Extensive Biliary Excretion of the Model Opioid Peptide [D-PEN^{2,5}] Enkephalin in Rats

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Purpose. This study was designed to test the hypothesis that the enzymatically stable opioid peptide, [D-pen^{2,5}] enkephalin (DPDPE), is excreted extensively into bile.

Methods. Following an i.v. bolus dose of DPDPE (10 mg/kg) to rats, concentrations of DPDPE in serum, bile, liver homogenate and urine were measured by a novel capillary zone electrophoresis method. Data were analyzed to recover the fundamental pharmacokinetic parameters (volumes of distribution; distribution and elimination rate constants governing DPDPE systemic and biliary disposition). Parallel *in vitro* experiments were performed to evaluate the partitioning of DPDPE between erythrocytes and plasma, as well as to assess the degree of binding of DPDPE to serum proteins.

Results. The majority of the administered dose (~80%) was recovered from bile as intact peptide. DPDPE disposition was best described by a two-compartment model with Michaelis-Menten elimination (K_m: 37.5 ± 11 μg/ml; V_{max}: 1143 ± 368 μg/min/kg) from the central compartment into bile, suggestive of an active hepatic transport system. DPDPE was associated with a distributional space of 486 ± 62 ml/kg. *In vitro* incubation of DPDPE with whole blood showed that ~65% of the peptide was associated with erythrocytes. The difference between concentrations of DPDPE in erythrocytes and plasma was statistically significant (29.2 ± 4.9 vs. 18.1 ± 3.1 μg/ml, $p < 0.05$), but not between whole blood and plasma (21.3 ± 2.8 vs. 18.1 ± 3.1 μg/ml, $p > 0.05$). Concentration-independent binding of DPDPE to serum proteins was evidenced between 10 and 100 μg/ml, with an unbound fraction of 0.517 ± 0.182.

Conclusions. DPDPE undergoes extensive biliary excretion after i.v administration in rats. The apparent nonlinearity in the biliary excretion of DPDPE revealed by the pharmacokinetic modeling strongly suggests the existence of an active transport system(s) in hepatocytes which may mediate the rapid disappearance of DPDPE from the systemic circulation.

KEY WORDS: DPDPE; opioid peptide; biliary excretion; CZE.

INTRODUCTION

Peptide drugs hold promise for treating a variety of diseases. For example, somatostatin peptide analogs have been used for the treatment of pancreatic endocrine and brain tumors (1,2), and cyclosporine is the mainstay in management of graft rejection (3). While peptides have the advantage of being very potent and highly specific, several issues must be addressed before their full therapeutic potential is realized. Among these limitations is the problem of adequate sojourn of peptides within the body so that the elicited effects are present for a suitably long period of time. One reason for the short biologic half-life of peptides is the instability of many of these compounds in the presence of endogenous enzymes (4,5). For example, dynorphine A 1–13 was extensively and rapidly metabolized by aminopeptidases and endopeptidases in human plasma and whole blood, with an *in vitro* half-life less than 1 min (6). Difficulties associated with proteolytic degradation by widely distributed peptidases may, in many instances, be overcome by appropriate chemical modification of the peptide (7,2); nevertheless, extensive biliary excretion of intact peptide may result in rapid disappearance from the systemic circulation and a short duration of action after i.v. administration (8), or a low systemic bioavailibility after oral administration due in part to first-pass hepatic extraction (9,10). Overcoming these difficulties will require additional understanding of the hepatic processing of enzymatically stable peptides.

[D-pen^{2,5}] Enkephalin (DPDPE; H-Tyr-D-Pen-Gly-Phe-D-Pen-OH, Pen = Penicillamine) is a [Met^5] enkephalin opioid pentapeptide first synthesized in 1983 (11). DPDPE was developed as an antinociceptive agent devoid of the multiple side effects commonly associated with opioids (12). The stability and distribution of ^{3}H-DPDPE has been examined in mice (12,13). DPDPE was relatively stable compared to endogenous enkephalin peptides, with an *in vitro* half-life up to 60 min when incubated with purified enkephalinase (NEP E.C.3.4.24.11) (14). However, these data should be interpreted cautiously due to the use of a relatively non-specific assay technique and the possibility of ^{3}H exchange during the experiment.

