A Sensitive and Specific Radioimmunoassay for LY309887, a Potent Inhibitor of Glycinamide Ribonucleotide Formyltransferase

Dennis L. Coleman, Nedjelko Canak, Gary D. Place, Chuan Shih, and Ronald R. Bowsher


ABSTRACT

LY309887, a reduced analogue of folic acid, is a potent inhibitor of glycinamide ribonucleotide formyltransferase and possesses a broad spectrum of antitumor activity. During preclinical studies using supplementation with oral folic acid, this second-generation inhibitor displayed both the desired safety profile and the pharmacology to warrant clinical investigation. A sensitive analytical method was needed to assess the pharmacokinetics of LY309887 due to the low doses planned for Phase 1 studies and the potential for low concentrations in plasma long after i.v. administration. We therefore undertook the development of a competitive RIA. A highly specific antiserum was raised in rabbits following immunization with LY309887 coupled to BSA. A RIA tracer was prepared by radioiodination of compound 389753, the adduct of LY309887 with p-tyramine. We developed a competitive-binding RIA procedure and used superparamagnetic particles coated with goat antirabbit IgG as a method for separating the bound and free forms of LY309887. The RIA is sensitive (0.5 ng/ml in serum and 25 ng/ml in urine), specific (negligible interference from endogenous folates), and reproducible (interassay coefficients of variation ranging from 8.1 to 15.4% and 7.6 to 8.3% for serum and urine controls, respectively). We used the RIA to assess the i.v. pharmacokinetics of LY309887 in both patients with metastatic cancer and dogs. The sensitivity of the RIA permitted the demonstration that serum concentrations of LY309887 decline in a multieponential manner with a prolonged terminal elimination phase. We conclude that the RIA is a valid method for quantifying LY309887 in biological fluids.

INTRODUCTION

LY309887, (R)-N-[[2-(2-amino-1,4,5,6,7,8-hexahydropyrazin-6-yl)ethyl]-2-thienyl[carbonyl]-L-glutamic acid (Fig. 1), is a potent inhibitor of GARFT (EC 2.1.2.2; Refs. 1 and 2). This enzyme catalyzes the first of two folate-dependent reactions in the de novo synthesis of purines. LY309887 follows lometrexol (Fig. 1), a previously identified first-generation GARFT inhibitor (2, 3). Clinical trials of lometrexol were limited due to an unexpected delayed and cumulative toxicity characterized by mucositis, thrombocytopenia, and leukopenia (4-7). LY309887, a second-generation inhibitor, is more potent than lometrexol, requires less polyglutamation for its antitumor activity, and is active in a broad spectrum of both murine and human xenograft tumor models (1, 2).

During preclinical studies, using supplementation with oral folic acid, LY309887 displayed both the desired safety profile and the pharmacology to warrant clinical investigation (2, 8). A sensitive analytical method was needed to evaluate the pharmacokinetics of LY309887 due to the low doses planned for Phase 1 studies and the potential for low concentrations in plasma long after i.v. administration. We, therefore, undertook the development of a competitive RIA for a number of reasons. (a) RIAs are sensitive analytical methods that are capable of detecting small molecules at nanomolar concentrations. (b) RIAs have been developed for a number of antifolate drugs, including lometrexol (9, 10) and methotrexate (11-15). (c) Preclinical metabolism studies have suggested that LY309887 is excreted largely unchanged in urine. Consequently, metabolite interference should not present a problem in the development of a RIA. (d) RIAs are cost effective and have a high level of analytical throughput.

