ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1994, p. 2134–2142
0066-4804/94/$04.00+0

Vol. 38, No. 9

Sensitive and Specific Radioimmunoassay for Fialuridine: Initial Assessment of Pharmacokinetics after Single Oral Doses to Healthy Volunteers

RONALD R. BOWSHER,[1,2]* JOYCE A. COMPTON,[1] JEFFREY A. KIRKWOOD,[1] GARY D. PLACE,[1] C. DAVID JONES,[3] THOMAS E. MABRY,[3] DAVID L. HYSLOP,[4] BARBARA L. HATCHER,[5] AND KARL A. DESANTE[5,6]

Departments of Drug Disposition & Bioanalytical Research,[1] Virology Research,[3] Clinical Pharmacology,[4] and Bioavailability and Pharmacokinetics,[5] Lilly Research Laboratories, Eli Lilly & Company, and the Departments of Biochemistry & Molecular Biology[2] and Pharmacology,[6] Indiana University School of Medicine, Indianapolis, Indiana 46202

Received 16 December 1993/Returned for modification 13 April 1994/Accepted 27 June 1994

Fialuridine (FIAU) is a halogen-substituted analog of thymidine that was undergoing clinical investigation as a drug for the treatment of chronic hepatitis B viral infection. However, clinical trials of FIAU were terminated after adverse events occurred following chronic oral administration. Prior to the termination of clinical trials, a sensitive assay was needed for the measurement of FIAU because of the anticipated low dose administered to patients. We therefore undertook the development of a radioimmunoassay (RIA). A specific antiserum was raised in rabbits following immunization with a 5′-*O*-hemisuccinate analog of FIAU coupled to keyhole limpet hemocyanin. Radiolabeled FIAU was synthesized by a destannylation procedure by using sodium [^{125}I]iodide. We developed a competitive-binding procedure and used precipitation with polyethylene glycol as the method for separating the bound and free forms of FIAU. The RIA is sensitive (0.2 ng/ml), specific (negligible interference from known metabolites and endogenous nucleosides), and reproducible (interassay coefficients of variation range from 5 to 19.7% for serum controls). We used the RIA to assess the pharmacokinetics of FIAU in healthy adult volunteers following administration of a single 5-mg oral dose. The sensitivity of the RIA permitted the detection of a prolonged elimination phase for FIAU in healthy volunteers and dogs, with mean elimination half-lives of 29.3 and 35.3 h, respectively. We conclude the RIA is a valid method for the quantification of FIAU in biological fluids.

Fialuridine (FIAU), 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil (Fig. 1, compound 2), is one of a series of 2′-fluoro-substituted arabinosyl pyrimidine nucleosides that have demonstrated potent antiviral activities against a number of clinically important viruses, including hepatitis B virus (HBV) (7, 11, 28, 47). Analogs of FIAU have been shown to inhibit viral replication in the woodchuck and duck models of HBV infection (18, 19). Although the mechanism of the anti-HBV activity is not well understood, evidence suggests that the triphosphate analog of FIAU is a potent inhibitor of HBV DNA polymerase activity (17, 23, 43). During early clinical investigation FIAU showed much promise as an anti-HBV drug because it markedly reduced the level of HBV DNA in the serum of patients with chronic hepatitis B (20). However, clinical trials were terminated after adverse events occurred following oral administration of FIAU (0.1 and 0.25 mg/kg of body weight per day) for more than 2 months (24, 29). The mechanism of the unexpected delayed toxicity is unresolved.

Prior to the termination of clinical trials an assay was needed to quantify FIAU in biological fluids. However, the anticipated low therapeutic dose in patients necessitated the development of a highly sensitive analytical method. The criterion of sensitivity precluded the use of an existing UV-based high-pressure liquid chromatographic (HPLC) method for FIAU because its lower limit of detection was 50 ng/ml. We used the following rationale to justify the development of a radioimmunoassay (RIA) for FIAU. First, RIAs are sensitive methods capable of detecting small molecules at nanomolar concentrations. Second, RIAs have been developed for a number of different nucleoside analogs (1, 10, 33–35, 38, 39, 42, 44, 45). Third, RIA has been reported to be the methodology of choice for quantifying zidovudine (AZT) in serum, especially when concentrations are less than 50 ng/ml (16, 49). Fourth, the presence of the halogen substituents on FIAU will increase its "foreignness," which should result in the formation of specific antibodies. Fifth, previous data suggest that FIAU is not metabolized in humans to a multiplicity of different compounds (15), which minimizes the potential for analytical interference from metabolites. Sixth, the iodine in the 5 position of the pyrimidine ring permits the direct introduction of radioactive iodine without any alteration in the antigenicity of FIAU. Finally, RIAs are cost-effective and have a high level of analytical throughput.

MATERIALS AND METHODS

Chemicals. FIAU (51), 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)uracil (FAU) (47, 48), and FIAU 5′-triphosphate (26) were synthesized in the Department of Virology Research at the Lilly Research Laboratories (Indianapolis, Ind.). 1-(2′-Deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-methyluracil (FMAU) was a generous gift from the Sloan-Kettering Institute (New York, N.Y.). Sodium phosphate, NaCl, sodium azide, EDTA, isobutyl chloroformate, bovine serum albumin (BSA), polyeth-

* Corresponding author. Mailing address: Lilly Laboratory for Clinical Research, Wishard Memorial Hospital, 1001 West Tenth Street, Indianapolis, IN 46202. Phone: (317) 276-6531. Fax: (317) 276-5495. Electronic mail address: bowsher_ronald_r@lilly.com.

