Pharmacokinetic Mechanisms for Obtaining High Renal Coelimination of Phencyclidine and a Monoclonal Antiphencyclidine Antigen-Binding Fragment of Immunoglobulin G in the Rat

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ABSTRACT

Our purpose was to determine mechanisms and methods for significantly increasing the renal coelimination of phencyclidine (PCP) and an anti-PCP monoclonal antibody binding fragment (anti-PCP Fab). To accomplish this goal, we performed a series of experiments to examine the dose-dependence of Fab elimination, mechanisms for enhancing PCP and Fab urinary coelimination and the antigenicity of repeated Fab administration. The results showed that urinary elimination of PCP and anti-PCP Fab was linear over a 30-fold range of doses. Anti-PCP Fab serum pharmacokinetics were best described using bi- or tri-exponential curves with a terminal elimination half-life of approximately 8 hr. Nevertheless, under all experimental conditions the early, nonterminal phase(s) were responsible for the majority (60%) of intact Fab elimination, with only 40% of the Fab eliminated during the terminal phase. These data suggest that the early rapid decline in Fab serum concentrations was primarily due to passive filtration and excretion of intact Fab, and not due to extravascular distribution as previously described. In comparison of methods for enhancing renal coelimination of Fab and PCP, systemic alkalization produced a significant increase in Fab urinary elimination, with 69% of the Fab dose and 41% of the PCP dose recovered intact in the urine. Finally, in studies of the antigenicity of Fab, repeated administration of Fab produced no significant immune response or renal impairment. Overall, these experiments suggest that careful attention to the physiological status of the kidney during early time periods is essential for maximum coelimination of Fab and bound chemicals.

The use of high affinity antibodies as therapeutic agents is becoming increasingly common. We have previously shown that an antiphencyclidine Fab (the antigen binding fragment of a monoclonal antiphencyclidine IgG) is effective in reversing PCP-induced behavioral effects (Valentine and Owens, 1996). Similar therapeutic effects are seen with other anti-drug Fab fragments against digoxin (Smith et al., 1976), digitoxin (Ochs and Smith, 1977), desipramine (Brunn et al., 1992) and colchicine (Sabouraud et al., 1991). In addition, antibody fragments have been used for the in vivo detection and treatment of various cancers (see review by Goldenberg, 1993) and as antithrombotic agents (Coller et al., 1991; Vermilyen, 1995). Although intact IgG is also used for these medical applications, the Fab fragment is preferable in many clinical situations due to its low antigenicity, more extensive extravascular distribution, and rapid elimination (Smith et al., 1979). The elimination of Fab fragments occurs primarily in the kidney through passive filtration followed by catabolism or urinary excretion (Spiegelberg and Weigle, 1965; Wochner et al., 1967; Arend and Silverblatt, 1975). Furthermore, high affinity Fab binding can produce enhanced urinary elimination of lipophilic compounds such as digitoxin (Ochs and Smith, 1977), PCP (Owens and Mayersohn, 1986; Valentine et al., 1994), colchicine (Sabouraud et al., 1992) and 2,2',4,4',5,5'-hexachlorobiphenyl (Keyler et al., 1994). However, in these previous studies the Fab-induced drug elimination is variable, and in most cases fairly low. Thus, the renal processes for producing high coelimination of Fab and Fab-bound chemicals are poorly understood.

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ABBREVIATIONS: AUC, area under the concentration-time curve; CLCR, creatinine clearance; CLR, renal clearance; CLS, systemic clearance; ELISA, enzyme-linked immunosorbent assay; Fab, antigen binding fragment of IgG; IgG, immunoglobulin G; terminal elimination rate constant; mol-eq, mole equivalent; PCP, phencyclidine; RIA, radioimmunoassay; T1/2, terminal elimination half-life; Vc, volume of the central compartment; VSS, volume of distribution at steady-state; HPLC, high-performance liquid chromatography; CV%, percent coefficient of variation.
The renal mechanisms involved in the Fab elimination processes could affect the success of antibody-based therapies in several ways. For instance, renal catabolism of antibody fragments used in the reversal of chemical toxicity (e.g., PCP) could potentially cause a release of previously bound toxin, thereby freeing the toxic substance to return to its sites of action or leading to renal toxicity (e.g., α-amanitin, Faulstich et al., 1988). Additionally, during diagnostic imaging studies the reabsorption and accumulation of radioconjugated antibody fragments in the kidney is potentially radiotoxic to the kidney, and can decrease imaging sensitivity in the abdominal area (e.g., Behr et al., 1996). In these cases, it would be beneficial to enhance the elimination of the intact (and functional) Fab by decreasing Fab reabsorption and catabolism. Furthermore, enhanced renal elimination of intact antibody fragments would also reduce the potential medical problems due to immune processing and subsequent antigenicity of the antibody.

Despite the apparent benefits associated with increasing urinary elimination of intact Fab, no single method is in widespread clinical use to enhance renal elimination. However, several studies have examined the coadministration of large doses of amino acids to competitively inhibit protein reabsorption in the kidney (e.g., DePalatis et al., 1995; Behr et al., 1996). Although this approach would presumably increase intact Fab urinary elimination, it may not be compatible with the use of Fab to reverse drug toxicity. This is because large doses of anti-drug Fab will probably be required to treat most drug overdoses and the resulting renal load of protein and amino acids could decrease renal function. Therefore, a more widely applicable method for increasing Fab elimination after high doses of Fab is needed.