MATERIALS AND METHODS

Chemicals. Reference standard LY309887 was obtained from the Lilly Research Center, Ltd., at Erl Wood, United Kingdom. The USP folic acid was obtained from the analytical reference standard Department at the Lilly Corporate Center (Indianapolis, IN). HEPES was purchased from Calbiochem (La Jolla, CA). Sodium chloride was obtained from Mallinckrodt (Paris, KY). Calcium chloride dihydrate was obtained from EM Science (Gibbstown, NJ). BSA was purchased from Boehringer Mannheim. BioMag goat antirabbit IgG (heavy- and light)-coated superparamagnetic iron oxide particles were obtained from PerSeptive Diagnostics (Framingham, MA). Control human serum was purchased from Biological Specialty Corp. (Lansdale, PA). A pool of control human urine was obtained from PerSeptive Diagnostics.
After dialysis for 24 h at 4°C, the conjugates were concentrated using cassettes with a 10,000 molecular weight cutoff (Slide-A-Lyzer; Pierce Chemical Co., Rockford, IL). Protein conjugates were dialyzed against 10 mM sodium phosphate-0.9% sodium chloride (two 4-liter volumes) using cassettes with a 10,000 molecular weight cutoff (Slide-A-Lyzer; Pierce Chemical Co., Rockford, IL). After dialysis for 24 h at 4°C, the conjugates were concentrated to 1 mg/ml and stored at −20°C.

Immunization. Production of rabbit anti-LY309887 antisera was carried out at Covance, Inc. (Denver, PA). Each immunogen solution was emulsified with an equal volume of Freund’s complete adjuvant. Four groups of six female New Zealand White rabbits were then immunized with 0.5 mg of an immunogen by multiple i.m. injections. For booster injections, each immunogen was emulsified with an equal volume of Freund’s incomplete adjuvant containing 1 mg/ml diphenhydramine. The rabbits received i.m. 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 ELISA in bleeds collected at 14-day intervals beginning at day 42.

Anti-LY309887 Antisera. Sera from two rabbits [EL961 (bleeds 3–11) and EL962 (bleeds 8–11)] immunized with the BSA-LY309887 conjugate (100:1) were selected for use in RIA applications. The pooled antiserum (~250 ml) was stored as 0.5-ml aliquots in plastic vials at −20°C.

Synthesis of Compound 389753, the p-Tyramine Adduct of LY309887. To a sample of 50 mg (0.1114 mmol) of LY309887 in 5.0 ml of dry DMSO at room temperature, 20°C, were added 26 mg of EDAC [1-(3-dimethylamino-propyl)-3-ethylcarbodiimide; 0.1356 mmol, 1.2 equivalent] and 52 mg of N-hydroxysuccinimide (0.4522 mmol, 4.0 equivalent). The reaction mixture was stirred overnight at 20°C before 76.3 mg of p-tyramine (0.557 mmol, 5.0 equivalent; free base) were added. The resulting reaction mixture was stirred at 20°C for 6 h and monitored by C18 reversed-phase HPLC. The chromatographic profile indicated the formation of the two mono-p-tyramine adducts (both the α- and the γ-p-tyramine adducts) with the glumatate portion of LY309887, a bis-p-tyramine adduct, and some unreacted (approximately 25%) LY309887. DMSO was removed under high vacuo at 70°C, and the resulting residue was dissolved in 30% CH3CN-70% 0.01 m ammonium acetate and purified on a preparative C18 reversed-phase HPLC column eluted with the same solvent system (30% CH3CN-70% 0.01 m ammonium acetate). The fractions of mono-p-tyramine adducts (both the α- and γ-conjugates) were collected and lyophilized to yield 45 mg of compound 389753 as a white, amorphous solid.

Preparation of Radiolabeled 389753. Radioiodinated 389753 was prepared bimonthly by the chloramine-T method at Amersham International Corp. (Arlington Heights, IL; Ref. 19). Radioiodinated 389753 was purified by C18 reversed-phase HPLC using gradient elution from 25 to 35% solvent B (solvent A, 0.1% trifluoroacetic acid in water; solvent B, 0.1% trifluoroacetic acid in 70% CH3CN). After purification, radioiodinated 389753 routinely had a specific activity of ≥2000 Ci/mmol and a radiochemical purity of >90%. The purified tracer was supplied in methanol at a concentration of 0.25 mCi/ml. We diluted the stock tracer to 0.1 ng/ml in assay buffer containing 25 ng/ml of folic acid for use in the RIA. The tracer solution was stable for at least 2 months when stored at 4°C.

