Sensitive, Specific Radioimmunoassay for Quantifying Pergolide in Plasma

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Pergolide, a synthetic ergoline with potent dopaminergic activity, is used to treat Parkinson disease. The low plasma concentrations of pergolide achieved during therapy complicate the development of a method for its analysis. Because radioimmunoassay successfully measures other structurally related ergolines in physiological fluids, we undertook the development of a radioimmunoassay of pergolide. The detection limit of the radioimmunoassay is 21 ng/L with an optimal working range from 100 to 1000 ng/L. We maximized assay specificity by using a monoclonal antibody that displayed low cross-reactivity with pergolide sulfoxide, a major metabolite found in animals. The radioimmunoassay has performed acceptably for >2 years during toxicology studies with rats and rhesus monkeys and in clinical studies involving patients with Parkinson disease. We consider the radioimmunoassay a valid method for quantifying therapeutic concentrations of pergolide in plasma.

Additional Keyphrases: ergolines - dopamine agonists - pharmacokinetics - Parkinson disease

Pergolide is a synthetic ergoline with potent dopaminergic activity (D1 and D2) (1, 2). The mesylate salt of pergolide, (8β)-8-[(methylthio)methyl]-6-propylergoline monomethanesulfonate (LY127809, Figure 1), is marketed under the tradename Permax® (Eli Lilly and Co., Indianapolis, IN) for use as adjunctive therapy in treatment of Parkinson disease (3, 4). Pergolide is more potent than other ergolines, e.g., bromocriptine (4–7), with a mean oral therapeutic dose of 3 mg/day administered in three doses (3, 4). The small therapeutic dosage of pergolide and its extensive metabolism in vivo result in low plasma concentrations of unchanged drug (7, 8). Consequently, sensitivity has been the major issue governing the development of an analytical method for pergolide. Because of their high sensitivity, radioimmunoassays (RIAs) have been the method of choice for measuring ergolines in physiological fluids (9–12). Here we report the development of a sensitive monoclonal antibody-based RIA that is suitable for quantifying therapeutic concentrations of pergolide in plasma. We have applied the RIA to toxicology studies involving rats and rhesus monkeys and to clinical studies of patients with Parkinson’s disease.

In this study we used pergolide sulfoxide, a major metabolite in animals (8), to screen for monoclonal antibodies that displayed selectivity for pergolide. Structural characterization of human metabolites has not been possible because of (a) the small dose of pergolide that is tolerated in normal volunteers and (b) the multiplicity of metabolites formed in humans. Tracer studies involving 14C-labeled pergolide indicate that, in humans, pergolide is transformed into highly polar metabolites, with the sulfoxide, sulfone, and N-despropyl metabolites serving as intermediates that undergo secondary metabolism (8); conjugation is not a major route of pergolide metabolism in humans (8). From the metabolic information and the in vitro structure activity data, we conclude that the RIA is specific for pergolide.

Materials and Methods
Materials

Reagents. Pergolide, pergolide mesylate (LY127809), and other pergolide derivatives were synthesized at the Lilly Research Labs., Indianapolis, IN (13). The derivatives of pergolide used in this study (depicted in Table 1) were (8β)-8-[(methylthio)methyl]ergoline-6-butanoic acid (LY150442), (8β)-8-[(methylthio)methyl]-6-pentylergoline (LY169166), (8β)-8-[(methylsulfonyl)methyl]-6-propylergoline (LY149945), 6-methyl-(8β)-8-[(methylthio)methyl]ergoline (LY106543), (RS)-8β-8-[(methylsulfinyl)methyl]-6-propylergoline (LY149082), (8β)-8-(methylthio)methyl]-d-ergoline (LY149670), (8β)-1-(methylthio)-8-[(methylthio)methyl]-6-propylergoline monochloride (LY243062), and 8-[(methylsulfinyl)-methyl]ergoline (LY202991). Sodium phosphate, RIA-grade bovine serum albumin, sodium azide, disodium EDTA, and neutralized activated charcoal (Norit-1) were purchased from Sigma Chemical Co. (St. Louis, MO). Scintisol® liquid scintilla-

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tion counting fluid was obtained from Isolab Inc. (Akron, OH). Dextran T-70 was purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Pooled plasma (EDTA or heparinized) from fasted normal adults was purchased from Biological Specialty Corp. (Lansdale, PA). Drug-free rhesus monkey plasma was obtained from Rockland (Gilbertsville, PA).