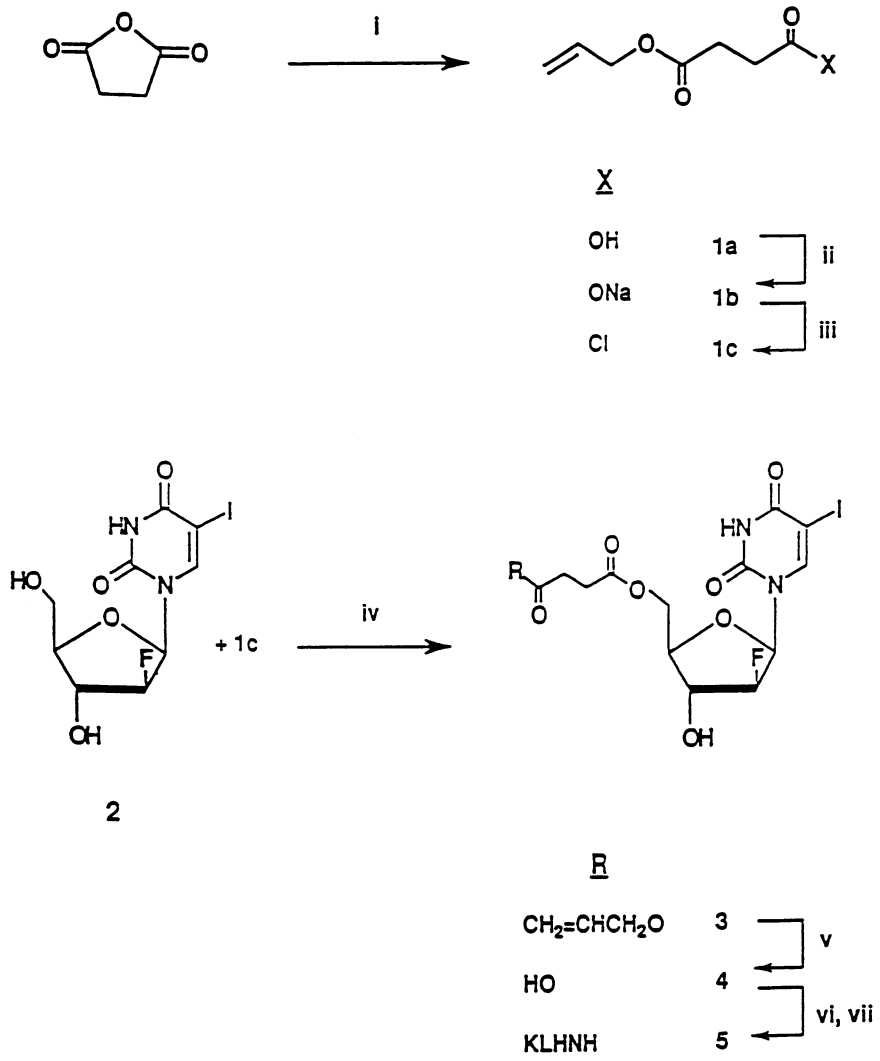

FIG. 1. Outline of the synthesis of the 5'-*O*-hemisuccinate analog of FIAU and the immunogen for antibody production. Reagents and conditions were as follows: i, allyl alcohol, DMAP, acetonitrile; ii, NaOH; iii, oxalyl chloride, CH_2Cl_2, 0°C; iv, pyridine, 0°C; v, sodium 2-ethylhexanoate, tetrakis(triphenylphosphine)palladium (O) [$(Ph_3P)_4Pd$], EtOAc; vi, isobutylchloroformate, triethylamine [Et_3N], DMF; vii, KLH, sodium carbonate, (pH 9.4), 0°C.

ylene glycol (PEG; molecular weight, 8,000), thymidine, uridine, cytidine, guanosine, and 5-iodo-uridine were purchased from Sigma Chemical Company (St. Louis, Mo.). Bovine gamma globulin was obtained from Calbiochem (La Jolla, Calif.). Tween 20, dimethylformamide (DMF), and keyhole limpet hemocyanin (KLH) were purchased from Pierce (Rockford, Ill.). Drug-free serum from fasted healthy adult male volunteers was obtained from Biological Specialty Corp. (Lansdale, Pa.). All other chemicals were reagent grade and were purchased from commercial vendors.

General synthetic methods. Nuclear magnetic resonance (NMR) spectra were determined at 300.135 MHz with a QE300 instrument (General Electric). Chemical shift values are expressed in δ values (parts per million) relative to tetramethylsilane. Elemental analyses were performed by the Department of Physical Chemistry at the Lilly Research Laboratories (Indianapolis, Ind.). Thin-layer chromatography was performed by using silica gel 60 F-254 plates (EM reagents). Merck silica gel (230-400 mesh) was used for flash chromatography. The reported yields reflect the amounts of purified compounds and are not optimized. Reversed-phase HPLC (RP-HPLC) was performed at a flow rate of 1 ml/min by using a Waters C_{18} Nova-Pak column (3.9 by 150 mm) and a mobile phase consisting of acetonitrile–0.5% ammonium dihydrogen phosphate buffer. Chromatography was monitored by UV detection at 254 nm.

Succinic acid, monoallyl ester (compound 1a). Allyl alcohol (4.3 ml, 63.6 mmol) and 4-(*N*,*N*-dimethylamino)pyridine (DMAP; 11.5 g, 95 mmol) were dissolved in acetonitrile (50 ml) at 23°C under an N_2 atmosphere and were treated with finely powdered succinic anhydride (9.5 g, 95 mmol). The reaction mixture was stirred for 3 h, the solvent was removed in vacuo, and the residue was diluted with ethyl acetate (EtOAc)–concentrated NH_4OH (1:1). The aqueous phase was washed with EtOAc, carefully neutralized to pH 4 with 2.0 M $NaHSO_4$, and treated with a second portion of EtOAc. Following phase separation, the organic layer was washed with a saturated solution of NaCl, dried over anhydrous Na_2SO_4, and distilled under reduced pressure (140 to 145°C at 7 mm Hg) to give 6.0 g (60%) of compound 1a (Fig. 1) as a colorless oil; ^1H

NMR ($CDCl_3$) 2.67 (m, 4H, $CH_2CH_2C{=}O$), 4.62 (m, 2H, OCH_2CH), 5.32 (m, 2H, $CH_2{=}$), 5.93 (m, 1H, $CH_2{=}CHCH_2$), 10.4 (bm, 1H, COOH).