Recently, we developed a capillary zone electrophoresis (CZE) method (15) that can be used for determination of DPDPE in serum. Slight modifications of this assay allow analysis of DPDPE in bile, liver homogenate, and urine. After i.v administration (15), DPDPE disappeared relatively rapidly from the systemic circulation, with a half-life (approximately 20 min) that was unexpectedly short for a peptide with enzymatic and metabolic stability (12,13). We hypothesized that DPDPE was removed rapidly from the blood stream by avid biliary excretion; whole body distribution studies with ^{3}H-DPDPE in mice showed that ~60% of the total radioactivity was associated with small intestine, intestinal flush and gallbladder, which indicated that DPDPE underwent extensive biliary elimination in mice (13). Accordingly, the present study was undertaken to examine the hepatobiliary disposition of DPDPE in rats, and to test the hypothesis that excretion of DPDPE into bile was responsible for the rapid removal of this peptide from the systemic circulation.

METHODS

Materials

DPDPE and DSLET (H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH) were provided generously by the National Institute on Drug Abuse (Baltimore, MD) and were used without further purification. All reagents used in this study were of the highest grade available from commercial sources.

Animals

Male Sprague-Dawley rats (250–300 g, Hilltop Laboratory Animals, Scottdale, PA) were housed individually in wire-mesh

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cages. Prior to the experiments, rats had free access to food and water and were maintained on a 12-hr dark/12-hr light cycle in a room with controlled temperature and humidity.

Hepatobiliary Disposition of DPDPE

The disposition of DPDPE in blood, bile, liver, and urine was examined *in vivo* in rats. Briefly, rats were anesthetized with urethane (1 g/kg), and the right jugular vein was cannulated with silicone rubber tubing (for DPDPE administration and blood sampling), the bile duct was cannulated with polyethylene (PE-10) tubing (for bile collection), and the urethra and bladder were cannulated with PE-50 and PE-60 tubing, respectively (for urine collection) (16). The jugular vein cannulae were filled with saline containing heparin (20 U/ml) to maintain patency. After two 5-min baseline bile collection, one 10-min baseline urine collection, and collection of a single pre-dose blood sample, DPDPE (5 mg/ml in saline, 10 mg/kg) was administered intravenously via the jugular vein cannula. The cannula was flushed immediately with heparinized saline (~200 μl) to avoid possible contamination by residual DPDPE. Blood samples (300 μl) were collected through the jugular vein cannula at 2, 4, 6, 8, 10, 15, 20, 30, 40 and 60 min after administration of DPDPE. Bile samples were collected at 5-min intervals for 20 min, 10-min intervals up to 40 min, a 20-min interval through 60 min, and two 30-min intervals through 2 hr. Urine samples were collected at 30- or 60-min intervals for 2 hr. Bile and urine volumes were determined gravimetrically assuming a specific gravity of 1 g/ml. Serum was obtained by centrifugation of blood at 15,000 *g* for 10 min. At the end of the experiment, the liver was isolated, blotted dry, and weighed. All samples were stored at −20°C until analysis.

In Vitro Protein Binding and Distribution of DPDPE in Rat Blood

Protein binding was determined with pooled serum from naive rats. DPDPE (10 mg/ml in water) was added to serum to obtain concentrations ranging from 10 to 100 μg/ml. Binding was assessed by ultrafiltration (YMT membrane, Amicon, Beverly, MA) after incubation at 37°C for 10 min. Preliminary experiments showed that DPDPE binding to the device was negligible. The unbound fraction was estimated by dividing the concentration of DPDPE in the filtrate by the total concentration in serum.

The partitioning of DPDPE between plasma and blood cells was determined by incubating DPDPE (200 μl of a 1-mg/ml solution) with whole blood (10 ml) from naive rats at 37°C. Two aliquots (200 μl each) were obtained at timed intervals for up to 6 hr. One aliquot was centrifuged (12,000 *g* × 10 min) to obtain DPDPE concentrations in plasma; the second aliquot was used for determination of DPDPE in whole blood after mixing with water (200 μl) to lyse the erythrocytes. Samples were stored at −20°C until analysis. Hematocrit was determined based on the ratio of weight of plasma to that of whole blood.

