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DANAZOL AMINO ACID PRODRUGS: IN VITRO AND IN SITU BIOPHARMACEUTICAL EVALUATION

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ABSTRACT

Three amino acid esters of danazol were evaluated to discern their potential as prodrugs to deliver high danazol concentrations to the systemic circulation. A different oral delivery mechanism was found for each of three danazol esters (lysinate, glycinate and sarcosinate) based upon hydrolysis data in biological fluids, enzymes, and portal vein and systemic concentrations following intraduodenal bolus doses and liver perfusions. Danazol lysinate was rapidly hydrolyzed to droplets of danazol in the intestinal fluid resulting in dose proportional absorption of danazol into the portal system. Danazol glycinate precipitates as amorphous particles in the intestinal tract where it is readily absorbed and partially hydrolyzed during absorption and by the liver resulting in both danazol and its glycinate being present in portal and systemic plasma. Danazol sarcosinate forms ester droplets in the intestinal tract which are very slowly hydrolyzed by intestinal enzymes. However, all of the

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sarcosinate ester is hydrolyzed during absorption resulting in only danazol being present in portal plasma. Each of the prodrugs, as oral dosages, resulted in increased systemic levels of danazol. This study clearly demonstrates how oral bioavailability can be improved with prodrugs, how metabolic mechanisms for prodrugs can be determined, how some animal models can mimic human drug disposition, how with three simple aminoacid esters, species differences can be demonstrated and what large differences can occur in physical form and hydrolytic reactivity.

INTRODUCTION

Danazol (**D**) is a synthetic, neutral, steroidal derivative of ethisterone, used in the treatment of endometriosis. It suppresses the pituitary-ovarian axis by inhibiting the output of gonadotropins from the pituitary gland (1). The normal and ectopic endometrial tissue is altered so that it becomes inactive and atrophic resulting in complete resolution of endometrial lesions in the majority of cases.

Doubling the dose of **D** only results in a 35% to 40% increase in blood levels (1). The lack of dose proportionality is probably due to the drug's low aqueous solubility of 5 mg/mL, an important factor in bioavailability, which could be increased by forming aminoacid prodrugs. The important factors in bioavailability of several compounds have been substantially improved by using the aminoacid prodrug as the delivery form.

The solubility problem of metronidazole, related to parenteral formulation, was overcome by preparation of amino acid esters of the drug (2). Amino acid esters were proposed as useful prodrugs in 1975 (3). The lysine ester of diethylcromoglycate resulted in a six fold increase in oral bioavailability in rabbits (4). A lysinate ester of hexadecanol significantly increased its uptake by rat mucosal cells (5). The absorption rate for estrone lysinate was shown to be up to five orders of magnitude greater than for estrone (6). The dissolution rate of hydrocortisone as a lysinate prodrug was increased and the drug was hydrolyzed rapidly at pH 7 (7). The synthesis and hydrolytic kinetics of the glycinate of acetominophen have been described (8). The alanyl ester of cephalosporin showed enhanced absorption and antibacterial action in mice (9).

Three amino acid ester prodrugs of **D** were synthesized by the Medicinal Chemistry Department of Sterling Winthrop Pharmaceuticals Research Division, in an attempt to improve its bioavailability, resulting in higher systemic danazol levels, by increasing its aqueous solubility. The oral absorption and hydrolytic characteristics of danazol lysinate (**DL**),

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danazol glycinate (**DG**), and danazol sarcosinate (**DS**) were investigated to evaluate each amino acid promoiety for suitability as a danazol delivery form in 1988. Hydrolytic rates in rat and dog fluids were compared to those in human fluids to find suitable animal models and demonstrate species differences. Porcine enzymes were used to determine specific enzyme effects in intestinal fluids. Lysine and glycine were chosen as promoieties because of the published successes mentioned above. Sarcosine was chosen to ascertain the effect of the N-methyl group on enzymatic stability. The prodrug structures are shown in FIGURE 1.

MATERIALS AND METHODS

Surgical Procedures: All animal care and use procedures were in accord with the Guide for Care and Use of Laboratory Animals (NIH Pub 86-23, 1985), and the Sterling Winthrop Pharmaceuticals Research Division, approved by the Institutional Animal Care and Use Committee.