The purpose of our studies was to maximize renal coelimination of anti-PCP Fab and PCP and to better define the pharmacokinetic mechanisms involved in this process. To accomplish these goals, we studied the effects of i.v. fluid loading and systemic alkalinization on PCP and anti-PCP Fab excretion. These procedures were chosen because they are readily available in clinical care settings, and they are consistent with currently accepted practices for treating stimulant toxicity (i.e., correction of metabolic acidosis; Roth et al., 1998). In addition, detailed anti-PCP Fab serum and urine pharmacokinetic studies were conducted in normal and alkalinized animals to better define the physiological and pharmacokinetic processes involved in Fab elimination. Finally, we studied the dose-dependency of Fab and PCP coelimination, and the safety of repeated anti-PCP Fab administration in rats.

Methods

General strategy. These studies consisted of a series of four experiments. In all of the Fab pharmacokinetic studies, PCP was included to examine the effect of Fab on PCP elimination, and as a probe to follow the functionality of the Fab. Although the Fab pharmacokinetics were extensively characterized in both serum and urine samples, PCP elimination was only monitored by quantitating PCP content in urine samples. In the first experiment, the urinary elimination of Fab and PCP in control, fluid loaded and alkalinized rats was studied. In the second experiment, anti-PCP Fab serum and urine pharmacokinetics were examined in PCP-treated control and alkalinized rats. In the third experiment, the dose dependence of Fab and PCP urinary elimination was studied. The fourth experiment examined the safety of Fab administration, and consisted of monitoring both renal function (using creatinine clearance, CLCr), and the rat immune response to Fab in selected rats from the three experiments above.

General experimental protocol. Our experimental strategy was to administer PCP via an indwelling venous cannula at time 0 followed 10 min later by a mole-equivalent (mol-eq) dose of anti-PCP Fab. For example, the 1 mol-eq Fab (50 kDa) dose for a 1-mg/kg dose (calculated as the free base) of PCP (MW = 243 g/mol) was 210 mg/kg. Fab was given 10 min after PCP to allow initial observation of the maximal PCP behavioral effects associated with the doses used (Valentine et al., 1996). PCP was administered at a dose of 1 mg/kg in most experiments, however a range of doses from 0.1 to 3.0 mg/kg was used in the anti-PCP Fab dose-dependence studies. These doses were chosen based on pharmacokinetic analysis (using the human PCP pharmacokinetic parameters of Cook et al. 1982) of human serum PCP concentrations determined in emergency room patients after PCP overdose (Walberg et al., 1983). We estimated that approximately 90% of these emergency room visits resulted from PCP doses of 1 mg/kg or less with doses ranging from approximately 0.2 to 3.5 mg/kg.

All drugs and solutions were manually administered via syringe into either the femoral or jugular venous cannula. PCP and anti-PCP Fab were always administered as a timed bolus injection over 10 to 20 sec. The cannula was flushed with 0.3 ml of saline after drug administration to ensure no drug or Fab remained in the cannula. During the experiments, animals were kept in Nalgene metabolic cages (Fisher Scientific, Springfield, NJ) from 1 to 2 hr before drug administration until the final urine samples were collected (no longer than 48 hr). Animals were prompted to void their bladder before the start of each experiment by letting them sniff ether for several seconds. All experiments began between 6 and 10 A.M. For the first 8 hr of the experiment, each urine void was promptly collected. Urine collection continued until elimination of Fab and PCP was essentially complete (at least 24 hr). Urine pH was measured, and samples were immediately placed on ice or refrigerated before centrifugation and freezing. Blood samples (for analysis of creatinine and for Fab serum pharmacokinetic experiments) were allowed to clot before centrifugation, after which the serum was collected and frozen until ready for analysis. Unless stated otherwise, all data for urinary excretion represent the percent of the dose appearing intact in the urine (expressed as the mean ± S.D.).

Drugs and chemicals. [3H]PCP (1-(1-[3H](n)cychoexyl)piperidine) and PCP HCl (1-(1-phenylcyclohexyl)piperidine hydrochloride) were obtained from the National Institute on Drug Abuse (Rockville, MD). The [3H]PCP (15.69 Ci/mmol) was used as a standard for determining PCP recovery after urine extraction and for determining PCP concentrations in urine extracts by RIA. All PCP concentrations were calculated as the free base. The [3H]Fab was synthesized from anti-PCP Fab as previously reported (McClurkan et al., 1993). Lactated Ringer’s solution was purchased from Baxter Healthcare Corp. (Deerfield, IL). Sodium sulfate, sodium azide and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were obtained from Fisher Scientific (Springfield, NJ), unless otherwise stated.

Production and purification of monoclonal anti-PCP Fab. Monoclonal anti-PCP IgG was produced in gram quantities from the hybridoma cell line MAb6B5 in a Cell-Pharm System II hollow fiber bioreactor (Unisyn Technologies Inc., Tustin, CA). The details of the anti-PCP IgG production are described elsewhere (McClurkan et al., 1993; Valentine et al., 1994, 1996; Hardin et al., 1998). Anti-PCP Fab was obtained by papain digestion of the monoclonal anti-PCP IgG followed by subsequent purification as described by Hardin et al. (1998). Anti-PCP Fab purity was determined by SDS-PAGE, and concentration was determined by spectrophotometry.