Analytical Procedures

Serum samples were pretreated and analyzed according to a method recently developed in this laboratory (16).

Urine samples (0.5–1 ml) were applied directly to a C_{18} solid phase extraction column with sample pretreatment as described for serum (15). Bile samples were diluted with water (1:50, v/v) and centrifuged (15,000 *g* × 5 min) before analysis by CZE. The amount of DPDPE remaining in the liver at the end of the experiment was determined after homogenization of the whole organ. Briefly, liver was homogenized in two volumes water with a blade homogenizor (Tekmar Co., Cincinnati, OH). Aliquots (200 μl) of homogenate were prepared and analyzed according to the method described for serum samples (15).

Electrophoretic separation conditions were as reported previously (15) except that samples were introduced into the capillary via gravity injection (50 mm, 30 sec). Data (peak areas of the analyte and internal standard) were acquired with Dionex CE software and recorded on an IBM-compatible personal computer. Recovery of DPDPE from serum, urine and liver homogenate was ~80%, and ~100% for bile (cv < 6%, n = 6). The concentrations of DPDPE in each matrice were calculated from the corresponding standard curves, which covered the range of concentrations encountered in samples. The limit of detection was 250 ng/ml.

Data Analysis

Estimation of Pharmacokinetic Parameters

The serum concentration-time and biliary excretion rate vs. time data for individual rats were analyzed with a series of compartmental models with the nonlinear least-squares regression program Scientist (Micromath, Salt Lake City, UT). Models that incorporated different compartmental structure (2 vs. 3 compartments) and different modes of elimination (first-order vs. Michaelis-Menten) were evaluated. For each model, the appropriate equations were fit to the serum concentration-time and biliary excretion rate vs. time data. Model selection and assessment of goodness-of-fit were based on the model selection criterion (MSC, a modified Akaike's information criterion), the degree of colinearity of parameters, standard error of parameter estimates, and the degree of bias in residual error.

Statistical Analysis

All data are presented as mean ± SD. Analysis of variance (ANOVA) and Student's t-test were used to analyze the protein binding and whole blood distribution data. The 0.05 level of probability was used as the criterion of significance.

RESULTS

Analysis of DPDPE in Various Biologic Fluids of Rats by CZE

DPDPE concentrations in serum, bile, liver and urine were determined by CZE after appropriate pretreatment for each matrix as described in the Methods section. Electropherograms for DPDPE in those matrices are displayed in Fig. 1. Baseline separation of DPDPE from DSLET and endogenous contaminants was achieved in serum and bile. Detectable concentrations of DPDPE were observed in serum for up to 40 min, and in bile for up to 2 hr; negligible DPDPE was observed in urine at all times during the experiment, and in liver tissue obtained at the end of experiment.