Albino, male, Sprague Dawley rats (220 to 477 g) were fasted four hours prior to all surgical procedures, but permitted water ad libitum. An I.P. injection of sodium pentobarbital at 50 mg/Kg was used to effect anesthesia. The animals were maintained on a 37°C slide warmer (Precision Scientific, Chicago, IL) throughout surgery and the subsequent experiment. Cannulations were made through a midline abdominal incision. All veinous cannulation sites are shown in FIGURE 2. All blood samples were heparinized (50 IU/mL) and centrifuged to separate the plasma fractions.

Rat Intestinal Absorption: An intraduodenal bolus was delivered from a glass syringe via teflon tubing (P.N. 26809, Waters Associates, Milford, MA), to a glass cannula positioned isoperistaltically in the duodenum, immediately posterior to the pyloris to eliminate gastric influences. The cannula was secured with a 4.0 silk suture ligation eliminating gastric influence. A 20 cm PE-50 cannula (Clay Adams, Parsippany, NJ), fitted with a 5 mm, 23 gauge needle tip was filled with heparinized normal saline (200 IU/mL), inserted into the hepatic portal vein and secured with instant adhesive, to allow blood collection. Plastic wrap (Reynolds Manufacturing, Richmond, VA) was placed over the opened abdomen to prevent dehydration during the experiment.

Rat Liver Perfusions: Heat molded J-shaped cannulas of PE-50 tubing with the same size needle tip, were placed in the hepatic portal vein.

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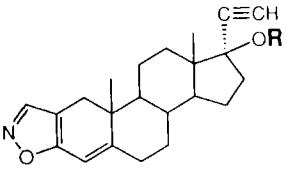

MOLECULE	R-GROUP
DANAZOL	H
DL	$COCHNH_2(CH_2)_4NH_2$
DG	$COCH_2NH_2$
DS	$COCH_2NHCH_3$

FIGURE 1

Chemical structures of danazol (**D**), danazol lysinate (**DL**), danazol glycinate (**DG**), and danazol sarcosinate (**DS**).

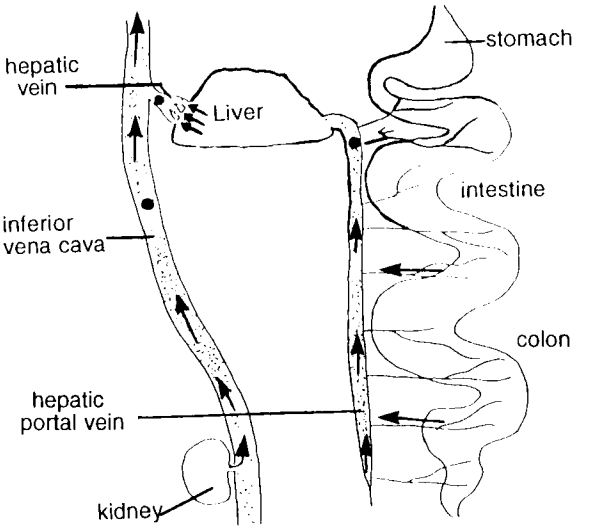

FIGURE 2

Diagramatic representaion of the digestive veinous vasculature. Veinous cannulation sites are identified by 0.
(Diagram provided by Shannon Simmons).

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Identical cannulas were placed in the hepatic vein directly posterior to the diaphragm, and in the Vena Cava inferior, superior to its junction with the renal vein, for blood collection (FIGURE 2). All catheterizations were secured with instant adhesive (Super Glue Corp., Hollis, NY). The drug solutions were perfused (Syringe pump, model 1341A, Sage Instruments, Cambridge, MA) for either 30 or 60 minutes at 0.1 mL/min. Hepatic and systemic (Vena Cava inferior) blood samples were collected at 10 minute intervals for 60 or 90 minutes, for analysis.