Animals. Adult male Sprague-Dawley rats (300 g) with indwelling jugular and femoral venous cannulae were purchased from Hilltop Laboratory Animals, Inc. (Scottsdale, PA). Each cannula (Dow
Corning silastic tubing, 0.020” ID × 0.037” OD) was surgically placed in either the right external jugular vein or the right femoral vein. Upon arrival from the vendor, each cannula was externalized from the subdermal space in which it was contained for shipping purposes. Cannulae were flushed with heparinized saline (25 U/flask) every other day to help maintain patency. Animals were allowed 1 wk to recover from surgery and travel before the start of experiments. At all times, animals were allowed free access to water and were fed enough food on a daily basis to maintain their body weight at approximately 300 g. All animal experiments in these studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Protocol for i.v. fluid loading and systemic alkalinization of rats. All rats received an i.v. bolus dose of 1.0 mg/kg of PCP in sterile saline (1 ml/kg) at time zero. Anti-PCP Fab (1 mol-eq) or saline (control) was administered in a total volume of 1 ml at 10 min. Creatinine clearance (CLCR) was determined using urine collected from 0 to 8 hr and a single serum sample collected at 4 hr (see below for details).

In the fluid loading experiment, three experimental conditions were used. These included PCP followed by saline and fluid loading (without Fab), PCP followed by Fab (without fluid loading) and PCP followed by Fab and fluid loading. Animals (n = 4) in this fluid loading experiment received these three treatment conditions in a repeated-measures, mixed-sequence design. Fluid loading consisted of an i.v. lactated Ringer’s solution manually infused at 0.5 to 1.0 ml/min to a final volume of 21 ml/kg. The infusion began immediately after the administration of anti-PCP Fab or saline treatments (approximately 11 min after PCP administration). The volume and rate of infusion were chosen to be consistent with human clinical practices.

For the systemic alkalinization experiments, a separate group of animals (n = 4) received PCP (1 mg/kg) followed by an i.v. dose of NaHCO3 (8 mEq/kg in 2 ml/kg administered over 1 min) at 8 min and either anti-PCP Fab or saline at 10 min. These animals received additional injections of NaHCO3 (2 mEq/kg in 1 ml/kg administered over 30 sec) at 55, 100, 145 and 190 min after PCP administration. This alkalinization regimen was chosen to determine if alkalinization altered PCP-induced effects and to be consistent with clinical therapy (i.e., correction of metabolic acidosis and the prevention of rhabdomyolysis; Roth et al., 1998). The conditions for this alkalinization regimen were optimized in preliminary experiments to maintain urinary pH > 8.0 for at least the first 8 hr of the experiment. The choice of pH 8.0 was somewhat arbitrary, but it allowed us to maintain a clearly alkalinized state.

Protocol for anti-PCP Fab serum pharmacokinetic studies. All animals received 1 mg/kg PCP followed 10 min later by 1 mol-eq unlabeled anti-PCP Fab and a tracer dose of [3H]Fab (approximately 3.2 × 107 dpm). Control animals (n = 4) received no additional treatment, while alkalinized animals (n = 4) received NaHCO3 as described for the systemic alkalinization experiments. In all cases, injections were made into one cannula (usually the femoral vein), and blood samples were obtained from the other cannula (usually the jugular vein).

Blood samples were obtained at 0, 5, 15 and 30 min and 1, 1.5, 2, 3, 4, 8, 12, 16 and 24 hr, and urine was collected for 48 hr as previously described (see General protocol). Following collection of each blood sample, the cannula was carefully filled with heparinized saline. The heparinized saline was removed from the cannula prior to collection of the next sample. The total blood collected during each experiment was less than 10% of each rat’s total blood volume.

Protocol for Fab and PCP dose-dependence studies. Animals (n = 6) received six treatments in a repeated-measures, mixed-sequence design. PCP (0.1, 0.3, 1.0 or 3.0 mg/kg) was administered at time 0 and followed 10 min later by a matching 1 mol-eq dose of anti-PCP Fab in a final volume of 2 ml (i.e., 21, 62, 210 and 620 mg/kg Fab, respectively). To determine the effect of PCP dose on PCP excretion in the absence of Fab, two additional treatments of PCP at a low and high dose (i.e., 0.3 or 3.0 mg/kg) were administered followed at 10 min by 2 ml sterile saline. CLCR was determined using urine samples collected from 0–8 hr, and a single serum sample obtained at 4 hr (see below for details).

Analysis of biological samples. Urine samples were assayed for both anti-PCP Fab and PCP content, except for the Fab serum pharmacokinetic studies. In the Fab serum pharmacokinetic studies, only anti-PCP Fab (not PCP) concentrations were determined in urine. The PCP extraction was similar to the method of Valentine and Owens (1996) which is capable of separating PCP from metabolites in plasma. However, because we were not sure of the specificity of this extraction in urine samples, which contain significantly greater concentrations of PCP metabolites than does serum, we assayed the extracted samples by a specific RIA for PCP. The anti-PCP goat antibody used for this RIA does not significantly cross-react with PCP metabolites (Owens et al., 1982; Owens, 1985). In addition, the accuracy of this RIA was previously validated in comparison with a gas chromatography procedure for PCP (Owens et al., 1982). The reproducibility of the RIA was determined using control blank urine samples spiked with known amounts of PCP representing a low and high PCP concentration in the linear range of the assay. These quality control samples (n = 4 per assay) were extracted along with the unknown urine samples.