RIA Reagents. All solutions except the stock solutions of LY309887 and antibody were stored at 4°C. The assay buffer used for diluting the tracer and antibody consisted of 50 mM HEPES, 0.9% sodium chloride, 0.25 mM calcium chloride, 0.1% sodium azide, 0.1% Tween 20, and 1% BSA, adjusted to pH 7.5. A 10 µg/ml stock solution of LY309887 was prepared in assay buffer without 0.25 mM calcium chloride. The stock standard was stable for at least 1 year when stored frozen in 1-ml aliquots in polypropylene tubes at −20°C. Fresh RIA standard curves were prepared weekly by diluting the stock solution of LY309887 in serum or plasma from the same species as the test samples.

Urine samples were analyzed following a dilution of at least 1:50 in assay buffer. Urine standard curves were prepared in assay buffer containing 2% drug-free urine from normal adult volunteers. Samples that had concentrations of LY309887 above the upper LOQ of the RIA were reanalyzed following dilution in assay buffer containing 2% drug-free human urine.

RIA Procedure. We developed a competitive-binding RIA for quantifying LY309887 in biological fluids. Each binding reaction (total volume, 650 µl) was performed in a 12 × 75-mm polypropylene tube and consisted of 500 µl of radioiodinated 389753 (50 pg/tube), 50 µl of serum or diluted urine (test sample or standard LY309887), and 100 µl of anti-LY309887 antisera (diluted 1:600). The level of nonspecific binding was determined by replacing antibody with assay buffer. After mixing, we incubated the binding reaction at room temperature for 18–24 h. The bound and free forms of the immunoreactive tracer were separated by adding 750 µl of assay buffer containing 2% drug-free human urine and 100 µl of anti-LY309887 antisera (diluted 1:600). The level of nonspecific binding was determined by replacing antibody with assay buffer. After mixing, we incubated the binding reaction at room temperature for 18–24 h. The bound and free forms of
LY309887 were then separated by adding 250 µl of goat anti-rabbit IgG superparamagnetic particles. Each tube was vortexed well and incubated at room temperature for 30 min. Following the addition of 1 ml of assay buffer, the tubes were vortexed and placed in a magnetic test tube rack. We decanted the liquid phase after 15 min and measured the radioactivity adhering to the beads in a gamma counter. A VAX computer was used to analyze the RIA data by a weighted four-parameter logistic algorithm.

### Table 1
Cross-reactivity of structurally related compounds in the RIA for LY309887

<table>
<thead>
<tr>
<th>Compound</th>
<th>ED_{50} (nm)</th>
<th>% relative potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY309887</td>
<td>1.53</td>
<td>100.0</td>
</tr>
<tr>
<td>Lometrexol</td>
<td>2.54</td>
<td>60.2</td>
</tr>
<tr>
<td>7,8-Dihydrofolate</td>
<td>4874.00</td>
<td>0.031</td>
</tr>
<tr>
<td>5,6,7,8-Tetrahydrofolate</td>
<td>6908.00</td>
<td>0.022</td>
</tr>
<tr>
<td>5-Methyl-5,6,7,8-Tetrahydrofolate</td>
<td>20339.00</td>
<td>0.008</td>
</tr>
<tr>
<td>Folic acid</td>
<td>&gt;10,000,000.00</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>&gt;10,000,000.00</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*Standard curves of LY309887 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} values were estimated by computer using a 4-PL algorithm. ED_{50} is defined as the concentration of analyte necessary to displace 50% of the bound radiolabeled LY309887. The percentage of relative potency was then calculated from the ratio of the ED_{50} for LY309887:ED_{50} for each test compound × 100.*

**RIA Validation.** The RIA validation experiments were conducted according to current bioanalytical recommendations (21, 22). We assessed interassay precision and accuracy by measuring the LY309887 concentration in controls that were prepared according to current bioanalytical recommendations 5,6,7,8-tetrahydrofobate, 5-methyltetrahydrofolate, methotrexate, and folic acid.