1-[5′-*O*-(Allylsuccinyl)-2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil (compound 3). Oven-dried FIAU (compound 2; 2.0 g, 5.37 mmol) was dissolved in freshly distilled pyridine and treated with 1.2 equivalents of compound 1c (prepared by treating the sodium salt (compound 1b) with oxalyl chloride in CH_2Cl_2 at 0°C) under an N_2 atmosphere. After stirring the reaction mixture for 1.5 h at 0°C, the solvent was removed in vacuo. The residue was diluted with EtOAc, washed with 0.1 N HCl (20 ml, two times), saturated with $NaHCO_3$ (20 ml, two times) and with a saturated solution of NaCl, and dried over Na_2SO_4. Flash chromatography ($CHCl_3$-methanol [MeOH]; 95:5) of the resulting foam yielded 0.82 g (30%) of compound 3 (Fig. 1) as a colorless solid; ^1H NMR (dimethyl sulfoxide [DMSO]-d6) 2.62 (m, 4H, $CH_2CH_2C{=}O$), 4.01 (m, 1H, H-4′), 4.23 (m, 1H, H-3′), 4.30 (m, 2H, H-5′), 4.52 (d, 2H, OCH_2CH), 5.01 (dd, 1H, H-2′, J = 35 Hz), 5.19 (dd, 1H, $CH_2{=}CH$, *trans*), 5.28 (dd, 1H, $CH{=}CH_2$, *cis*), 5.89 (m, 1H, $CH_2{=}CH$), 6.12 (dd, 1H, H-1′), 7.86 (s, 1H, H-6); mass spectra (MS) (field desorption [FD]) *m/z* 514 (m+2). Analysis for $C_{16}H_{18}FIN_2O_8$. Calculated: C, 37.52; H, 3.54; N, 5.47. Found: C, 37.67; H, 3.63; N, 5.41. Infrared (IR) ($CHCl_3$) 1722 cm^{-1}, 1695 cm^{-1}. The HPLC (30:70) retention time was 4.71 min.

5′-*O*-(Succinyl)-2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil (compound 4). A degassed solution of compound 3 (0.260 g, 0.51 mmol), and oven-dried sodium 2-ethylhexanoate (0.21 g, 1.26 mmol) in dry EtOAc was treated with $(Ph_3P)_4Pd$ (0.058 g, 0.05 mmol) at 23°C. After stirring the reaction mixture for 2 h, the resulting precipitate was filtered and rinsed with EtOAc. The solid was dissolved in 10 ml of EtOAc-H_2O (1:1) and was carefully acidified to pH 3 with 1.0 N HCl. After phase separation, the aqueous layer was back extracted with 15 ml (three times) of EtOAc. The organic phases were combined, dried over Na_2SO_4, and concentrated to a foam. The product was purified by flash chromatography ($CHCl_3$-MeOH-HOAc; 95:5:0.5) and yielded 0.095 g (41%) of compound 4 (Fig. 1) as a slightly colored foam; ^1H NMR (DMSO-d6) 2.42 (m, 4H, $CH_2CH_2C{=}O$), 3.98 (bs, 1H, H-4′), 4.25 (m, 1H, H-3′), 4.31 (m, 2H, H-5′) 5.02 (d, 1H, H-2′, J = 35 Hz), 6.1 (dd, 1H, H-1′), 7.89 (s, 1H, H-6); MS (FD) *m/z* 474 (m+2). Analysis for $C_{13}H_{14}FIN_2O_8$: Calculated: C, 33.07; H, 2.99; N, 5.93. Found: C, 33.16; H, 3.16; N, 5.93.

Preparation of the immunogen (compound 5). Compound 4 was coupled to KLH by a modified mixed anhydride reaction (13, 14, 36, 39). The 5′-*O*-hemisuccinate of FIAU (compound 4) (60 mg, 0.127 mmol) was dissolved in 0.68 ml of ice-cold, dry DMF in a silanized 12-by-75-mm borosilicate glass tube. Following the addition of 27 μl of triethylamine (0.191 mmol), 42 μl of isobutyl chloroformate (0.318 mmol) was slowly added below the level of the meniscus. The reaction mixture was then incubated for 45 min with continuous mixing in an ice bath.

A 100-μl aliquot of the mixed anhydride reaction (0.017 mmol) was added to a 15-ml polypropylene tube that contained 7.5 ml of a 5-mg/ml solution of KLH (7.6 nmol) in 50 mM sodium carbonate (pH 9.4). The reaction mixture was incubated for 3 h at 4°C on an orbital mixer. During the incubation the pH of the reaction mixture was maintained by adding 1 M sodium carbonate (pH 9.5). The reaction was quenched by adding 15 ml of 25 mM sodium phosphate–0.9% NaCl (pH 7.5). The 5′-*O*-hemisuccinate–FIAU conjugate (compound 5 [Fig. 1]) was then dialyzed for 2 days at 4°C against 5 liters (two times) of the same buffer. Following dialysis, the conjugate solution was concentrated to 2 mg/ml and was stored as 1-ml aliquots at −20°C. The conjugation efficiency was estimated to be approximately 70% for the available amino groups on the basis of UV spectroscopy and reaction with trinitrobenzenesulfonate (2, 46).

Immunization. The production of rabbit anti-FIAU antibodies was carried out at HRP, Inc. (Denver, Pa.). The immunogen solution was emulsified with an equal volume of Freund's complete adjuvant. Two groups each of six female New Zealand White rabbits were then immunized with 0.5 mg of the immunogen by multiple intramuscular injections. For booster injections the immunogen was emulsified with an equal volume of Freund's incomplete adjuvant containing 1 mg of diphenhydramine per ml. The rabbits received intramuscular booster injections containing 0.1 mg of the immunogen at days 14 and 28 and subsequently at 28-day intervals. Antibody titers were measured by RIA in bleeds collected at 14-day intervals beginning at day 42. The fifth bleed from rabbit EL738 was selected for use in RIA applications. The antiserum (~25 ml) was diluted 1:2 with assay buffer and was stored as 0.5-ml aliquots at −20°C.