Anti-PCP Fab concentrations were determined by HPLC using a molecular weight sizing column (TSK-GEL column G3000SWXL, Tosohaas, Montgomeryville, PA; flow rate = 1 ml/min). The HPLC mobile phase consisted of 50 mM Na2HPO4, 50 mM NaH2PO4, H2O and 100 mM Na2SO4 (pH 6.7). The HPLC system (Waters Corp., Milford, MA) consisted of an autoinjector in series with a multi-solvent delivery system, a UV absorbance detector and a fraction collector. The Millenium software package (Waters Corp., Milford, MA) was used to control the HPLC system and to collect all HPLC data. After centrifugation at 3500 rpm for 5 min, 20-μl urine aliquots were injected, and the UV absorbance at 254 nm was monitored. Quantitation of Fab by peak height was based on standard curves of anti-PCP Fab prepared in HPLC mobile phase. This method of detection provided a linear standard curve over a range of 10 μg/ml to 30 mg/ml of Fab. It should be noted that no similarly sized proteins were present in the normal rat urine to interfere with anti-PCP Fab quantitation. Reproducibility was monitored using blank urine samples spiked with a known amount of anti-PCP Fab representing a low and high amount of Fab. These quality control Fab samples were analyzed with each batch of urine samples (i.e., every 10–15 injections).

A tracer dose of [3H]Fab was needed for quantitation of Fab concentrations in serum. In contrast with the urine, the presence of other similarly sized serum proteins made the quantitation of unlabeled anti-PCP Fab in serum impossible by UV absorbance detection. In the urine and serum pharmacokinetic studies, [3H]Fab concentrations were used for pharmacokinetic calculations to assure the most consistent results. The [3H]Fab (in urine or serum) was separated from metabolic products using the HPLC sizing column and then collected in a 3-ml fraction corresponding to the 50-kDa Fab peak. The [3H]Fab in each fraction was then quantitated by liquid scintillation spectrometry. The specificity and accuracy of this technique was confirmed in preliminary experiments. In addition, the unlabeled Fab and [3H]Fab content in urine samples from animals receiving both unlabeled Fab and [3H]Fab were quantitated using both the UV absorbance method as described above, and the liquid scintillation spectrometry method (after HPLC separation). This allowed a direct comparison and validation of the two techniques used to quantify Fab in this study.

Renal passive filtration rate was monitored as a measure of renal function by determining CLCR in selected animals. Midway through a timed urine collection, a single blood sample was obtained and CLCR was determined using the equation: 

$$CLCR = \frac{UCR \times Q}{PCR};$$

where UCR is the concentration of creatinine in the urine, Q is the flow rate, and PCR is the creatinine clearance.
average urine flow rate, and $S_{CR}$ is the concentration of creatinine in the serum. Serum and urine creatinine concentrations were determined using a commercially available diagnostic kit (Sigma).

**Serum and urine pharmacokinetic calculations.** Both model-dependent and model-independent methods, as described by Gibaldi and Perrier (1982), were used to analyze serum [3H]Fab concentration-time data. All analysis was carried out using the pharmacokinetic software package WinNonLin (Scientific Consulting, Inc., Cary, NC). Model-dependent analysis was used to help determine the complexity of the early changes in the concentration-time curves, and consisted of fitting a nonlinear regression curve to the plasma [3H]Fab concentration-time data. Biexponential and triexponential curves were fit to the data using both $1/y$ and $1/y^2$ weighting functions to obtain the best-fit curve. The selection of the best-fit curve for each individual data set was based on visual comparison of the fits, the statistical variance of the pharmacokinetic parameters, analysis of the residuals plot and a statistical F ratio test as described by Boxenbaum et al. (1974).

Pharmacokinetic parameters derived from model-independent analysis were used to compare treatment groups. The pharmacokinetic calculations included determination of the terminal elimination rate constant ($\lambda_e$), the terminal elimination half-life ($T_{1/2e}$), the systemic clearance ($CL_{sys}$) and the volume of distribution at steady state ($V_{SS}$) calculated as the product of mean residence time and $CL_{sys}$. Renal clearance ($CL_R$) was calculated as systemic clearance times the fraction of the [3H]Fab dose appearing in the urine. $CL_R$ was also determined over specific time intervals (0–3 and 3–12 hr after PCP administration) using the following formula: $CL_R = \frac{U_{Fab} \times Q}{S_{Fab}}$, where $U_{Fab}$ is the average urinary concentration of Fab over the specified time interval, Q is the average urine flow rate over the interval and $S_{Fab}$ is the predicted concentration of Fab in the serum at the midpoint of the interval. This is analogous to the approach used to determine endogenous $CL_{sys}$. In all cases, urine samples for the $CL_{sys}$ and interval $CL_R$ analysis were collected over at least a 2-hr period, which has been shown to be sufficient for accurate determination of $CL_{sys}$ (Sladen et al., 1987).

**Immunization and serum collection for studies of Fab antigenicity.** For production of rat antisera against the murine monoclonal anti-PCP Fab, rats (n = 3) were immunized subcutaneously at multiple injection sites with a total of 50 $\mu$g of anti-PCP Fab in 1.5 ml saline emulsified with an equal volume of Freund's complete adjuvant. The rats were boosted with subcutaneous injections at multiple sites 3 wk later using 50 $\mu$g anti-PCP Fab in 1.5 ml saline emulsified with an equal volume of Freund's incomplete adjuvant. Immune serum was collected 2 wk after the booster injection. Preimmune serum was obtained from these rats before the immunizations. This immunization schedule was chosen to mimic the time-frame of the multiple Fab injections used for the dose-dependence experiment, where the first and last i.v. doses were approximately 3 wk apart.