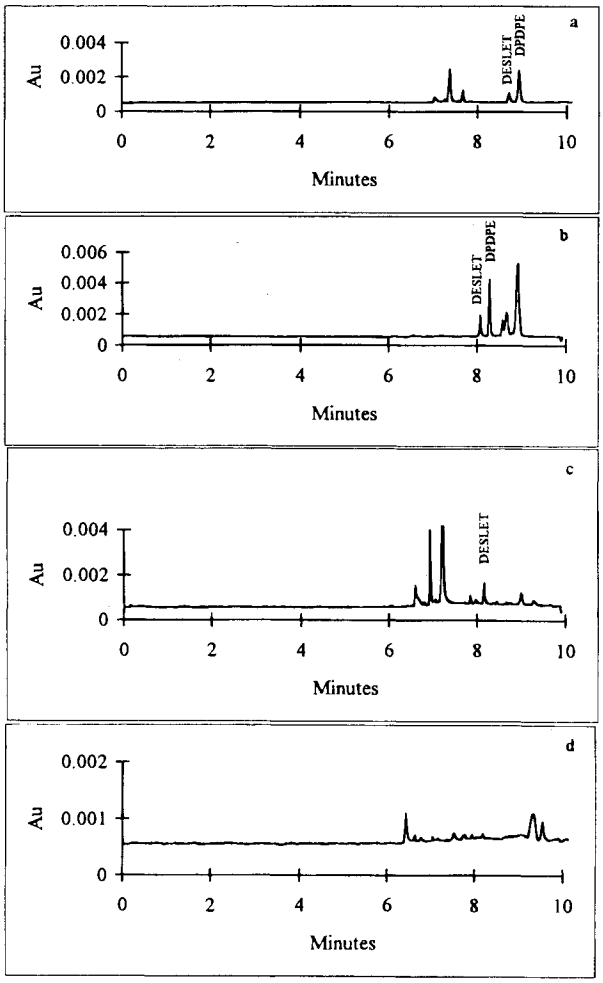

Fig. 1. Electropherogams of DPDPE and the internal standard DSLET in rat serum (a), bile (b), liver homogenate (c) and urine (d). Serum (10 min), bile (40–60 min collection), and urine (30–60 min collection) were obtained after DPDPE administration (10 mg/kg) to rats. Liver homogenate was obtained from a naive animal, and shows that peaks interfering with DPDPE and DSLET were absent. No DPDPE was observed in liver obtained from rats 2 hr after DPDPE administration. The experimental conditions were as described in the text.

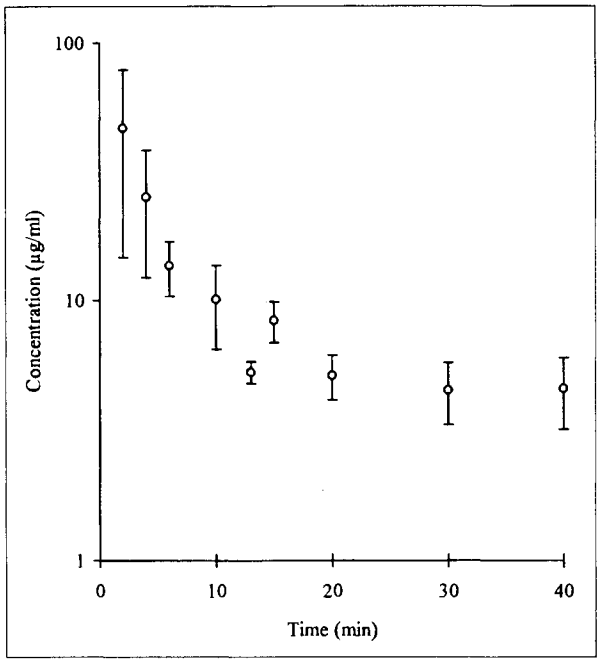

Fig. 2. Serum concentration-time data for DPDPE after a 10-mg/kg i.v. bolus dose to rats. Data are expressed as mean ± SD (n = 5).

Disposition of DPDPE in Serum and Bile

The serum concentration-time profile of DPDPE in rats (n = 5) after a 10-mg/kg i.v. bolus dose was consistent with a polyexponential function (Fig. 2). Therefore, at least a two-compartment model was required to describe DPDPE disposition in serum. A large quantity of DPDPE appeared in bile; ~80% of the administered dose was recovered in bile during the sampling period (Fig. 3). The kinetics of DPDPE excretion into bile appeared to be complex based on the excretion rate vs. time profile (Fig. 3). The apparent mode (linear vs. nonlinear) of biliary excretion of DPDPE was explored by analyzing the relationship between DPDPE biliary excretion rate and DPDPE serum concentration at the mid-point of the bile collection period (Fig. 4). The mid-point serum concentrations were approximated by averaging the two serum concentrations that bracketed the mid-point. These data were fit with both first-order and Michealis-Menten equations. The biliary excretion rate vs. concentration data were described better by the Michealis-Menten equation than a first-order function based on the model selection criteria discussed previously.

A total of thirteen compartmental models (two vs. three compartments with different elimination modes [linear or nonlinear] from either compartment) were compared for the analysis of serum concentration and biliary excretion rate vs. time data obtained from individual animals. A two-compartment model with nonlinear elimination from the central compartment best described the data based on statistical criteria (Fig. 5). The microrate constants governing transfer of DPDPE between the central and peripheral compartments (k_{12}, k_{21}) and the Michealis-Menten constants (K_m and V_{max}) associated with DPDPE elimination from the central compartment, as well as volumes of distribution of the two compartments, are shown in Table I.