Radioiodinated pergolide. (8β)-8-{[Methylthio(methyl)]-6,12'-3H2}propylergoline mesylate (lot TRQ.5770) was prepared at Amersham (Arlington Heights, IL) by reducing the 6-allyl derivative of pergolide with tritium gas (14). After isolation by preparative thin-layer chromatography, the tritiated pergolide was >98% pure (specific activity 42 kCi/mol). The stock labeled pergolide was stored in ethanol containing mercaptoethanol, 2 mL/L, under argon at -20 °C to minimize generation of oxidation products. We assessed the purity of tritiated pergolide periodically by using thin-layer chromatography with LK5DF silica-gel plates (Whatman Inc., Clifton, NJ) and a mobile phase of chloroform/methanol/acetonitrile/conc. ammonium hydroxide (60:10:27:3, by vol). The migration of authentic unlabeled pergolide was determined by visualization with ultraviolet light; radiolabeled pergolide and its oxidation products were detected by fluorography performed at -70 °C for 16 h.

Monoclonal antibody to pergolide. The immunogen was prepared by conjugating pergolide-6-butanoate, LY150442, to keyhole limpet hemocyanin by coupling with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. The efficiency of conjugation was estimated by the recovery of labeled pergolide-6-butanoate. Production of monoclonal antibody hybridomas was carried out at Hybridoma Sciences Inc. (Atlanta, GA). The specificity of antibodies produced by positive clones was evaluated by competitive RIA. Antibody from clone 12H.1.5 was selected for use in the pergolide RIA because of its low cross-reactivity with pergolide sulfoxide. Implanted clone 12H.1.5 in 50 mice generated 150 mL of monoclonal antibody-containing ascites fluid. The ascites fluid was stored in 1-mL portions at -20 °C. The antibody was diluted 50 000-fold with assay buffer for use in the RIA.

Radioimmunoassay reagents. The assay buffer for diluting tritiated pergolide and monoclonal antibody consisted of 50 mmol/L sodium phosphate buffer, pH 7.5, containing 1 g/L each of EDTA, sodium azide, and bovine serum albumin. For use in the RIA, we diluted the labeled pergolide to 1 μg/L (~100 000 counts/min per milliliter) with assay buffer. We prepared a stock solution of pergolide standard at 0.2 g/L by dissolving pergolide mesylate (LY127809) in ethanol containing mercaptoethanol, 2 mL/L. Stock pergolide was stored at -20 °C in containers purged with argon. We prepared fresh standard curves weekly by diluting the stock pergolide in human plasma. A suspension of dextran-coated charcoal was prepared by adding 5 g of charcoal to 100 mL of assay buffer that contained 1 g of Dextran T-70. We stored all RIA reagents at 4 °C.

Procedures

Radioimmunoassay. Each binding reaction (total volume 700 μL) was carried out in a 12 × 75 mm polystyrene culture tube by adding 100 μL of tritiated pergolide solution, 100 μL of monoclonal antibody reagent, and 500 μL of plasma (either standard pergolide or sample). Standards and samples were analyzed in duplicate or triplicate. Nonspecific binding was determined by replacing antibody with assay buffer in the incubation. After mixing, we incubated the reaction mixture for 20 h at 4 °C. The bound and free forms of pergolide were then separated by adding 0.5 mL of cold dextran-coated charcoal to each assay tube. The samples were vortex-mixed, allowed to stand for 15 min at room temperature, and then centrifuged at 3500 × g for 15 min at 4 °C. We transferred 1 mL of the supernate to a scintillation vial containing 15 mL of Scintisol and counted the radioactivity for 5 min with an LS-3800 scintillation counter (Beckman, Palo Alto, CA). Assay data were analyzed by a VAX computer with use of a weighted four-parameter logistic model algorithm (15). The pergolide concentration in plasma test samples was estimated from a standard curve covering the concentration range from 5 to 5000 ng/L.