**Pharmacokinetic Study.** A dose-escalating, open-label Phase I study of LY309887 was conducted in patients with metastatic cancer. In this study, LY309887 was administered at doses ranging from 1 to 12 mg/m² by i.v. injection once every 21 days with oral folic acid supplementation 2 days prior to, on the day of, and 2 days after administration of LY309887. Samples of whole blood were collected as serum at specified times for up to 96 h after dosing and then at weekly intervals until the administration of the next i.v. dose. The serum samples were stored frozen at −20° C prior to RIA analysis.

**RESULTS**

**Antiserum Characterization.** We successfully raised a specific antiserum to LY309887 by immunizing 24 rabbits with LY309887 coupled to BSA, ovalbumin, and keyhole limpet hemocyanin. Following immunization, rabbit sera were screened for the presence of anti-LY309887 antibodies by ELISA using plates coated with LY309887 coupled to RSA. Selectivity for LY309887 was evaluated by comparing the antibody binding to LY309887-RSA and folic acid-RSA. On the basis of the ELISA results, all rabbits produced antibodies against LY309887. However, sera from only 12 of 24 rabbits displayed the desired selectivity for LY309887. These sera were evaluated further for their ability to bind radiolabeled 389753, the radiolabeled p-tyramine adduct of LY309887. Antisera from two rabbits immunized with the BSA-LY309887 conjugate (1:100) were selected for use in the development of a RIA based on their affinity and selectivity for LY309887.

We evaluated the cross-reactivity of a number of structurally related compounds in competitive-binding experiments to characterize the specificity of the antiserum. These compounds included both endogenous folates and structurally related antifolate drugs. The compounds evaluated included lometrexol, 7,8-dihydrofolate, 5,6,7,8-tetrahydrofolate, 5-methyltetrahydrofolate, methotrexate, and folic acid.

**Effect of Divalent Cations on Antibody Binding.** During RIA development, we discovered that binding of radiolabeled 389753 by antibody was increased substantially by the presence of divalent cations. As reported in Table 2, all divalent cations tested increased antibody binding relative to the level in buffer only. Although Zn^{2+} and Cu^{2+} produced the greatest enhancement in antigen binding at low concentrations, we selected Ca^{2+} for use in the RIA due to its superior solubility. Accordingly, 0.25 M Ca^{2+} was included in the assay buffer used for diluting both the tracer and antiserum.

**RI A Validation.** Typical standard curves of LY309887 in serum and urine are shown in Fig. 2. The validated range of
the serum RIA was 0.5–5 ng/ml. We collected standard curve data from 39 RIAs over a period of more than 10 months. For these assays, the level of nonspecific binding was 1.32% ± 0.03% (mean ± SE), with a maximum binding of 36.8% ± 0.86%. The slope and ED\textsubscript{50} values were 1.66 ± 0.02 and 1.56 ± 0.03% (mean ± SE), with a maximum binding of 36.8% ± 1.03% for 26 assays. The slope and ED\textsubscript{50} values were 1.68 ± 0.02 and 1.84 ± 0.08 ng/ml, respectively.

We evaluated interassay precision (%CV) and accuracy by measuring the LY309887 concentration in serum and urine control samples. In all cases, the zero serum and diluted urine control samples produced responses that were below the LOQ of the RIA. For serum controls (n = 75), the interassay %CVs were 15.4% (0.5 ng/ml), 9.2% (1 ng/ml), and 8.1% (5 ng/ml), respectively, with mean recoveries that ranged from 92.0 to 108%. LY309887 was determined to be stable in human serum for at least 1 year when stored frozen at a temperature of -20°C or lower. The interassay %CVs for the urine controls (n = 48) were 8.3% (25 ng/ml), 7.6% (50 ng/ml), and 8.2% (250 ng/ml), respectively, with mean recoveries that ranged from 99.2 to 121%. Assay parallelism (dilutional linearity) was demonstrated by the reproducible recovery of immunoreactive LY309887 following dilution of either serum or urine test samples (data not shown).