1-(2′-Deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-trimethylstannyluracil (compound 6). A degassed solution of FIAU (compound 2; 0.250 g, 0.67 mmol) and hexamethylditin (0.436 g, 1.34 mmol) in dry dioxane (10 ml) was treated with $(Ph_3P)_4Pd$ (0.038 g, 0.03 mmol), and the reaction mixture was stirred for 2 h at 60°C under an N_2 atmosphere. The solvent was then removed in vacuo and the residue was dissolved in CH_3CN-pentane (1:1). After washing several times with pentane to remove the tin residue, the CH_3CN phase was concentrated and the resulting foam was subjected to flash chromatography (SiO_2, $CHCl_3$-MeOH-NH_4OH; 95:5:0.5) and yielded 0.122 g (44%) of compound 6 (Fig. 2) as a colorless powder; ^1H NMR ($CDCl_3$) 0.19 (s, 9H, $SnMe_3$), 3.59 (m, 2H, H-5′), 3.75 (m, 1H, H-4′), 4.22 (m, 1H, H-3′), 5.06 (m, 1H, H-2′), 5.13 (m, 1H, 5′-OH), 5.87 (d, 1H, 3′-OH), 6.15 (dd, 1H, H-1′), 7.44 (s, 1H, H-6); MS (FD) *m/z* 409 (m+1). Analysis for $C_{12}H_{19}FIN_2O_5Sn$: Calculated: C, 35.24; H, 4.68; N, 6.85. Found: C, 35.25; H, 4.98; N, 6.56.

1-(2′-Deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-[^{125}I]uracil (compound 7). Radioiodinated FIAU was synthesized bimonthly by the following procedure at DuPont NEN Products (Boston, Mass.). An aqueous solution of sodium [^{125}I]iodide (10 mCi, 4.5 nmol) was mixed in a glass vial with 0.2 ml of 0.5 M sodium phosphate (pH 7.5) and 0.1 ml of acetonitrile that contained 0.24 μmol of compound 6. The reaction was initiated by adding 0.1 ml of an aqueous solution of chloramine-T (0.36 μmol). After 5 min the reaction was quenched by the addition of 0.1 ml of 5.3 mM sodium meta-bisulfite. Compound 7 (Fig. 2) was purified by RP-HPLC by using a C_{18} column (250 by 4.6 mm) and gradient elution from 0 to 100% solvent B (solvent A, 0.05 M ammonium acetate [pH 7.6]; solvent B, solvent A and acetonitrile [1:1]). After purification by RP-HPLC radiolabeled FIAU routinely had a specific activity of >2,000 Ci/mmol and a radiochemical purity of >95%.

RIA reagents. All solutions except the stock solutions of FIAU and radiolabeled FIAU were stored at 4°C. The assay buffer used to dilute radiolabeled FIAU and antibody consisted of 100 mM sodium phosphate–1% EDTA (wt/vol)–0.1% sodium azide–0.05% Tween 20–0.25% BSA (pH 7.5). For use in the RIA, stock radiolabeled FIAU was diluted to 250 pg/ml. The working tracer solution was stored at 4°C and was usable for a period of at least 10 weeks. A 100-μg/ml stock solution of FIAU in assay buffer was stable for more than 9 months when stored frozen in 1-ml aliquots in polypropylene tubes at −20°C. Standard curves were prepared weekly by diluting the stock FIAU in serum that contained 0.02% sodium azide. A 1%

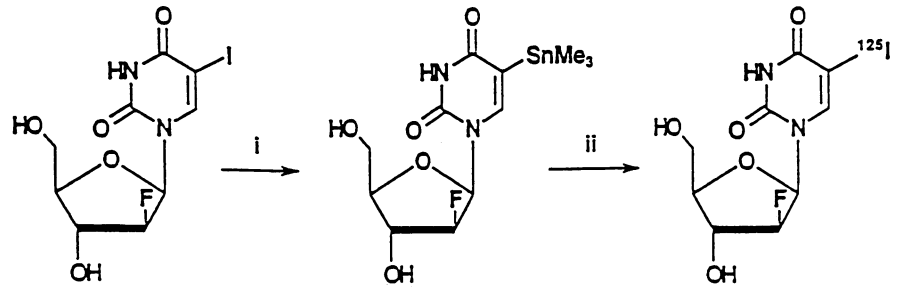

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FIG. 2. Outline of the synthesis of radiolabeled FIAU. Reagents and conditions were as follows: i, $(Me_3Sn)_2$, $(Ph_3P)_4Pd$, dioxane, 60°C; ii, Na [^{125}I]iodide, chloramine-T, acetonitrile.

solution of bovine gamma globulin was prepared in 25 mM sodium phosphate–0.9% NaCl–0.1% sodium azide (pH 7.5). A solution of 20% PEG–0.02% sodium azide was used for precipitating the antibody-bound fraction of FIAU.

RIA procedure. We developed a competitive-binding RIA method for quantifying FIAU in biological fluids. Each binding reaction (total volume, 500 μl) was performed in a 12-by-75-mm polystyrene tube and consisted of 100 μl of radioiodinated FIAU (25 pg), 300 μl of serum (test sample or standard FIAU in drug-free serum), and 100 μl of anti-FIAU antiserum (diluted 1:450). The level of nonspecific binding was determined by replacing antibody with assay buffer in the incubation mixture. After mixing, the binding reaction mixture was incubated for 18 to 24 h at 4°C. The bound and free forms of FIAU were then separated by adding 100 μl of cold 1% bovine gamma globulin; this was followed by the addition of 1 ml of cold 20% PEG. Each tube was vortexed thoroughly and was centrifuged at ~3,500 × *g* for 15 min at 4°C. After decanting the supernatant, the radioactivity in the precipitate was measured in an Apex 10/600 automatic gamma counter (Micromedic Systems, Inc., Horsham, Pa.). We used a VAX computer to analyze RIA data by a weighted four-parameter logistic model algorithm (12). The FIAU concentration in the test samples was estimated from a standard curve for concentrations ranging from 0.006 to 1,000 ng/ml.

RIA validation. The detection limit (sensitivity) of the RIA method was calculated statistically by the method of Rodbard (41). We assessed interassay precision and accuracy by measuring the FIAU concentration in controls that were prepared by adding reference FIAU to drug-free human serum at concentrations of 0, 0.5, 5, and 50 ng/ml. Serum controls were stored frozen in aliquots at −20°C. The specificity of the RIA was investigated by comparing the cross-reactivity of FIAU with a number of structurally related nucleosides. These compounds included FAU, FMAU, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-bromouracil (FBrAU), the 5′-triphosphate of FIAU, 5-iodo-uridine, and the endogenous nucleosides thymidine, uridine, cytidine, and guanosine.