RIA validation. RIA sensitivity was estimated by the method of Rodbard (16). The antibody affinity constant was calculated by the computer program LIGAND (17). We evaluated RIA specificity by systematically examining the cross-reactivity of pergolide metabolites found during preclinical animal studies (7, 8) and of several synthetic derivatives of pergolide. After preparing standard curves of pergolide and the test compounds in

### Table 1. Structure of Pergolide Derivatives Studied

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
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<tr>
<td>LY127809</td>
<td>-CH₂SCH₂</td>
<td>-CH₂CH₂CH₃</td>
<td>-H</td>
</tr>
<tr>
<td>LY150442</td>
<td>-CH₂SCH₂</td>
<td>-CH₂CH₂CH₃</td>
<td>-H</td>
</tr>
<tr>
<td>LY189166</td>
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<td>-CH₂CH₂CH₂CH₃</td>
<td>-H</td>
</tr>
<tr>
<td>LY149945</td>
<td>-CH₂SCH₂</td>
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<td>-H</td>
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<td>LY100543</td>
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<td>-H</td>
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<td>-H</td>
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<td>-H</td>
</tr>
<tr>
<td>LY243062</td>
<td>-CH₂SCH₂</td>
<td>-CH₂CH₃</td>
<td>-CH(CH₂)₂</td>
</tr>
<tr>
<td>LY202991</td>
<td>-CH₂SCH₂</td>
<td>-H</td>
<td>-H</td>
</tr>
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</table>
drug-free human plasma, we analyzed their displacement curves statistically by the computer program ALLFIT (18). In this program, the 50% effective dose (ED_{50}) is defined as the concentration of analyte necessary to displace 50% of the bound labeled pergolide. The relative potency estimates were then calculated from the ratio of the ED_{50} for pergolide to the ED_{50} value for each test compound.

We assessed interassay precision and recovery by adding pergolide standards to plasma from humans, rats, and rhesus monkeys at 0, 50, 100, 500, and 2500 ng/L. Interassay precision was then estimated by measuring the concentration of pergolide in the control samples in multiple assays. Analytical accuracy was assessed two ways. First, we determined the dilutional recovery of pergolide in a sample of pooled rat plasma obtained between 0.5 and 6 h after oral administration of pergolide at 10 mg/kg body wt. Second, we used C_{18} reversed-phase HPLC to characterize the identity of immunoreactive pergolide present in rat plasma 1.5 h after an oral dose of 5 mg/kg. The elution profile of pergolide, pergolide sulfoxide, pergolide sulfone, N-despropylpergolide, and N-despropylpergolide sulfoxide was characterized by absorbance detection at 254 nm. The order of elution, from earliest to latest, was N-despropylpergolide sulfoxide, pergolide sulfoxide, pergolide sulfone, N-despropylpergolide, and pergolide. We then assessed the elution profile of immunoreactive pergolide in rat plasma after oral dosing. After injecting 50 mL of the plasma onto the HPLC column, we eluted the column at a flow rate of 1 mL/min with a mobile phase of 50 mmol/L ammonium acetate buffer, pH 4.6, and a gradient of acetonitrile from 50 to 900 mL/L in a total volume of 30 mL, collecting 0.3-mL fractions. After removing the mobile phase by centrifugation under reduced pressure, we reconstituted each dried residue with 1 mL of drug-free human plasma and measured the pergolide concentration by RIA.

Pharmacokinetics Studies

Pergerolide in patients with Parkinson disease. We studied 15 patients whose total daily dose of pergolide ranged between 0.75 and 3.0 mg, given three times a day. Informed consent was obtained from all study participants. On the study day, the patients received their usual morning dose: either 0.25 (n = 8), 0.5 (n = 5), or 1 mg (n = 2) of pergolide. A 5-mL sample of antecubital venous blood was obtained before administration of pergolide. Blood samples were then drawn into EDTA at 0.5, 1, 2, and 4 h after dosing. We stored the plasma samples frozen in plastic tubes at −20 °C.

Pergerolide in rhesus monkeys. Groups (n = 4 to 20) of domestically reared monkeys (Macaca mulatta), weighing 4.3 ± 0.63 kg, were administered pergolide (1-mg tablets) at single oral doses of 1, 2, 5, or 10 mg. The monkeys were fasted for 14–16 h before dosing, and food was withheld for 4 h after dosing. Venous blood samples were collected and converted to heparinized plasma before dosing and at 0.5, 1, 1.5, 2, 3, 4, 8, 12, 24, and 48 h after administration of pergolide. We stored the plasma samples frozen in plastic tubes at −20 °C.