Protein Binding and Distribution of DPDPE in Rat Whole Blood

DPDPE was bound to serum proteins in a concentration-independent fashion over the range of concentrations examined; no statistical difference was observed between the unbound fraction of DPDPE at 10, 20, or 100 μg/ml. The overall unbound fraction of DPDPE was 0.52 ± 0.18 (n = 9).

The *in vitro* concentration-time profile for DPDPE in blood cells and plasma during incubation in whole blood is displayed in Fig. 6. The concentration of DPDPE in cells was higher than that in plasma at all time points examined. Since there was no significant degradation of DPDPE over the 6-hr incubation period, the partitioning data were combined across sampling times. A significant difference in overall mean DPDPE concentrations between cells and plasma was observed (29.2 ± 4.9, vs. 18.1 ± 3.1 μg/ml, $p < 0.05$). In contrast, the difference in the overall mean DPDPE concentration between whole blood

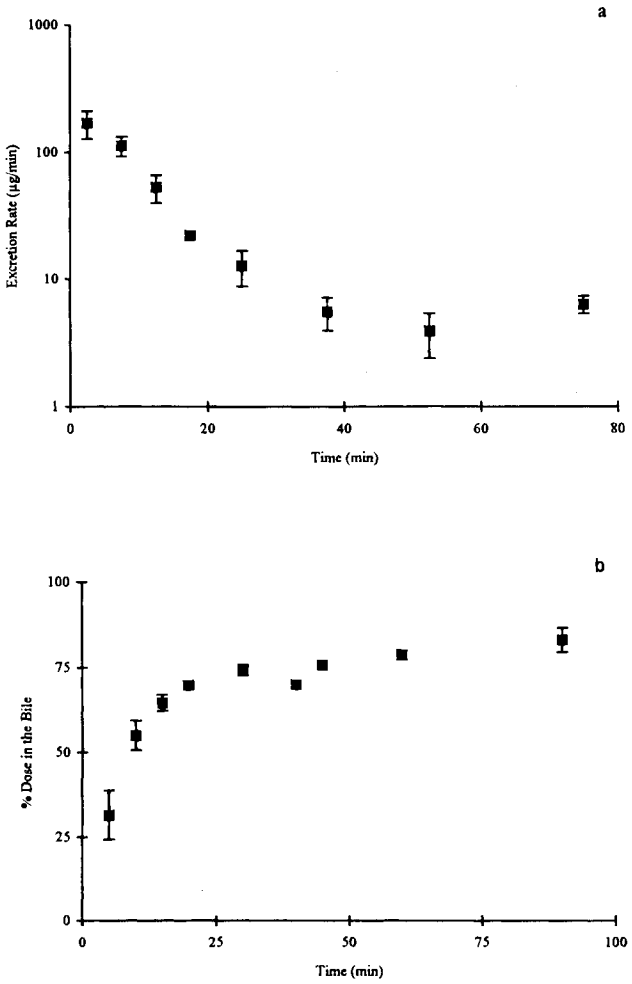

Fig. 3. Time course of DPDPE biliary excretion rate (a) and the cumulative percent of the dose recovered in the bile (b) after a 10-mg/kg i.v. bolus dose of DPDPE. Data are expressed as mean ± SD (n = 5).