Human pharmacokinetic study. Protocol H3X-LC-PPPG(a) was performed at the Lilly Laboratory for Clinical Research during the period from March through May 1993. This study was approved by our institution's Human Subjects Review Committee, and written informed consent was obtained from all participants. The primary objective of the study was to compare the bioequivalency of three different FIAU formulations (1 mg/ml of syrup and 1- and 5-mg tablets) in fasting subjects. The syrup formulation was supplied by Oclassen Pharmaceuticals, Incorporated (San Rafael, Calif.). The study was an open-label, randomized, modified Latin square design and included 16 healthy adult male volunteers between the ages of 27 and 54 years. One volunteer did not complete the study and was replaced with another subject. On the study day a 5-ml sample of venous blood was collected prior to the administration of drug. All doses of FIAU were given orally with 180 ml of water at 8:00 a.m. Blood samples were then obtained at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, and 24 h after dosing. Serum samples were stored frozen at −20°C prior to analysis by RIA. All doses of FIAU were administered following a 5-day washout period.

Drug concentration in serum data were analyzed by computer by using noncompartmental linear pharmacokinetic methods. The terminal (log-linear) phase of the declining concentration in serum was defined by selecting points from a semilogarithmic plot of each individual's serum concentration-versus-time data. A linear regression (ln*C* versus *t*, where *C* is concentration and *t* is time) incorporating these points was used to estimate the elimination rate constant (β; slope of the regression line). The elimination half-life in serum ($t_{1/2\beta}$) was calculated from the relationship 0.693/β. Areas under the serum concentration-time curves (AUCs) were calculated by trapezoidal rule from time zero through the last observed concentration in serum obtained at time *t* (AUC_{0-t}). The AUC_{0-t} was extrapolated through infinite time ($AUC_{0-\infty}$) by dividing the concentration predicted from the regression line for the last observed sampling point by β. The amount excreted unchanged in the urine was determined by measuring the concentration over a timed interval and multiplying this by the volume. Cumulative amounts were subsequently recorded.

Several pharmacokinetic variables that were calculated may have been influenced by the fraction of the dose of FIAU that ultimately reached the systemic circulation, the so-called bioavailability first-pass effect. Variables that may have been so affected are clearance from serum, nonrenal clearance, and volume of distribution. The apparent clearance from serum was calculated by dividing the dosage by $AUC_{0-\infty}$, the apparent renal clearance was determined by dividing the amount excreted unchanged in the urine by AUC_{0-24}, and the apparent nonrenal clearance was calculated as the difference between clearance from serum and renal clearance. The apparent volume of distribution was calculated by dividing the clearance from serum by β.

To obtain an estimate of half-life, we used data beyond the original 24-h sampling scheme. Because of the sensitivity of the RIA, we were able to quantify the concentration of FIAU in most of the serum samples obtained 144 h after administration of the previous dose. Therefore, we used the predose sample (0

TABLE 1. Cross-reactivities of structurally related compounds in the RIA for FIAU[a]

Compound	ED_{50} (nM)	% Relative potency
FIAU	6.05	100.0
FBrAU	14.2	42.6
FIAU 5'-triphosphate	50.1	12.1
FAU	4,000	0.15
FMAU	4,900	0.12
5-Iodo-uridine	11,019	0.05
Thymidine	250,000	0.002
Uridine	>1,000,000	<0.001
Cytidine	>1,000,000	<0.001
Guanosine	>1,000,000	<0.001
Potassium iodide	>1,000,000	<0.001

[a] Standard curves of FIAU and the test compounds were prepared in assay buffer. Each standard curve concentration was analyzed in duplicate by the RIA method described in Materials and Methods. ED_{50}s were estimated by computer by using a 4-PL algorithm. ED_{50} is defined as the concentration of analyte necessary to displace 50% of the bound radiolabeled FIAU. The percent relative potency was then calculated from the ratio of the ED_{50} for FIAU to the ED_{50} for each test compound × 100.

h) of subsequent doses to estimate the long half-life. It should be noted that one subject in whom we estimated an 8-h half-life was the only individual who had no measurable FIAU concentrations at 144 h following the administration of any treatment.

RESULTS

Antiserum characterization. We successfully raised a specific antiserum to FIAU by immunizing 12 rabbits with the 5'-*O*-hemisuccinate analog of FIAU coupled to KLH (Fig. 1, compound 5). All rabbits produced antibodies against FIAU. Comparable antibody titers were achieved when rabbits were immunized with BSA and ovalbumin conjugates of 5'-*O*-hemisuccinate–FIAU. For synthesis of KLH conjugates, we used molar hapten presentation ratios of 2,000:1 and 10,000:1. The highest antibody titers occurred in rabbits immunized with the FIAU-KLH conjugate (2,000:1). The antibody titer for rabbit EL738 did not reach a maximum until bleed 8. However, we used the fifth bleed from this rabbit in the development of the RIA because it displayed the desired selectivity for the parent compound.

To characterize the specificity of the antiserum, we evaluated the cross-reactivities of a number of structurally related nucleosides in competitive-binding experiments. The percent cross-reactivity was calculated from the ratio of the 50% effective dose (ED_{50}) of FIAU to the ED_{50} of each compound. All of the compounds tested were substantially less potent than FIAU at displacing radiolabeled FIAU (Table 1). Modification of FIAU at either the 5 position of the pyrimidine ring or the 2' position on the furanose ring markedly reduced cross-reactivity with the antiserum. In particular, the animal metabolite FAU competed only 0.15% as well as FIAU. All of the endogenous nucleosides evaluated had cross-reactivities of <0.002%. The 5'-triphosphate of FIAU, a potential intracellular metabolite, competed 12.1% as well as the parent compound.

RIA validation. A typical standard curve for the FIAU RIA is shown in Fig. 3. Although the detection limit (sensitivity) of the RIA was estimated to be 0.07 ng/ml, we assigned 0.2 ng/ml as a lower limit of quantitation to decrease the potential for false-positive results resulting from differences in the sample matrix. The optimal working range of the RIA was 0.5 to 50 ng/ml. For 10 validation assays the level of nonspecific binding was 4.3% ± 0.18% (mean ± standard error of the mean [SEM]), with a maximum binding of 24.7% ± 0.54%. The slope and ED_{50}s were 0.87 ± 0.01 and 2.21 ± 0.04 ng/ml, respectively, with coefficients of variation (CVs) for all standard curve parameters ranging from 3.3 to 14.5%. We evaluated interassay precision (coefficients of variation) and accuracy by measuring the FIAU concentration in serum control samples. In all cases the zero control generated a response that was below the limit of quantitation of the RIA. For the serum controls the interassay coefficients of variation were 19.7% (0.5 ng/ml), 5.0% (5 ng/ml), and 9.4% (50 ng/ml), respectively, with mean recoveries that ranged from 81 to 109%.