Table 2. Cross-Reactivity of Compounds in Pergolide Radioimmunoassay

<table>
<thead>
<tr>
<th>Compound</th>
<th>LY no.</th>
<th>Relative potency, %</th>
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</thead>
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<tr>
<td>Pergolide</td>
<td>127809</td>
<td>100</td>
</tr>
<tr>
<td>Pergolide-6-butanolate</td>
<td>150442</td>
<td>32.1</td>
</tr>
<tr>
<td>N-Pentylpergolide</td>
<td>169166</td>
<td>18.0</td>
</tr>
<tr>
<td>Pergolide sulfoxide</td>
<td>148945</td>
<td>14.5</td>
</tr>
<tr>
<td>N-Methylpergolide</td>
<td>106543</td>
<td>6.5</td>
</tr>
<tr>
<td>Pergolide sulfoxide</td>
<td>149082</td>
<td>4.2</td>
</tr>
<tr>
<td>N-Despropylpergolide</td>
<td>148670</td>
<td>1.0</td>
</tr>
<tr>
<td>1-Isopropylpergolide</td>
<td>243062</td>
<td>0.4</td>
</tr>
<tr>
<td>N-Despropylpergolide sulfoxide</td>
<td>202991</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Bromocriptine</td>
<td></td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Results

Monoclonal antibody characterization. Isotype analysis revealed that the monoclonal antibody was an IgG2a immunoglobulin. Scatchard analysis indicated that the antibody contained a single population of binding sites with an affinity constant of 5.04 × 10⁸ L/mol. To characterize the specificity of the antibody, we evaluated the cross-reactivity of the pergolide derivatives shown in Table 1 in competitive-binding experiments. All of the compounds evaluated were less potent than pergolide for displacing radiolabeled pergolide (Table 2). Modification of the pergolide molecule at either position six or eight markedly reduced the reactivity with the antibody. The pergolide derivative that was modified at both position six and eight, LY202991, failed to displace labeled pergolide at concentrations as great as 1 mg/L. The addition of an isopropyl group at position one also reduced cross-reactivity with the antibody. Bromocriptine failed to cross-react with the antibody at concentrations as great as 1 mg/L.

RIA development. After analyzing pergolide standard curves with different dilutions of the monoclonal antibody, we selected a dilution of 50 000-fold for use in the RIA We determined that a 50 g/L suspension of charcoal was optimal after comparing the effect of different amounts of charcoal on the nonspecific binding and the maximum binding. When the optimized conditions were used, we obtained the pergolide displacement curve shown in Figure 2. The standard curve parameters (mean ± SE; n = 11) for the pergolide RIA were 1.32% ± 0.09% for the nonspecific binding, 40.6% ± 1.67% for the maximum binding, 1.04 ± 0.02 for the slope, and 173 ± 10.5 ng/L for the ED_{50}.

RIA validation. We estimated the detection limit (sensitivity) of the RIA to be 21 ng/L. We compiled over 22.5 years interassay precision data for plasma control samples from four different studies involving humans, rats, and rhesus monkeys. As shown in Figure 3, the interassay CV was <15% for measurements of pergolide concentrations between 100 and 1000 ng/L. The CV for plasma controls at 50 and 2500 ng/L was 30%. The mean analytical recovery of pergolide added to the control...
samples was 93% at 50, 102% at 100, 100% at 250 and 500, and 87% at 2500 ng/L.

To assess analytical accuracy, we showed that the immunoreactive pergolide in rat plasma after oral administration yielded dilution curves parallel to that for authentic pergolide (Figure 2). We also demonstrated that the immunoreactive pergolide in rat plasma after a single oral dose of 5 mg/kg was coeluted with standard pergolide during HPLC with a C18 column.