To confirm the identity of immunoreactive LY309887 as a parent compound, we compared the plasma concentrations of immunoreactive LY309887 to those determined by HPLC and \(^3\)-counting after i.v. administration of a 0.25 mg/kg dose of \(^{14}\)C-LY309887 to female beagles. As shown in Fig. 3, a–c, the concentrations of immunoreactive LY309887 agreed closely with the levels determined by HPLC for all samples with concentrations greater than 10 ng/ml, the limit of detection of the HPLC method. Thus, these data support the view that the RIA is specific for LY309887. However, the RIA method permitted the demonstration of a prolonged elimination phase for LY309887 due to its superior sensitivity. Immunoreactive LY309887 was detectable in the serum of all dogs for a period of at least 72 h. In one dog, immunoreactive LY309887 was detected in plasma 120 h after i.v. administration. Plasma concentrations determined by \(^3\)-counting only agreed with the results of RIA and HPLC in samples collected immediately following i.v. administration. The levels LY309887 determined by \(^3\)-counting were consistently higher than either the HPLC or RIA methods beyond 4–6 h.

**Phase I Pharmacokinetic Study.** We used the new RIA to evaluate the pharmacokinetics of LY309887 after i.v. administration of single doses to patients with metastatic cancer. Representative data from four patients are depicted in Fig. 4 for doses ranging from 1 to 12 mg/m\(^2\). Serum concentrations of LY309887 declined in a multieponential manner upon termination of the bolus infusion. LY309887 concentrations reached a nadir at about 24 h and then persisted at that level for at least 72 h. At 72 h, the serum concentrations of immunoreactive LY309887 ranged from 0.5 to 4.2 ng/ml. LY309887 was detected in samples collected 96 h after dosing in two of four patients.

**DISCUSSION**

In this report, we described the development of a sensitive and specific RIA for the quantification of LY309887, a second-generation GARFT inhibitor. We used mixed anhydride chemistry to prepare protein conjugates of LY309887. By coupling LY309887 to proteins through its glutamate carboxyl groups, the hapten was oriented to maximize formation of antibodies that would recognize the pteroic acid portion of LY309887. This conjugation strategy, which has been used to produce antibodies against folic acid and related antifolate drugs (23–25), minimized the potential for cross-reactivity with endogenous folates.

Antibody specificity was evaluated in cross-reactivity experiments involving a number of structurally related compounds (Table 1). Modifications at either the pteridine or thiophene portions of LY309887 reduced cross-reactivity with the antisemum. In particular, the high level of cross-reactivity found with lometrexol and not seen with other naturally occurring folates suggests that the 5,10-dideaza pteridine moiety is critical for defining the antigenic epitope. Of the endogenous folates tested, only 7,8-dihydrofolate and 5,6,7,8-tetrahydrofolate cross-reacted greater than 0.01% as well as LY309887. Thus, we concluded that the antiserum is specific for LY309887 and that naturally occurring folates will not cross-react in the RIA. This conclusion is supported by the observation that serum samples collected from patients prior to the administration of LY309887 consistently produce a response below the lower LOQ of the RIA.