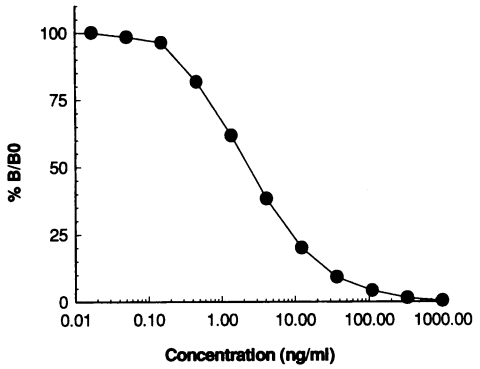

FIG. 3. Standard curve of FIAU. *B*/*B*0, binding of radiolabeled FIAU (*B*) relative to maximum binding (*B*0), the level of binding in the absence of unlabeled FIAU.

For the measurement of the FIAU concentration in serum, all samples were analyzed undiluted and following a 1:5 and 1:50 dilution with drug-free human serum. Assay parallelism was demonstrated by the reproducible recovery of immunoreactive FIAU following dilution (data not shown). We also confirmed the identity of immunoreactive FIAU as the parent compound by two different procedures. First, we evaluated the elution behavior of immunoreactive FIAU during C_{18} RP-HPLC. Samples of serum and urine were extracted, dried under vacuum, resuspended in the mobile phase, and chromatographed. Thirty 1-ml fractions were collected beginning immediately after injection. The isocratic elution conditions that we used completely resolved FIAU, FAU, FMAU, thymidine, and uridine, with FIAU having the longest retention time. In all cases we found only one peak of immunoreactive ^{1}FIAU, and the peak coeluted with standard FIAU. Second, FIAU concentrations were confirmed in selected serum samples by a PE-Sciex API III LCMS method with a sensitivity of 0.5 ng/ml.

Pharmacokinetic study in healthy volunteers. We used the new RIA to evaluate the pharmacokinetics of FIAU after administration of a single 5-mg oral dose to 16 healthy adult male volunteers. Only data following the administration of the syrup formulation of FIAU are reported, since this is the only formulation that was used in other clinical studies. Mean pharmacokinetic variables are summarized in Table 2, and mean concentration data are depicted graphically in Fig. 4. The sampling scheme was designed on the basis of an estimated $t_{1/2\beta}$ of 3 to 4 h. This estimate was obtained after the administration of radiocarbon-labeled FIAC, the deoxycytidine analog of FIAU, and subsequent analysis by RP-HPLC (15). The RIA used in the present study is at least 250 times more sensitive than the UV-based HPLC assay. Thus, the RIA allows the quantification of FIAU concentrations in serum samples collected at much later times, when the concentrations

TABLE 2. Model-independent kinetic variables of FIAU after administration of a single 5-mg oral dose to 16 healthy subjects

Variable[a]	Mean	Standard deviation	Range
Body wt (kg)	72.0	7.5	60.6–87.7
C_{max} (ng/ml)	238	75.5	143–425
T_{max} (h)	0.5	0.1	0.5–1.0
Elimination rate (h^{-1})	0.0266	0.0157	0.0194–0.0852
$t_{1/2\beta}$ (h)	29.3	6.3	8.1–35.7
AUC_{0-24} (ng · h/ml)	516	127	310–811
AUC_{0-144} (ng · h/ml)	830	254	421–1443
$AUC_{0-\infty}$ (ng · h/ml)	858	244	544–1475
Clearance from serum (liters/h)	6.27	1.73	3.39–9.19
Volume of distribution (liters)	258	78.0	108–390
Amount (mg) excreted in urine at:			
0–4 h	1.51	0.53	0.12–2.17
0–8 h	1.76	0.57	0.40–2.45
0–12 h	1.92	0.61	0.52–2.66
0–24 h	2.12	0.68	0.69–3.27
Percent dose from 0 to 24 h	42.5	13.6	13.8–65.4
Renal clearance (liters/h)	4.21	1.37	1.55–6.58
Nonrenal clearance (liters/h)	2.06	1.66	0–5.40

[a] C_{max}, maximum concentration of drug in serum; T_{max}, time to maximum concentration of drug in serum; the other abbreviations are defined in the text.

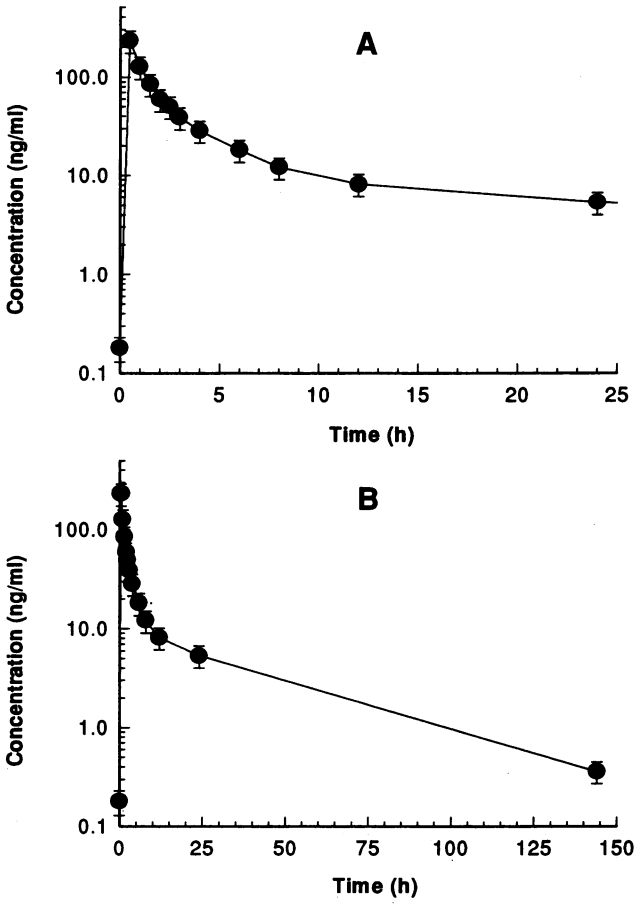

FIG. 4. Mean FIAU serum concentration-time profile in fasted healthy adult volunteers following oral administration of a single 5-mg dose (1 mg/ml of syrup × 5 ml). Data are plotted as the means ± standard errors of the means for 16 subjects. (A) Concentrations in serum 0 to 24 h after dosing; (B) concentrations in serum 0 to 144 h after dosing.