ELISA was used to test for the presence of rat antibodies against anti-PCP Fab in randomly selected animals that had received either one (210 mg/kg) or four (21, 62, 210 and 620 mg/kg) i.v. injections of anti-PCP Fab. These animals were chosen from the serum pharmacokinetic and dose-dependence experiments, respectively. From these two groups of animals, serum was obtained 2 wk (n = 3 from each experiment) and 4 wk (n = 2 from each experiment) after the final anti-PCP Fab injection. Preimmune serum was obtained from seven of the experimental animals used in this study for comparison purposes.

ELISA. Microtiter plates (96 wells) were coated with anti-PCP Fab (100 ng/well) in 0.1 M carbonate buffer (pH 9.6) for 3 hr at 37°C. The plates were then washed five times with phosphate-buffered saline containing 0.1% Tween-20 (v/v) and stored at 4°C. Serial dilutions of rat serum were prepared in PBS containing 0.1% Tween-20, and 100 $\mu$l/well was added to the microtiter plate in duplicate. The plates were incubated overnight at 4°C. Plates were again washed five times as previously described. A 100-$\mu$l aliquot of alkaline phosphatase conjugated goat anti-rat Ig (IgG and IgM, heavy and light chain specific) was diluted 1:15,000 and added to each well. The plates were incubated at room temperature for 1 hr and then washed. A 100-$\mu$l aliquot of p-nitrophenyl phosphate (30 mg/50 ml of 10 mM diethanolamine containing 0.5 mM MgCl$_2$ · 6H$_2$O) was then added to each well. After a color development period, the amount of chromogenic product formation was determined using an ELISA plate reader at 410 nm. To ensure consistent color development among several plates, the immune serum from a selected immunized rat was included as a sample on each microtiter plate, and color development was allowed to proceed until the absorbance of the 1:800 serum dilution reached 1.5.

**Statistical analysis.** All values are reported as the mean ± S.D. All statistical analyses were conducted with the computer software SigmaStat (Jandel Corporation, San Rafael, CA). Student's t test was used when comparing two groups. A repeated-measures one-way analysis of variance followed by a Student-Neuman-Keuls post hoc test was used to compare differences between more than two treatment groups within the same experiment. Overall comparisons among all treatment conditions (e.g., control, fluid loading and alka-lininization) were made using a one-way analysis of variance followed by a Student-Neuman-Keuls post hoc test. Statistical significance was considered to be achieved at a level of P < .05.

**Results**

**General experimental observations.** PCP and Fab administrations, as well as fluid loading, were well tolerated in all animals. Adverse PCP-induced behavioral effects observed in the 10-min interval after PCP administration (but before Fab treatment) ranged from slight head weaving after a 0.1-mg/kg dose, to severe ataxia and immobility after a 3.0-mg/kg dose. Although no attempt was made to quantitate animal behavior in these experiments, anti-PCP Fab, administered at 10 min after PCP, completely reversed the PCP-induced behavioral effects within minutes.

The assays used for quantitation of PCP (RIA), Fab (HPLC separation with UV absorbance detection) and [3H]Fab (HPLC separation with liquid scintillation spectrometry detection) were found to be sensitive and reproducible. PCP concentrations in two urine control samples containing known amounts of PCP (25 and 100 ng/ml) were 26.1 ± 7.4 ng/ml (CV, % = 28.5; n = 53) and 102.9 ± 27.0 ng/ml (CV, % = 26.2; n = 55). This yielded an RIA analytical recovery of 104 and 102%, respectively. Urine anti-PCP Fab concentrations determined by UV absorbance after HPLC separation in two control urine samples were 1.5 ± 0.2 mg/ml (CV, % = 13.3; n = 21) and 13.9 ± 1.2 mg/ml (CV, % = 8.4; n = 12). This yielded an HPLC/UV analytical recovery of 100 and 93%, respectively. Analysis of two control serum [3H]Fab samples by HPLC separation and liquid scintillation spectrometry also produced excellent results (1,676 ± 144 dpm; CV, % = 8.7, n = 8; and 33,000 ± 4,900 dpm; CV, % = 14.9, n = 7). This yielded an HPLC/liquid scintillation spectrometry analytical recovery of 92 and 109%, respectively.

In addition to the above quality control measures, we also validated the two analytical techniques needed for quantitation of Fab. Although quantitation of [3H]Fab using HPLC separation followed by liquid scintillation spectrometry has been used previously in this laboratory (McClurkan et al., 1993), we thought that quantitation of unlabeled Fab by UV detection would be a more accurate reference method for determining absolute urine Fab concentrations after in vivo administration. However, we also know that quantitation of
unlabeled Fab in the serum by this method was not possible due to interference from similarly sized proteins. $[^3H]$Fab in urine samples from animals receiving anti-PCP Fab and a tracer dose of anti-PCP $[^3H]$Fab was first separated from Fab metabolites and other proteins by HPLC and then quantified by UV absorbance detection (as the Fab eluted from the column). In addition, the Fab peak at 50 kDa was collected and analyzed by liquid scintillation spectrometry for quantification of $[^3H]$Fab. In eight animals, the percentage of the anti-PCP Fab dose appearing in the urine was 65.1 ± 7.3% (by UV absorbance), and the percentage of the anti-PCP $[^3H]$Fab dose in the urine was 60.7 ± 11.5% (by liquid scintillation spectrometry). Consequently, the results from these two techniques were in excellent agreement.