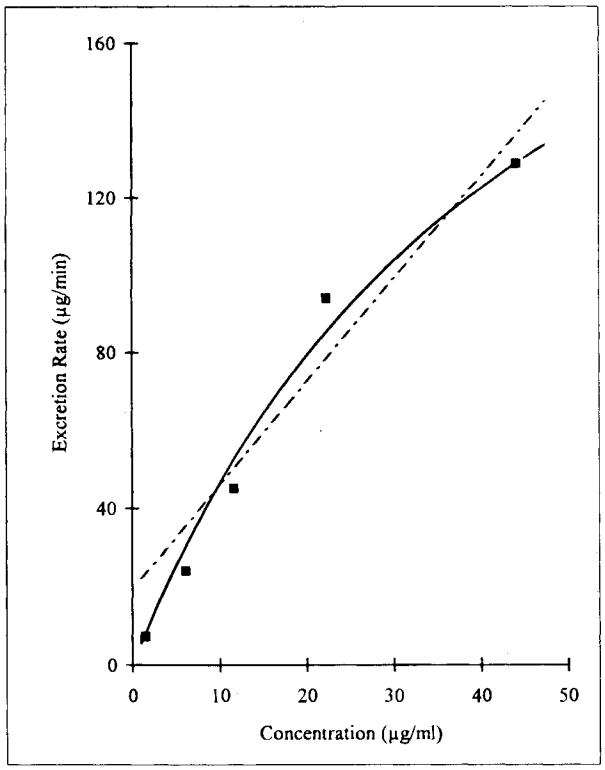

Fig. 4. Representative DPDPE biliary excretion rate vs. serum concentration at the mid-point of bile collection. Symbols represent observed data; solid line is the fit of the Michaelis-Menten equation to the data; and broken line is the fit of a first-order equation to the data.

and plasma was not statistically significant (21.3 ± 2.8 vs. 18.1 ± 3.1 μg/ml, $p > 0.05$).

DISCUSSION

Previous experiments (12–13,15) have shown that DPDPE is eliminated rapidly from the systemic circulation, even though this peptide appears to be metabolically stable. Extensive biliary excretion of peptides (17,18) limits the potential utility of these compounds in clinical arena. The present study was designed to test the hypothesis that rapid removal of DPDPE from the systemic circulation is due to avid biliary excretion.

Hepatic elimination via biliary excretion of intact DPDPE was the major route of DPDPE clearance from the systemic circulation, accounting for 83 ± 4% of the administered dose. Renal excretion of DPDPE did not appear to contribute to systemic clearance, as no detectable peptide was recovered in urine. The fate of the remaining ~20% of the dose is unknown, but may represent metabolic processes and/or sequestration of the peptide in organs and tissues.

Compartmentally-based pharmacokinetic analysis of the serum concentration and biliary excretion rate vs. time data was employed to characterize the hepatobiliary disposition of DPDPE. The rationale for incorporating a nonlinear elimination process into the compartmental models was based on the fact that concentrations of DPDPE were significantly higher in bile than those in blood. Bile to blood concentration ratios ranged from 70 to 110, suggesting an active transport process must have been involved in net DPDPE translocation from blood into bile; uptake across the basolateral membrane and/or efflux across the canalicular membrane may serve as a rate-limiting step in DPDPE elimination. The relationship between biliary excretion rate and serum concentration of DPDPE at the mid-point of bile collection period (Fig. 4) also was suggestive of a capacity-limited process for the biliary excretion of DPDPE. The nonlinear hepatobiliary disposition of DPDPE was further supported by the modeling of serum and bile data for individual animals; a two-compartment model with nonlinear elimination from central compartment was optimal based on standard statistical criteria. The nonlinearity in DPDPE hepatobiliary disposition encountered in this study, even though the results of the present experiment cannot be used to determine whether the active process occurs during uptake or excretion, indicates that DPDPE might be an excellent substrate to elucidate the mechanism of hepatobiliary disposition of enzymatically stable peptides. Only limited studies have addressed this issue thus far (19).

The binding and/or partitioning of therapeutic agents between cellular components (including erythrocytes, leukocytes and platelets) in whole blood and plasma can exert a significant influence on disposition and biologic activity, espe-

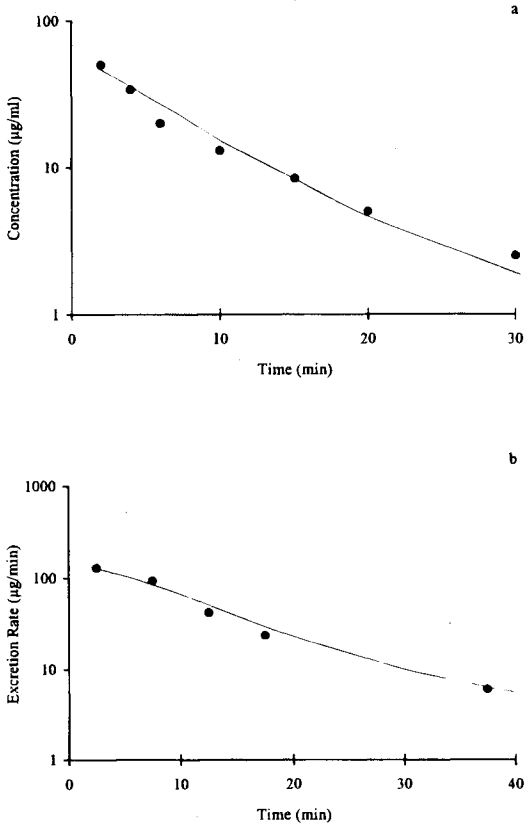

Fig. 5. Representative serum concentration-time profile (a) and DPDPE biliary excretion rate (b) after a 10-mg/kg i.v. bolus dose of DPDPE. Symbols indicate observed data; lines represent the fit of the optimal model.

