 L of water for 48 h twice and then lyophilized. From spectrophotometric analysis, a coupling ratio of 15 mol of formononetin ether/mol of BSA was calculated.

Preparation of Anti-Formononetin Antibodies. Four randomly bred rabbits received intradermal injection of an emulsified mixture of Freund's complete adjuvant and 1 mg of formononetin ether–BSA. This preimmunization was followed by a monthly intradermal boost with 0.5 mg of the formononetin conjugate emulsified in Freund's noncomplete adjuvant for 5 months. Blood was collected for 2 weeks after the boost to obtain serum, which was stored at −20 °C. 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 $K_a = 6.8 \times 10^9$ L/mol was calculated.

Synthesis of (^{3}H)Formononetin Derivative. The (^{3}H)-leucine derivative of formononetin was also prepared by the mixed anhydride reaction. Fifteen millligrams (46 μmol) of formononetin-7-*O*-(carboxymethyl) ether 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 100 mCi (5 μmol) of (^{3}H)leucine (specific activity 20 Ci/mmol, Shanghai Nuclear Institute, Shanghai, China). The coupling of carboxyl and amino group was allowed to proceed at 4 °C for 24 h, and the major product was isolated by chromatography on silica gel plates (Merck, Rahway, NJ) developed in methanol–chloroform (11:89, MC) [R_f of formononetin-7-*O*-(carboxymethyl) ether = 0.40; R_f 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 (Chervu and Murty, 1975).

Schedule of Radioimmunoassay. The radioimmunoassay procedure was as follows: Each assay tube consisted of 0.5 mL of either 0.1% gelatin PBS or sample, 0.1 mL of tracer [2.1 pmol of (^{3}H)formononetin derivative, ca. 10 000 cpm at 14% counting efficiency], and 0.1 mL of either antiserum or PBS (for unspecific binding determination). Samples were assayed in triplicate. The tubes were incubated at 4 °C for 48 h, and the bound from free formononetin was separated by adding 0.4 mL of dextran-coated charcoal (Sigma, St. Louis, MO) at 4 °C. Tubes were mixed and incubated for 30 min at 4 °C and centrifuged at 4000 rpm for 10 min at 4 °C. The supernatants were collected and mixed with 10 mL of scintillation cocktail and counted in a liquid scintillation counter (Beckman LS 9800 or LS 5000TD, Fullerton, CA).

Extraction of Plant Material. A total of 1 g of grass or red clover tissue was extracted twice in 5 mL of 95% EtOH and refluxed for 2 h. The extracts were decanted and the solutions brought to a known volume with water. For the analysis of assay specificity by immunohistogram (the distribution of immunoreactive compounds on a thin-layer chromatogram of a red clover extract), the undiluted samples were applied to 10 × 10 cm of silica gel and developed in MC. After development, 0.5-cm sections of the entire plate were cut out and extracted in 1 mL of MeOH overnight. The samples were then diluted with water and assayed.

RESULTS

Radioimmunoassay. The synthetic scheme for both hapten and tritiated tracer is summarized in Figure 1.

The binding of tritiated tracer to the antibodies was competitively inhibited by the addition of increasing

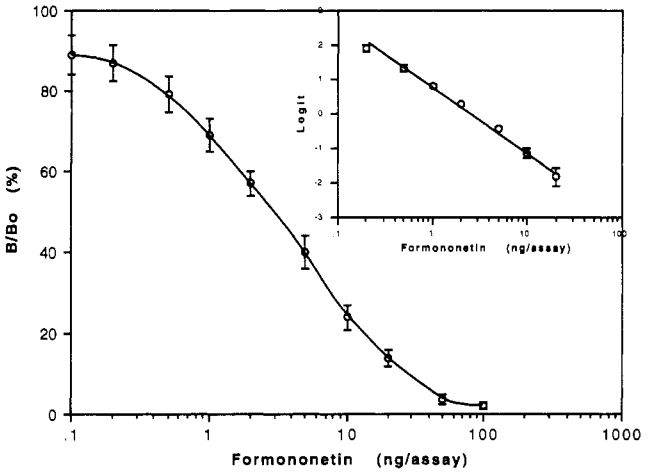

Figure 2. Standard curve for formononetin radioimmunoassay using the (^{3}H)formononetin derivative as the tracer. Bars indicate standard deviations for triplicate samples. B = binding of the (^{3}H)formononetin derivative to antibodies in the presence of unlabeled formononetin; B_0 = binding in the absence of unlabeled formononetin. Insert shows the linear transformation of the standard curve using the logit plot [logit(B/B_0) = ln$[(B/B_0)/(100 - B/B_0)]$.