**Effect of fluid loading and NaHCO$_3$ treatment on Fab and PCP elimination.** The total percentage of Fab (and PCP) excreted over 48 hr in the urine of control, fluid loaded and alkalinized animals was 64.1 ± 10.3% (38.3 ± 10.6%), 55.7 ± 6.1% (39.0 ± 14.3%) and 69.1 ± 4.1% (41.4 ± 7.2%), respectively. However, urinary elimination of both Fab (fig. 1) and PCP (results not shown) was essentially complete within 3 hr. Consequently, we carefully analyzed the dramatic changes occurring during the first 3 hr, when fluid loading and alkalinization produced the greatest effects on urine volume and Fab elimination (fig. 2).

The urinary excretion of PCP was previously shown to account for elimination of 2.5 ± 0.5% of the PCP dose (Valentine et al., 1994). In our study, PCP excretion in the absence of Fab was in agreement with the previous study, and it was unaffected by fluid loading (1.5 ± 1.0%) or alkalinization (1.6 ± 0.4%). Administration of anti-PCP Fab after PCP administration resulted in a dramatic increase in the amount of PCP appearing in the urine (fig. 2). However, fluid loading or alkalinization did not further improve this dramatic effect of Fab therapy on PCP urinary excretion.

Figure 3 shows representative plots of Fab excretion rate plotted at the midpoint of each urine collection interval. These plots suggested an increasing excretion rate as the therapy progressed from control treatment to fluid loading to alkalinization. Furthermore, there appeared to be a change from biphasic urinary elimination in control and fluid loaded animals to rapid monophasic elimination in alkalinized animals.

Alkalinization produced no significant behavioral effects in preliminary studies. However, when NaHCO$_3$ was administered to rats 8 min after PCP administration, it immediately precipitated a pronounced increase in PCP-induced behavioral effects. Rats which moments before exhibited symptoms...
typical of the 1 mg/kg dose of PCP (e.g., head weaving and hyperlocomotion), almost immediately showed signs associated with higher doses of PCP (e.g., severe ataxia and anesthesia). Three of these animals stopped breathing temporarily (for about 30–45 sec); however, in no case was the combination of PCP and NaHCO3 fatal. When these NaHCO3-treated rats received anti-PCP Fab at 10 min (2 min after NaHCO3) they quickly recovered as indicated by a decrease in PCP-induced behavioral effects. In contrast, the NaHCO3-treated rats which were given saline at 10 min were slower to recover, requiring approximately 20 min for the anesthetic effects to dissipate and an additional 30 to 45 min for all PCP-induced behavioral effects to dissipate.

**Effect of systemic alkalinization on anti-PCP Fab pharmacokinetics.** Blood sampling was well tolerated in all rats, as indicated by hematocrit values obtained before (0.49 ± 0.03) and immediately after (0.39 ± 0.02) each experiment. Figure 4 shows a representative serum anti-PCP [3H]Fab concentration vs. time plot superimposed on a plot of the cumulative Fab urinary excretion for the same animal. Model-dependent analysis of concentration-time data from control animals (n = 4) was best described by a triexponential function with either a 1/y (n = 1) or 1/y² (n = 3) weighting. Data from alkalinized animals was best described by either a biexponential (n = 2) or triexponential (n = 2) function with 1/y² weighting in all cases. Pharmacokinetic values obtained from model-dependent analysis differed by no more than 12% from values obtained from model-independent analysis. Consequently, we only report the pharmacokinetic values from the model-independent analysis (table 1). Figure 5 summarizes the effects of alkalinization on CL_{CR} and CL_{F} during an early (i.e., 0–3 hr) and late (i.e., 3–12 hr) time interval.

**Effect of Fab dose on Fab and PCP coelmination.** The amount of anti-PCP Fab excreted intact in the urine increased linearly with the dose of Fab administered (fig. 6, upper panel), representing an average of 60.0 ± 9.4% of all Fab doses appearing in the urine. In addition, the amount of PCP appearing in the urine was directly related to the amount of Fab in the urine (fig. 6, lower panel), corresponding to an average of 27.9 ± 8.2% of all PCP doses. In contrast, renal excretion of PCP after administration of 0.3 or 3.0 mg/kg PCP, without Fab treatment, resulted in only 2.3 ± 0.2% and 2.3 ± 0.7% of the dose appearing in the urine, respectively. Although each animal received four different doses of Fab over a 4-wk period, passive filtration (as assessed by CL_{CR}) was normal over the entire course of the experiment (data not shown).

**Immune response after single and multiple doses of anti-PCP Fab in rats.** Animals subcutaneously immunized with anti-PCP Fab and adjuvant, with booster injections at 3 wk, produced a substantial titer that was present 2 wk after the booster injections (fig. 7). In contrast, animals receiving anti-PCP Fab on one or four separate occasions did not show a significant titer at 2 or 4 wk after the final anti-PCP Fab administration (fig. 7).

**Discussion**

Recognizing the importance of the kidney in the clearance of antibody fragments, these experiments were designed to study conditions that enhance anti-PCP Fab and PCP coelmination. Results from previous reports of Fab urinary elimination in the rat vary considerably, showing 15, 36, 16, 21, 8, 6 and 53% of the Fab dose eliminated in the urine (Arend and Silverblatt, 1975; Pentel et al., 1988; Sabouraud et al., 1992; McClurkan et al., 1993; Moran et al., 1994; Valentine et al., 1994; K eyler et al., 1995, respectively). In our experiments, total anti-PCP Fab urinary excretion was generally higher than found previously, and ranged from 41.2 to 79.8% of the dose with a grand average of 61.0 ± 9.5% (data from all experiments). We think that the variability in the previously reported values could be due to differences in analytical techniques, the species studied, the species isotype of the Fab fragment and differences in experimental conditions.