**Measurement of plasma concentrations of pergolide.**
We investigated the utility of the RIA for quantifying therapeutic concentrations of pergolide in plasma from patients with Parkinson disease. Mean steady-state concentrations of pergolide are depicted in Figure 4 for patients who received total daily doses of 0.75, 1.5, or 3 mg three times a day. We detected pergolide in all samples obtained before the next administration of drug, the respective mean concentrations being 275, 569, and 1167 ng/L. Although the plasma mean concentrations were highly variable (CVs >28%), in all cases the concentrations exceeded the detection limit of the assay by at least sevenfold. Thus, the RIA is sufficiently sensitive to measure pergolide in patients at steady state doses of \( \geq 0.75 \text{ mg/day} \).

Because single-dose pharmacokinetic studies of pergolide are not feasible in normal healthy adult volunteers, we attempted to assess the pharmacokinetics of pergolide in rhesus monkeys. Mean plasma concentrations of pergolide are shown in Figure 5 after oral administration of pergolide at 1, 2, 5, and 10 mg. The maximum concentration in plasma (mean ± SE) was 2.09 ± 0.22 \( \mu \text{g/L} \) for the 1-mg dose (n = 20), 4.57 ± 2.04 \( \mu \text{g/L} \) for the 2-mg dose (n = 5), 10.2 ± 3.35 \( \mu \text{g/L} \) for the 5-mg dose (n = 5), and 26.0 ± 13.8 \( \mu \text{g/L} \) for the 10-mg dose (n = 4). For all doses of pergolide, the time to maximum drug concentration in plasma ranged between 2.4 and 2.7 h.

**Discussion**

**Analytical Considerations**

Radioimmunoassays have been used widely for quantifying ergolines in physiological fluids (9–12). How-
ever, routine measurement of therapeutic concentrations of pergolide in plasma is difficult for several reasons. First, pergolide is more potent than other ergolines, such as bromocriptine (4–7), so that a small therapeutic dose is sufficient. The maximum plasma concentration of bromocriptine was reportedly 691 ng/L after oral administration of 5 mg to eight healthy fasted adult male volunteers (19); in 10 Parkinson patients at steady state, the mean plasma concentration of bromocriptine was 1850 ng/L 1.5 h after a 5-mg dose (20).

Second, in humans, pergolide undergoes extensive biotransformation to ≥ 10 different highly polar metabolites (8). This further decreases the plasma concentration of unchanged drug. Rubin et al. (8) reported a mean peak plasma concentration of 1.8 μg/L for pergolide equivalents after oral administration of 138 μg of radiolabeled pergolide to three healthy adult volunteers. Thus, a requisite for any assay to measure therapeutic concentrations of pergolide is the ability to quantify the parent compound in the nanograms per liter range. Our initial lack of success at developing a chromatographic-based assay for pergolide led us to undertake the development of an RIA for pergolide.

We systematically investigated the specificity of the monoclonal antibody by evaluating the cross-reactivity of pergolide metabolites and several synthetic derivatives of pergolide. Any modification of pergolide at position one, six, or eight reduced cross-reactivity with the monoclonal antibody (Table 2). Thus, we postulate that the overall stereochemistry of pergolide is important for defining the antibody-binding epitope. Of the previously characterized metabolites of pergolide tested, all but the sulfone were <5% as potent as pergolide at displacing radiolabeled pergolide. In humans, pergolide is transformed into highly polar metabolites, with the sulfoxide, sulfone, and N-despropyl metabolites probably serving as intermediates that undergo secondary metabolism (8). Thus, based on the specificity of the monoclonal antibody (Table 2) and the nature and multiplicity of human metabolites, we conclude that it is unlikely that any of the pergolide metabolites can accumulate in the circulation in sufficient concentration to interfere in the RIA.

We selected an antibody dilution of 50,000-fold for use in the RIA. Even though we can detect displacement of tritiated pergolide at concentrations ≤ 10 ng/L, we established 2 ng/L as the routine quantification limit of the RIA. We determined empirically that a 50 g/L suspension of charcoal was optimal for use in the RIA. At lower percentages of charcoal the nonspecific binding increased, presumably because of increased binding of tritiated pergolide to low-affinity sites on plasma proteins.

We assessed RIA precision by compiling data for plasma control samples over 2.25 years. The interassay CV of the RIA was <1.5% for concentrations of pergolide between 100 and 1000 ng/L, increasing to 30% for pergolide concentrations <100 ng/L or >1000 ng/L (Figure 3). For all studies the recovery estimates for the plasma controls ranged from 87% to 102%. Thus, we conclude the optimal working range of the RIA extends from 100 to 1000 ng/L.