Table 1. Cross-Reactivity of Various Compounds Structurally Related to Formononetin with the Antiserum

compound	pmol needed for 50% displacement of the tracer	% cross-reactivity
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

amounts of formononetin standard. A typical standard curve is shown in Figure 2. The assay has a measuring range of 0.4–40 ppb (0.7–75 pmol) as determined from the logit linear transformation of the standard curve data. The sensitivity of this assay (at the 99.5% confidence limit) is 0.2 ng (0.7 pmol) of formononetin.

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 are shown in Table 1. (Equol was a gift from Dr. H. Adlercreutz, Department of Clinical Chemistry, University of Helsinki, Helsinki, Finland; coumestrol was donated by Dr. Adrian Franke, Cancer Research Center of Hawaii, Honolulu, HI; quercetin, matheucinol, and 2′-methoxyformononetin were kindly provided by Prof. Gongyu Han, Department of Plant Chemistry, The Second Army Medical University of Shanghai, Shanghai, China; other compounds tested for cross-reactivity were purchased from ICN, Costa Mesa, CA.) Further experiments for the specificity of the antisera were conducted by the distribution of immunoreactive compounds on the thin-layer chromatography (immunohistogram) of the extracts of red clover, grass, and rumen fluid. The analysis of these extracts showed that the only immunoreactive peak was associated with the area of the plate having R_f values identical with that of the formononetin reference.

The precision and reproducibility of this assay were characterized by an intra-assay coefficient of variation for

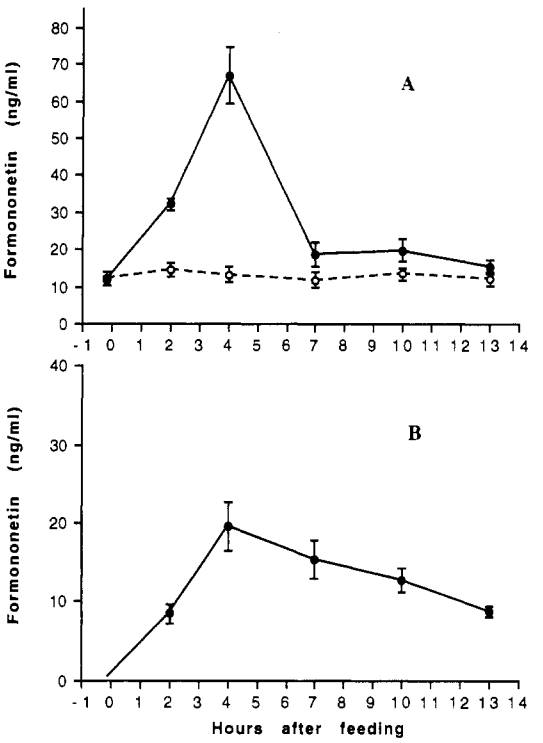

Figure 3. Formononetin concentration in rumen fluid (A) and blood plasma (B) of wethers at different times after feeding on red clover (●) or control grasses (○). No detectable amounts of formononetin were found in the blood plasma of wethers fed the control grasses. Data represent means ± SD from two animals in triplicate.

triplicate values of 6.5 ± 0.2% (n = 13) and by an inter-assay coefficient of variation of 11.9 ± 1.8% (n = 11). Formononetin added to rumen fluid samples was recovered at approximately 75%. The lower recovery in rumen fluid may be due to the insolubility of formononetin in PBS. The recoveries of formononetin addition in the plasma and plant extracts were about 95% and 98%, respectively.