Indeed, a previous study from our laboratory shows that anti-PCP Fab excretion in rats varies from 3 to 37% of the Fab dose, with an average of 21.0 ± 15.3% (McClurkan et al., 1993). Because there was a direct relationship between urine volume and the amount of Fab eliminated in the urine in this previous study, we suspected that the level of hydration of the animals was a major reason for differences in Fab elimination. These previous data suggested that Fab excretion could potentially be increased by enhancing urine output, and provided a major reason for focusing the current studies on optimizing conditions for Fab renal elimination.

The approximate 15-fold increase in PCP urinary excretion observed in our study (i.e., from 1–3% with no treatment to 30–40% with Fab treatment) was substantially greater than increases in urinary drug excretion found with other anti-drug antibodies. For instance, antidesipramine Fab produces a 7-fold increase in desipramine excretion (from 2.1–14.2%; K eyler et al., 1995), although urinary excretion of colchicine increases 4-fold after anticolchicine Fab administration (from 9.0 to 38.0%; Sabouraud et al., 1992). Also, urinary elimination of digoxin is enhanced by anti-digoxin Fab (from 5.0 to 16.3%; Johnston et al., 1987), and hexachlorobiphenyl excretion is increased by anti-hexachlorobiphenyl Fab (from 1.3 to 12.2 ng/24 hr; K eyler et al., 1994). Furthermore, the increase in PCP urinary excretion observed in our study was substantially higher than the 4-fold increase in urinary PCP excretion (from 2.5% without anti-PCP Fab to 10.3% with a 1 mol-eq dose of anti-PCP Fab), found in our previous studies.
The greater increase in PCP excretion found in our studies appeared to be due to the substantial increase in the total amount of anti-PCP Fab in the urine. Another potential cause of the variability in Fab urinary excretion is the possibility of dose-dependent, nonlinear urinary elimination. The Fab doses used previously in this and other labs have varied considerably (i.e., from 0.9 to 7500 mg/kg). However, over the 30-fold range of Fab doses used in our study, the amount of intact Fab (and PCP) appearing in the urine increased in a linear fashion with the increasing Fab dose (fig. 6). This direct relationship between Fab dose and amount of Fab excreted in the urine indicated that urinary Fab elimination for this monoclonal Fab is a first-order process over the range of doses studied. However, it would not be surprising if urinary elimination was nonlinear at much smaller doses (i.e., lower than the 21-mg/kg dose used in these studies), because the renal processing of Fab can involve saturable reabsorption in the proximal tubule. The reabsorption of proteins is a high capacity process under physiological conditions but may become saturated when the protein load increases (Maack et al., 1979; Christensen and Nielsen, 1991). It is possible that the reabsorption of Fab is capacity-limited, and was already saturated at the 21-mg/kg dose of Fab. Although Fab elimination appears to be linear with respect to Fab dose, the most encompassing definition of linearity is with respect to dose and time. In these studies, although elimination is linear with respect to dose, it appears to be nonlinear with respect to time, because CLR is substantially lower at times after 3 hr (table 1).

Although fluid loading and alkalinization both produced a significant increase in urine output for at least 3 hr, alkalinization had the greatest effect on Fab urinary excretion. This could be explained by the fact that alkalinization produced a significantly greater increase in urine volume compared to fluid loading. Nonetheless, it is not clear whether the increase in Fab excretion produced by alkalinization was due to the effect of bicarbonate on urinary pH or its effect on urine...
Because alkalinization produced increases in Fab urinary excretion (figs. 1–3 and 5), additional experiments were performed to extensively analyze Fab pharmacokinetics in the serum and urine of control and alkalinized rats. Although no differences in $T_{1/2a}$, $V_{SS}$ or $CL_{SS}$ were found between control and alkalinized animals (table 1), $CL_{R}$ over the interval from 0 to 3 hr was increased approximately 50% in alkalinized animals (fig. 5). This pharmacokinetic parameter is the best measure of the rate of Fab elimination, because it takes into account urine and serum Fab concentrations as well as urine flow rate. Analysis of $CL_{CR}$ over the same 0 to 3-hr interval allowed us to directly compare this measure of glomerular filtration rate with Fab $CL_{R}$ over a short, discreet time-span. Alkalinization produced a significant increase in $CL_{CR}$ that was comparable to the increase in $CL_{R}$ produced during this same time interval. Taken together, the serum and urine data clearly showed that urinary alkalinization significantly increased the rate of anti-PCP Fab excretion, and this change appeared to be due to an increase in glomerular filtration.

The fact that Fab $CL_{R}$ was significantly lower in the terminal elimination phase (fig. 5), and the fact that more than 90% of urinary excretion was complete within 3 hr indicated that a different process of elimination, other than urinary excretion, was responsible for the long terminal elimination phase (i.e., an $8 \, hr \cdot t_{v,\lambda a,b}$). This other mechanism accounted for elimination of approximately 30 to 40% of the dose in all animals in these studies. It is not clear how this fraction of the Fab dose bypassed the highly effective renal excretion process; however, at least two possibilities exist. The first possibility is that although the Fab is passively filtered in the glomerulus, it is efficiently reabsorbed in the proximal tubule and catabolized in the kidney or returned intact to the circulation to be eliminated by nonrenal routes. Indeed, some evidence for trans-tubular transport exists (Maack et al., 1979; Christensen and Nielsen, 1991). The second possibility is that the remaining Fab completely bypasses passive filtration by the kidney and is eliminated by nonrenal metabolic processes.