are below the sensitivity of the HPLC assay. As noted from Table 2, the half-life estimates for FIAU ranged from 8.1 to 35.7 h, with a mean of 29.3 h. The peak concentration of FIAU ranged from 143 to 425 ng/ml, with a mean of 238 ng/ml. In general, these peak concentrations agreed with the data obtained by RP-HPLC. The absorption of FIAU was rapid, with a time to the maximum concentration in serum of 0.5 h. The time to the peak of 0.5 h is only an estimate, because this was the first sample collected following the administration of FIAU. The mean AUC for FIAU during the first 24 h after dosing was 516 ng · h/ml, with a range of from 310 to 811 ng · h/ml. When extrapolated to infinity, the mean AUC was 858 ng · h/ml and ranged from 544 to 1,475 ng · h/ml. The area calculated in the first 24 h was 60% of the total AUC, while the area calculated to 144 h represented approximately 96% of the total AUC. Approximately 40% of the FIAU was excreted in urine within the first 24 h, and 70% of that was excreted within the first 4 h. Following the peak at 0.5 h, there was a rapid decline in the concentration in serum through the first 8 h. Thereafter, the decline occurred more slowly, with a terminal $t_{1/2\beta}$ of about 29 h. The mean clearance from serum was 6.3 liters/h, whereas the renal clearance was 4.2 liters/h. A comparison of the clearance from serum and urine excretion data suggested that two-thirds of the dose is excreted in the urine as parent compound.

DISCUSSION

In this report we described the development of a sensitive and specific RIA for the quantification of FIAU, a halogen-substituted analog of thymidine. For the following reasons we used the 5′-*O*-hemisuccinate analog of FIAU to synthesize protein conjugates for the development of a specific antiserum against FIAU. First, the presence of a hemisuccinate substituent facilitated the synthesis of protein conjugates by appending a carboxyl-bearing moiety on FIAU. Second, bridging groups are frequently added to small molecules to enhance the formation of hapten-specific antibodies (8, 53). Third, protein conjugates of 5′-*O*-hemisuccinate derivatives have successfully been used to produce rabbit antibodies against the nucleosides 1-β-D-arabinofuranosylcytosine (ara-C) and 1-β-D-arabinofuranosyluracil (33–35, 45). Finally, a spatial model indicated that the 5′ position on the arabinofuranosyl ring was most distal from the iodine substituent at position 5 on the pyrimidine ring. This was important, because previous data indicated that the des-iodo-FIAU, FAU, was a major metabolite in animals and humans (15, 37). Therefore, according to Landsteiner's principle (3), immunization with protein conjugates of 5′-*O*-hemisuccinate-FIAU was predicted to result in the formation of antibodies that recognize the 5-iodo region of the pyrimidine ring and that do not cross-react with FAU.

For the present study we wanted to use an analog of FIAU bearing a 5′-*O*-hemisuccinic acid moiety for coupling to protein ε-amino groups by mixed-anhydride chemistry. The most direct approach, which involved the treatment of FIAU with succinic anhydride, gave inseparable mixtures of 3′-mono-, 5′-mono, and 3′,5′-bis-hemisuccinic acids. A mono-2,2,2-trichloroethyl ester of succinyl chloride has been used to form an ester at the 5′-hydroxyl of cytidine derivatives, including ara-C

(21, 33–35). However, the harsh conditions required for removing the trichloroester prompted us to use an alternative strategy involving an allyl protected ester. Thus, as shown in Fig. 1, the succinic acid monoallyl ester was prepared efficiently by reacting allyl alcohol with succinic anhydride in the presence of 4-(*N*,*N*-dimethylamino)pyridine; this was followed by a short-path distillation (50). Regioselective 5′-*O* monoacylation was not trivial, but an acceptable amount of the desired ester was obtained upon treatment of FIAU with the corresponding acid chloride in pyridine at 0°C. Under these reaction conditions the 3′,5′-bisester and 3′-monoester could also be isolated in small quantities by normal-phase chromatography. The synthetic sequence was completed by using the palladium (0)-catalyzed exchange deprotection method of Jeffrey and McCombie (25). Oxidative insertion of Pd (0) into the C-5–I bond of the pyrimidine ring did not compete with the formation of the Π-allyl complex and subsequent deprotection. Additionally, the yield and reproducibility of this reaction were improved substantially by the use of oven-dried sodium 2-ethylhexanoate and freshly prepared catalyst. The structure of compound 4 was confirmed by elemental analysis, mass spectrometry, and ^{1}H NMR studies. The signal of the 5′-CH_2 in compound 4 appears 0.7 ppm down field relative to that of FIAU (4.32 and 3.62 ppm, respectively). The chemical shift of the 3′-CH remains virtually unchanged for both molecules. Thus, the major acylation product was the 5′-*O*-hemisuccinate analog of FIAU.

Immunization of rabbits with a 5′-*O*-hemisuccinate analog of FIAU coupled to KLH resulted in the production of a specific anti-FIAU 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 1). Modifications at either the 5 position of the pyrimidine ring or the 2′ position of the furanose ring markedly reduced the cross-reactivity with the antiserum. Removal of the iodine substituent resulted in a dramatic reduction in the level of cross-reactivity. FIAU was more than 500 times more potent than FAU, and free iodine failed to displace radiolabeled FIAU at concentrations as high as 1 mM. Therefore, the presence of the iodine substituent is critical for recognition by the antibody. This conclusion was supported by the high potency found for the 5-bromo analog of FIAU. In contrast, the 5-methyl analog of FIAU, FMAU, was nearly 400 times less potent than the bromo analog, even though the methyl and bromine moieties have similar Van der Waal's radii. These data suggest that the antigenic determinant of FIAU comprises both steric and electronic components. All endogenous nucleosides evaluated, including thymidine, uridine, cytidine, and guanosine, had a negligible level of cross-reactivity in the RIA. Even though 5-iodo-uridine was 100 times more potent than uridine, it was nearly 2,000-fold less potent than FIAU. Therefore, the 2′-fluoro moiety on the arabinofuranosyl ring is also important for defining the antibody-binding epitope. Thus, we concluded that the antiserum is highly specific for FIAU and that full cross-reactivity requires the presence of both the 5-iodo and 2′-fluoro substituents.