Serial dilutions of extracts of rumen fluids or plants resulted in an inhibition of a tracer binding curve that was parallel to the formononetin standard curve.

Formononetin Concentrations in Wether Blood Plasma and Rumen Fluid at Different Times after Feeding on Red Clover. The contents of formononetin in the control grasses and red clover as determined by this RIA were 0.062 ± 0.003 (n = 6) and 1.305 ± 0.050 g/kg of dry weight (n = 12), respectively. This led to a daily intakes of 372 mg/day of formononetin for the wethers in the control period and 2237 mg/day in the experimental period.

The formononetin found in wether blood plasma and rumen fluids at different times after red clover feeding is shown in Figure 3 after correction for losses by recovery. No detectable amounts of formononetin were found in the blood plasma of the wethers fed the control grasses. However, formononetin was increased rapidly in the rumen fluid and reached the maximum level within 4 h after red clover intake. At the same time, the formononetin in plasma was increased in a similar pattern as that in the rumen fluid and also reached a peak at 4 h after feeding. At the time of the peak, the plasma and rumen fluid levels were 19.6 ± 4.1 and 66.9 ± 7.8 ng/mL, respectively. After this peak, the formononetin declined rapidly in rumen fluid but gradually in plasma. The biological half-lives ($T_{1/2}$) of formononetin concentration in rumen fluid and plasma were 2 and 8 h, respectively.

DISCUSSION

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 C_7. 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 ∂-amino group of leucine. The latter derivative can serve as a tracer when leucine is labeled with tritium (see Figure 1).

To determine whether the measurement of formononetin by RIA compared favorably with another method of formononetin analysis, six extracts of red clover pasture were analyzed for formononetin content by both RIA and HPLC (Wang, G., et al., 1990). The values from both methods showed good correlation ($r = 0.928$). The values of formononetin content in the red clover pasture were 1.307 ± 0.061 g/kg of dry weight by RIA and 1.253 ± 0.054 g/kg of dry weight by HPLC. It is not known why RIA values were on the average 5% higher than HPLC values, although this may be due to the cross-reactivity of formononetin glucosides to the antisera. Formononetin occurs in plant tissues mostly in the bound form of glucosides, which are hydrolyzed in the rumen by the microorganisms (Nilsson et al., 1967). However, because the immunohistogram of the extract of red clover showed that immunoreactivity was confined only to the formononetin band, there might be some other cross-reactivities which were not tested.

To verify that the immunoreactivity detected in plasma was in fact formononetin, several plasma samples were applied and fractionated by thin-layer chromatography (TLC) according to our previous method (Wang and Han, 1989), and the fractions were then extracted and analyzed by RIA. RIA analysis of fractions from the TLC showed that the characterization of the immunoreactive material in plasma was only formononetin.

The biological action of formononetin in animals is poorly understood due to two reasons: one is that formononetin shows no to little estrogenic activity when tested in mice (Mohsin and Pal, 1975), and the other is that formononetin in rumen is metabolized to equol (Nilsson et al., 1967). Equol in blood plasma of sheep fed clover pastures could reach levels of 3–5 μg/mL (Shutt, 1976) or 1–2 μg/mL (Lundh et al., 1990). Thus, it looks reasonable that the high blood concentration of equol (about 100 times that of formononetin concentration) is responsible for infertility in sheep.

On the contrary, formononetin has been shown to have estrogenicity of the same order as that of other isoflavonic phytoestrogens by the vaginal smear method in ovariectomized mice (Wang, 1990). In addition, formononetin has been found to promote the mammary DNA synthesis in the animal model and to competitively bind cytosolic estrogen receptors in mouse mammary glands (unpublished data). Therefore, the potential biological action of formononetin in vivo should be closely examined.

The experimental results reported in this paper have demonstrated that a new RIA method for formononetin has been developed. The levels of formononetin in wether rumen fluid and blood plasma monitored by this RIA method may prove to be extremely valuable in understanding the relationship among formononetin in pastures, its digestion, metabolism, and absorption, and its potential activities in sheep and other animals.

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