Detailed analysis of the area under the curve of each component of the bi- and tri-exponential curves from the control and alkalinized rats (i.e., $A/\lambda_1$, $B/\lambda_2$ and $C/\lambda_3$) showed that the terminal elimination phase accounted for only 39.3 ± 5.5% of the total area, although the early elimination phase(s) ($A/\lambda_1$ for two-compartment models or $A/\lambda_1 + B/\lambda_2$ for three-compartment models) accounted for 61.2 ± 5.2% of the total plasma AUC. The percentage of the plasma AUC accounted for by this early elimination phase (i.e., 61.2%) is in excellent agreement with the percentage of the dose appearing in the urine from these animals over the first 3-hr interval (i.e., 6.85%). This is in also in agreement with pharmacokinetic data from an earlier classic study of the various routes of Fab elimination in the rat (Arend and Silverblatt, 1975) which shows that the first phase of the biexponential concentration vs. time curve accounts for 61.3% of the total AUC. However, as with other studies of Fab renal elimination, these investigators obtained low amounts of intact Fab in the urine (14.3% of the total dose). When we considered the overall significance of these data, we concluded that the total AUC minus the terminal AUC, divided by the total AUC may be a good predictor of the maximum amount of Fab that can be eliminated unchanged in the urine under optimal conditions.

The description of the serum anti-PCP $[^3H]$Fab concentration vs. time data in these studies as bi- or tri-exponential functions is consistent with other reports on Fab pharmacokinetics in the rat (Sabouraud et al., 1992; McClurkan et al., 1993). However, these previous studies suggest that the initial rapid decline in blood concentrations (i.e., the first or first and second phases of the two- and three-compartment models, respectively) result from Fab extravascular distribution, with a half-life ranging from 0.2 to 2.4 hr. They also report terminal elimination half-lives of 1.3 to 16.3 hr. In addition, studies using other species have defined a distribution phase for Fab as well, with distribution half-lives of 0.3 hr in the baboon (Smith et al., 1979), and 0.2 and 0.7 hr in the rabbit (Timsina and Hewick, 1992; Rivière et al., 1997, respectively).

However, based on the current studies, we think that the previous reports describing a distribution phase for Fab have made an incorrect physiological interpretation of their two- and three-compartment pharmacokinetic models. Urinary excretion profiles from the current study clearly showed that urinary elimination of intact Fab (representing 60% of the Fab dose) was complete within 2–3 hr (figs. 1 and 2). At approximately this time, the monoexponential, linear terminal-elimination phase in the serum begins (fig. 4). Therefore, we think that this early rapid phase is primarily due to renal excretion of intact Fab, although Fab distribution undoubtedly makes some contribution to the early decline in Fab serum concentrations. This interpretation of our data is not necessarily surprising because it has been estimated that efficient elimination of peptides and proteins by renal filtration would result in plasma half-lives of 30 to 60 min (McMartin, 1992).

Although systemic alkalinization did increase the rate of Fab urinary excretion, a potentially desirable effect in immunotherapy, several gross observations from these experiments raise questions as to the safety of this practice. NaHCO$_3$, administered 8 min after PCP but before Fab administration, precipitated a dramatic surge in PCP-induced effects, which appeared in some cases to be life threatening. We think that slight alterations in serum pH after the bolus
NaHCO₃ administration altered the ionization characteristics of PCP (pKₐ = 9), allowing more nonionized drug to pass into the brain. It is possible that this effect could be avoided by administering Fab before NaHCO₃. However, symptomatic treatment of metabolic acidosis with NaHCO₃ will often occur in the emergency room before treatment of a drug overdose. Nevertheless, we think that maintaining high urine output, particularly during the first 3 hr after Fab administration, is the major factor for achieving effective elimination of intact Fab (and PCP) in the urine. Consequently, other procedures, such as administration of an appropriate diuretic, might achieve the same goals.

Fab administration appeared to be safe and well tolerated in our studies. CL_CRT was not adversely affected in any of the experiments, and was actually increased above normal values in alkalized animals. Also, the immunogenicity of Fab appeared to be minimal, because the serum from rats after passive administration of our murine monoclonal Fab showed only a slight immune response compared to control pre-treatment serum and serum from rats actively immunized with the same Fab. The minimal immune response in the rats used in this study is consistent with reports from humans that show only a 0.8% incidence of allergic reaction to ovine-derived digoxin-specific Fab (Hickey et al., 1991).

In summary, these studies showed that Fab urinary excretion was a first-order process over a 30-fold range of doses. Rapid urinary excretion accounted for approximately 60% of Fab elimination, and was essentially complete within 3 hr. The rate of Fab excretion from 0 to 3 hr was enhanced by urinary alkalization, but was unaffected by fluid loading. In addition, the time-course of Fab disposition in urine and serum indicated that Fab pharmacokinetics are best explained by a biphasic or triphasic curve. The early phase(s) involve(s) a rapid renal elimination process (which is complete within 3 hr) that is followed by a much slower terminal elimination phase. Finally, all of our measures showed that even repeated Fab administration was safe in these animals.

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References


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