During RIA development, we discovered that the binding of radiolabeled 389753 by antibody was increased significantly by the presence of divalent cations (Table 2). All divalent cations tested enhanced antigen binding. Although Zn\(^{2+}\) and Cu\(^{2+}\) were found to produce the greatest stimulation at low concentrations, we selected Ca\(^{2+}\) for use in the RIA due to its superior solubility. A final concentration of about 0.2 mM Ca\(^{2+}\) in the binding reaction was determined to be optimal. Presumably, the presence of divalent cations induces a conformational...
Concentration-time profile of immunoreactive LY309887 in the serum patients with metastatic cancer after bolus i.v. administration at doses of 1, 4, 8, and 12 mg/m². Serum concentrations of LY309887 were determined by RIA in samples collected up to 96 h after dosing. The results are plotted as follows: 1 mg/m² (○); 4 mg/m² (△); 8 mg/m² (□); and 12 mg/m² (▼).

change in radiolabeled LY309887 or anti-LY309887 antibodies, which results in an increase in antigenicity. Calcium-dependent antigen-antibody reactions have been reported for polypeptides with a high glutamic acid content and acidic polysaccharides (26, 27). Because the glutamic acid moiety is common to all endogenous folates and antifolate drugs, we speculate that divalent cations may also increase the antigenicity of these compounds. Thus, the use of buffers containing calcium or other divalent cations may be beneficial in RIAs of folic acid and related antifolate drugs.

Assay sensitivity was maximized by using a radiolabeled ligand of high specific activity and an efficient method for separating the bound and free forms of LY309887. In contrast to a number of published RIAs for folic acid and antifolate drugs that used tritium as the radiolabel (11–14, 23, 28), we used a radioiodinated p-tyramine adduct of LY309887 as the tracer. Histamine and tyrosine methyl ester adducts have also been used as radioiodinated tracers in RIAs of folic acid analogues (23, 29, 30). Because of its high specific activity (≥2000 Ci/mmol), we added only 50 pg of 125I-labeled 389753 to each in vitro binding reaction. Superparamagnetic iron oxide particles coated with goat antirabbit IgG (heavy and light) were used to separate the bound and free forms of LY309887. This reagent produced efficient separation, eliminated the need for centrifugation, and yielded a nonspecific binding of only about 1.5%.

Fig. 3  a, serum concentration-time profile of LY309887 in a female beagle (dog 261882) after bolus i.v. administration of a 0.25 mg/kg dose of [14C]LY309887. Serum concentrations of LY309887 were determined by HPLC (△), β-counting (○), and RIA (▼) in samples collected up to 96 h after dosing. b, serum concentration-time profile of LY309887 in a female beagle (dog 261902) after bolus iv. administration of a 0.25 mg/kg dose of [14C]LY309887. Serum concentrations of LY309887 were determined by HPLC (△), β-counting (○), and RIA (▼) in samples collected up to 96 h after dosing. c, serum concentration-time profile of LY309887 in a female beagle (dog 261942) after bolus i.v. administration of a 0.25 mg/kg dose of [14C]LY309887. Serum concentrations of LY309887 were determined by HPLC (△), β-counting (○), and RIA (▼) in samples collected up to 96 h after dosing.
The new method was used to assess the pharmacokinetics of LY309887 in both beagles and patients with metastatic cancer (Figs. 3, a–c, and 4). For both species, the serum concentrations of LY309887 decreased in a multiexponential manner upon termination of the bolus infusion. LY309887 concentrations reached a nadir at about 24 h and then persisted at that level for at least 72 h. Serum levels of lomotrexol have also been reported to decline in a multiexponential manner with a terminal half-life of 55 h (32, 33). Thus, our preliminary human data suggest that the i.v. pharmacokinetics of LY309887 are qualitatively similar to those reported previously for lomotrexol and the related antifolate drug, methotrexate.

In summary, we report the development of a sensitive and specific RIA method for the quantification of LY309887 in biological fluids. The new RIA has been used successfully to measure the concentrations of immunoreactive LY309887 in sera of humans and dogs and in human urine. The sensitivity of the assay permitted the demonstration that serum concentrations of LY309887 decrease in a multiexponential manner with a prolonged terminal elimination phase. We conclude the RIA is a valid method for quantifying LY309887 in biological fluids. The new method will be useful in future studies to further characterize the disposition of LY309887 in humans and animals.

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References


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