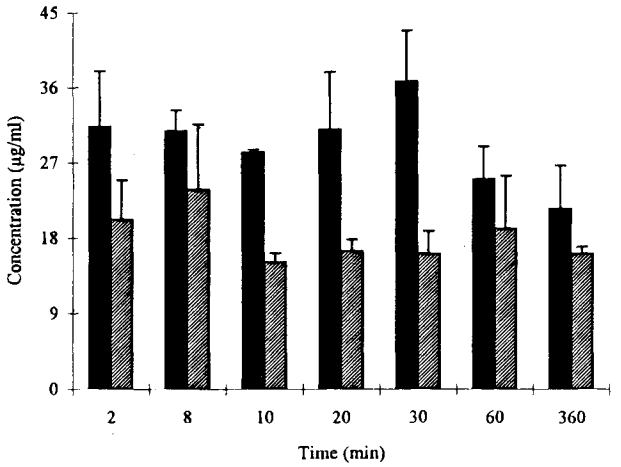

Fig. 6. Distribution of DPDPE in rat whole blood. Solid and hatched bars represent concentrations of DPDPE in erythrocytes and plasma, respectively. Error bars indicate SD (n = 4).

cially for compounds with a high degree of protein binding and/or high affinity towards erythrocytes. For example, the distribution of the cyclic peptide cyclosporine is affected substantially by protein (especially lipoprotein) concentration in plasma, as well as by hematocrit, since it is highly bound to lipoprotein in plasma and also to red blood cells; 58% of the circulating CsA is associated with red blood cells (20). The present study represents the first time that these aspects of DPDPE disposition have been addressed. DPDPE was not highly bound in rat serum; the unbound fraction was ~0.5. The results of the *in vitro* distribution in rat blood indicated that DPDPE was associated with cellular components to a greater extent than with plasma, as in the case of cyclosporine A (20). The overall mean concentration of DPDPE in whole blood did not differ statistically from those in plasma (21.6 ± 2.8 vs. 18.1 ± 3.1 μg/ml, $p > 0.05$), even though a significant difference between the concentrations of DPDPE in red blood cells and plasma was observed.

Consistent with the literature (12), no detectable metabolites (i.e., no unidentified peaks) were observed in the electropherograms in the present study. There was no difference in retention times or number of peaks between the serum samples from naive animals with DPDPE added *in vitro* and serum samples obtained from rats after DPDPE administration (data not shown). However, the fact that metabolites were not observed by CZE does not necessarily indicate that metabolites were not formed. Biotransformation products may have been excluded during sample pretreatment or may not have been observed due to detection limitations. However, based on mass balance the majority (83 ± 4%) of the dose was excreted as intact peptide, suggesting that metabolic degradation of DPDPE is a minor contributor to systemic clearance.

In summary, the present study tested the hypothesis that DPDPE was excreted extensively into bile, and that biliary excretion was responsible for the rapid elimination of DPDPE from the systemic circulation. A saturable process appeared to be involved in the hepatic uptake and/or biliary excretion of DPDPE. Further elucidation of the mechanism(s) underlying DPDPE disposition is the focus of ongoing efforts.

Table I. Pharmacokinetic Parameters[a] Associated with DPDPE Disposition in Rats (n = 5)

Parameters	Mean ± SD
K_{12} (min^{-1})	0.130 ± 0.010
K_{21} (min^{-1})	0.054 ± 0.004
V_{max} (μg/min/kg)	1143 ± 368
K_m (μg/ml)	37.5 ± 11.0
V_1 (ml/kg)	486 ± 62
V_2 (ml/kg)	89 ± 31

[a] Serum concentration-time and biliary excretion rate-time data were fit for each animal to recover individual parameters.

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