Test samples having a high concentration of pergolide are reanalyzed after dilution with drug-free plasma. We validated the accuracy of the RIA by demonstrating that the dilution curves for immunoreactive pergolide in rat plasma parallel those for authentic pergolide (Figure 2). Also, we determined that immunoreactive pergolide in rat plasma is coeluted with standard pergolide during C18 reversed-phase HPLC.

Hemolysis quenches the scintillation counting, producing a false-positive result in the RIA. Therefore, hemolyzed plasma samples are not appropriate for analysis by this method. The lack of an alternative method with sufficient sensitivity to measure therapeutic plasma concentrations of pergolide precluded any systematic comparison of the RIA with another methodology.

Pharmacokinetics

We used the RIA to measure steady-state concentrations of pergolide in plasma from patients with Parkinson disease. For all patients the pergolide concentrations that we detected before the next drug administration suggested that the plasma concentrations were proportional at steady state. At all doses, the plasma concentrations of pergolide varied substantially between individuals. Despite extensive enterhepatic extraction (21), bromocriptine is detectable by RIA in plasma of Parkinson patients 12 h after dosing (20). In a single-dose study in normal adult volunteers, the mean plasma concentrations of pergolide peaked 1–2 h after dosing (19), suggesting that pergolide and bromocriptine have a similar time to maximum concentration in plasma (7). In this study, we found no correlation between the plasma concentrations of pergolide and the drug efficacy, presumably because of the high between-subject variability. High between-subject variability and poor bioavailability are pharmacokinetic features common to ergolines (7). Although the study design and the high CV (>28%) did not allow estimation of pharmacokinetic variables, the data indicate that the sensitivity of the RIA is sufficient to permit routine measurement of plasma concentrations of pergolide when patients are at steady state on doses of ≥ 0.75 mg/day.

Single-dose pharmacokinetic studies involving the therapeutic doses of pergolide are not feasible in normal healthy adult volunteers, owing to the severe nausea and vomiting that are frequent side effects of dopamine agonists (7, 19, 22). Pergolide therapy is initiated in patients at a daily dose of 0.05 mg, being gradually increased over a period of several weeks until an optimal efficacious dose is achieved (3). It is unlikely that any analytical method can detect pergolide with sufficient sensitivity to permit estimation of human pharmacokinetic variables after a dose of only 0.05 mg. An alternative method for estimating pharmacokinetic variables would be to dose to steady state and then calculate the half-life from the decline in plasma concentrations after withdrawing the pergolide. However,
this approach is not feasible in either normal volunteers or Parkinson patients, for ethical considerations.

Because the emetic response to dopamine agonists is attenuated in rhesus monkeys (23), we attempted to assess the pharmacokinetics of pergolide in this species. Although the pharmacokinetic analysis was complicated by the high degree of between-subject variability, the data displayed a general linear increase in plasma concentrations with increasing doses of pergolide (Figure 5). Plasma pergolide concentrations declined in parallel, suggesting that plasma clearance was constant, and the pharmacokinetics remained linear between doses of 1 and 10 mg. The data from monkeys appeared to be comparable with the steady-state data from Parkinson patients, which suggests that rhesus monkeys may serve as a useful pharmacokinetic model for pergolide in humans.

In summary, we report the development of a sensitive RIA method for determining pergolide in plasma. Assay specificity was maximized by using a monoclonal antibody that displayed low cross-reactivity with pergolide metabolites. We used the RIA to measure pergolide in plasma from rats, rhesus monkeys, and humans. We conclude that the RIA is sufficient for routine measurement of therapeutic plasma concentrations of pergolide in Parkinson patients at steady state at doses of ≥0.75 mg/day.

Several individuals at Eli Lilly and Co. contributed to this project. We gratefully acknowledge John Schaus for providing us with the derivatives of pergolide, C. John Parli and Eric Evenson for performing the HPLC experiments with rat plasma, and Jimmie Shouffer and his associates for their help during the pharmacokinetics studies with monkeys. We also thank William J. Wheeler for coordinating the preparation of tritiated pergolide at Amersham and acknowledge Ross Crabtree for his contribution to the initial phase of this project and Louis Lemberger for his helpful discussions.

References