An unknown glucuronide, most likely at the 5′ position, has been reported to be a minor metabolite of FIAU in humans following intravenous administration of radiolabeled FIAC (15). Cross-reactivity experiments were not performed with the 5′-glucuronide because of the lack of its availability. However, we determined that modification of FIAU at the 5′ position of the arabinofuranosyl ring also reduces cross-reactivity, because the 5′-triphosphate analog was only 12% as potent as FIAU (Table 1). Thus, the reduced cross-reactivity of the 5′-triphosphate analog suggests that the presence of a 5′-glucuronide metabolite in biological fluids will not interfere with the specific measurement of FIAU.

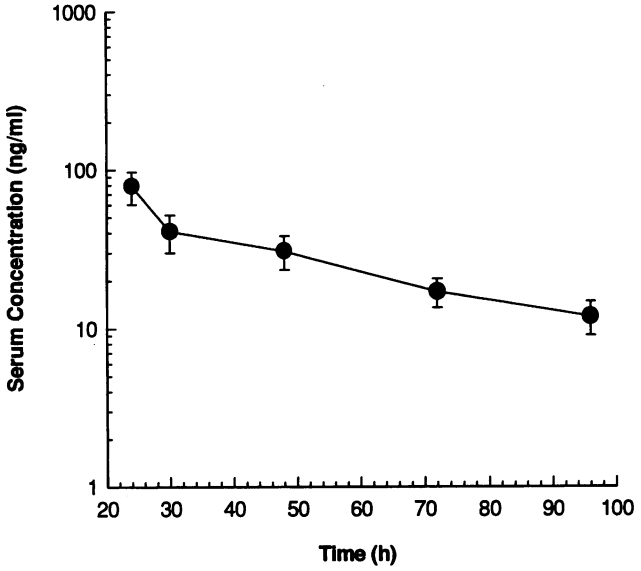

FIG. 5. Terminal elimination phase for concentrations of FIAU in sera of dogs following oral administration at 3 mg/kg for 3 months. Data are plotted as the means ± standard errors of the means (n = 5).

Assay sensitivity was maximized by the use of a radiolabeled ligand of high specific activity and the addition of the test sample at 300 μl. In contrast to a large number of published RIAs for nucleosides that used tritium as the radiolabel (1, 33–35, 39, 42, 44, 45), we used radioiodinated FIAU as the tracer. The preparation of a radioiodinated tracer was facilitated by the presence of the iodine substituent on the pyrimidine ring of FIAU. Radioiodinated FIAU was synthesized to a high yield by a destannylation procedure with sodium [^{125}I]iodide (52). Because of its high specific activity (>2,000 Ci/mmol), we added only 25 pg of [^{125}I]FIAU to each binding reaction. For freshly prepared material, this quantity of tracer routinely produced >200,000 cpm. The hydrophilic nature of FIAU permitted the use of PEG as a method for separating the bound and free forms of radiolabeled FIAU. PEG is widely used as a separation reagent in RIAs (4), and it has been used in RIAs of AZT (10, 16). The combined use of a radioiodinated tracer and PEG precipitation resulted in a separation procedure that required minimal manipulative effort and that was applicable for the processing of large numbers of samples.

We used the new RIA method to assess the pharmacokinetics of FIAU following administration of a single 5-mg oral dose to healthy volunteers. The RIA permitted the characterization of an elimination phase for FIAU with a mean $t_{1/2}$ of 29.3 h (Fig. 4). The prolonged elimination phase was not unique to humans, because we found a similar $t_{1/2\beta}$ of 35.3 h in dogs following oral administration of FIAU at 3 mg/kg for 3 months (Fig. 5). Other antiviral nucleoside analogs such as AZT have been reported to have $t_{1/2}$s in the range of 1 to 3 h following oral dosing (6, 9, 27, 32). Recently, Morse et al. (30, 32) reported that they were able to detect the presence of a prolonged elimination phase for AZT ($t_{1/2\beta}$ = 13.9 h) in patients infected with human immunodeficiency virus by a sensitive RIA method but not by HPLC. They also proposed that a multiexponential decay curve should more accurately describe the oral disposition of AZT than a monoexponential

decay curve, assuming an intracellular site of action (31, 32). FIAU has been detected in the tissues and DNA of mice following acute intraperitoneal administration of radiocarbon-labeled 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (5, 22). Recently, FIAU was found to accumulate in the DNAs of several different animal species following chronic oral administration (40). Thus, we hypothesize that the RIA permits the detection of FIAU in plasma and urine following the efflux of drug from tissue depots, and this contributes to the prolonged elimination phase.

In summary, we reported the development of a sensitive and specific RIA method for the quantification of FIAU in biological fluids. The sensitivity of the RIA permitted demonstration of a prolonged elimination phase for FIAU. The RIA has been used successfully to measure the concentrations of FIAU in serum from a number of different species, in addition to humans and dog, including rats, monkeys, and woodchucks. Thus, we conclude the RIA will be useful in further studies of the disposition of FIAU in animals and should facilitate the investigation of the mechanism of delayed toxicity of FIAU.

ACKNOWLEDGMENTS

We recognize the following individuals at the Lilly Research Laboratories for contributions to this project. We gratefully acknowledge William J. Wheeler for coordinating the synthesis of radioiodinated FIAU at DuPont, NEN Research Products. We thank Todd A. Gillespie and Anthony Murphy for performing the Sciex API III LCMS analyses and Pat Dhahir, Tamara Priest, and Charlotte McKinney for performing the RP-HPLC to assess the specificity of the RIA. We recognize the efforts of Jeffery A. Engelhardt and the animal studies group in the dog study of FIAU. We also thank Richard F. Bergstrom, Joseph M. Colacino, and Jennifer L. Stotka for helpful discussions.

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