Hydrolysis of the Esters in Rat, Dog, and Human Blood Plasma: Whole blood was collected during aspiration of the intestinal fluid, was heparinized with 50 mL of 10,000 IU/mL of heparin sodium for each 10 mL of blood, and the plasma fraction separated by centrifugation. A 3 mL portion of each plasma was heated to 37°C in a circulating water bath (Model D1-G, Haake Buchler Instruments, Inc., Saddle Brook, NJ), then spiked to about 20 nmoles/mL of prodrug in aqueous solution. The experimental solutions were maintained at 37°C for the duration of the sampling period. Prodrug hydrolysis in the plasma was quenched with acetonitrile. The sample was then centrifuged and analyzed as described in the HPLC section.

Hydrolysis of the Esters in Porcine Esterase and Porcine Pancreatin: A pH 8.0, phosphate buffer solution (0.05 molar, Fisher Scientific, Fair Lawn, NJ) was heated to 37°C in 9.75 mL aliquots with the circulating water bath. Porcine esterase (Sigma Chemical Co., St. Louis, MS) was added in 3 drop portions to the buffer, and was vortex mixed. Immediately, 250 ml of a 1.0 mmole/mL prodrug solution was added.

A pH 7.0 phosphate buffer (0.05 molar, Fisher Scientific, Fair Lawn, NJ) was heated to 37°C in a 40 mL volume, and 500 mg of porcine pancreatin (Sigma Chemical Co., St. Louis, MS) was added. The solution was mixed and subsequent 1:1 dilutions with the buffer were prepared, centrifuged, and the clear supernatant heated to 37°C in 9.75 mL aliquots. The 1.0 mmole/mL prodrug solutions were added in 250 mL volumes to the individual enzyme-buffer preparations. Samples were taken from both preparations at regular timed intervals.

Particle Size Determinations: Particle size measurements were made with a Malvern 2600c laser particle sizer, operated with an AT & T 6300 PC computer having a M3.0 version software, and using a 14.3 mm beam, 63 mm lens and a 15 mL cell volume (Malvern Instruments, Malvern, United

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Kingdom). The instrumental measurements were used to verify the precipitations observed microscopically.

Hydrolysis in Rat Intestinal Fluids: Rat intestinal fluid was collected from a 20 cm length of the gut using the duodenal canulation described in the rat intestinal absorption method. The gut was washed with 1 ml of normal saline and up to 3 ml volume was collected at the exit teflon cannula (1 cm length of P. N. 26809, Waters Associates, Milford, MA). The fluid was diluted 1:1 with normal saline, mixed and centrifuged. The supernatant was heated to 37°C, and spiked to about 16 nmoles of prodrug in aqueous solution. Samples were taken at regular intervals for HPLC analysis.

Rat intestinal fluid was collected with a 3 mL rinse of 5% dextrose, and centrifuged. The clear supernatant and an equal volume of an aqueous prodrug solution (1 mg/mL) were mixed in a 10 mL glass vial and examined microscopically at 630X with a Leitz Laborlux 12 polarizing microscope (Upstate Technical, Syracuse, NY). Photomicrographs of the precipitates were taken with an Olympus Model 2S 35 mm camera (Olympus Corp., Woodbury, NY).

Hydrolysis in Human Gastro-Intestinal (GI) Fluids

Surgically sterile healthy female volunteers, ranging in age from 28 to 44 years and within 20% of normal body weight, with no known history of gastrointestinal disorder or substance abuse, and free of prescribed medications for the preceeding two weeks were admitted to the Sterling Winthrop Pharmacology Study Unit at Albany Medical Center Hospital in Albany, New York. Following an overnight fast, cetacaine spray and viscous lidocaine were used to facilitate oral intubation with a triple lumen catheter. Placement of the distal end of the catheter beyond the Ampulla of Vater was confirmed by fluoroscopy. After an initial collection of intestinal fluid, Kinevac (cholecystekinin) was administered to stimulate the gall bladder, simulating the fed condition. A second fluid sample was then collected. Intestinal perfusion of normal saline was necessary in six of the 14 subjects during the aspiration.

Gastric fluid was obtained from fasted volunteers using a standard clinical nasogastric tube. Placement of the tube was verified by ascultation, and Kinevac was not administered.

The GI fluids were diluted 1:1 with distilled water, mixed and centrifuged. Diluted intestinal fluids were assayed for pancreatic lipase and amylase activity with Delta test kits (Biomedix, Division of Electro-Nucleonics, Inc., Cranbury, NJ). A 3 mL portion of each fluid was heated

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to 37°C, and spiked with an aqueous prodrug solution. Aliquots of the incubates were quenched in acetonitrile as previously described for HPLC analysis.

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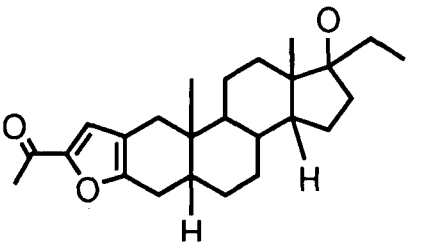
HPLC Analysis:

Internal Standard:



1-[(5a,17a)-17-Hydroxypregn-2, 20-dienol [3,2-b]furan-5'-yl]-ethanone; 6 mg/mL in acetonitrile

Standards: Stocks - 1 mg/ mL in methanol

1. Danazol (D)
2. Danazol Lysinate (DL)
3. Danazol Glycinate (DG)
4. Danazol Sarcosinate (DS)

To 1.9 mL of unmedicated blood plasma, 100 mL of drug stock was added, to make plasma concentrations of 50 mg/mL. Further dilutions were made in plasma to obtain a standard series of 10, 8, 6, 4, 2, and 0.5 mg/mL for each drug. This procedure was repeated with each fluid type i. e. , gastric, intestinal fluid. Blood plasma and GI fluid samples were added in 100 or 200 mL volumes to 400 mL of acetonitrile to quench drug decomposition, extract the compounds and precipitate biological materials interfering with the HPLC analysis. All standards and samples were extracted with acetonitrile containing the internal standard, as described above, and assayed by one of the methods outlined below:

	DL and DG	DS
Mobile Phase:	Water-Acetonitrile-THF (450 : 400 : 150) + 4 g Sodium Dihydrogen Phosphate + 200 mg Octanesulfonic Acid	Acetonitrile-0.05M Ammonium Acetate Buffer (pH 4.7), (725 : 275)

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Flow: 1.5 mL/min 1.0 mL/min
(Model 501 pump, Waters Associates, Milford, MA)

Retention Times: **D**-7.9; **DL**-4.8; **DG**-5.1 **D**-10.6; **DS**-6.1
(min)

Injection: 50 mL, (WISP 712, Waters Associates, Milford, MA)

Guard Column: RP-18 Newguard 15 x 3.2 mm, 7 mm
(Brownlee Labs, Inc., Santa Clara, CA)

Analytical Column: C-18, 250 x 4.6 mm, 10 mm
(ODS-3, Whatman Inc., Clifton, NJ), for analysis of animal fluids(Econosil, Alltech Associates, Inc., Deerfield, IL), for analysis of human fluids

Detection: 284 nm, 0.01 aufs, (Spectroflow 757 Variable Wavelength Detector, Kratos Analytical, Ramsey, NJ)

Integration: Peak area (745 Data Module, Waters Associates, Milford, MA) Peak height (Fisher Recordall Series 5000, Fisher Scientific, Fair Lawn, NJ)

Calculations; Linear regression analysis of the peak area or peak height ratio of drug/internal standard was used to interpolate sample drug concentrations.

RESULTS AND DISCUSSION

HPLC Analysis: Correlation coefficients for all assays were greater than 0.99. Sample chromatograms of prodrugs and **D** extracted from human blood plasma are shown in FIGURE 3. **D** and all the prodrugs were detectable to at least 0.2 nmoles/mL.

Rats, rat fluids, dog plasma and porcine enzymes were used to model the hydrolytic profile of the prodrugs and to compare with results in human blood plasma and gastrointestinal fluids. Species differences were demonstrated as well as how three simple aminoacid ester promoieties can result in delivery, absorption and metabolism differences of **D**. The results are summarized in TABLE 1, and discussed below.

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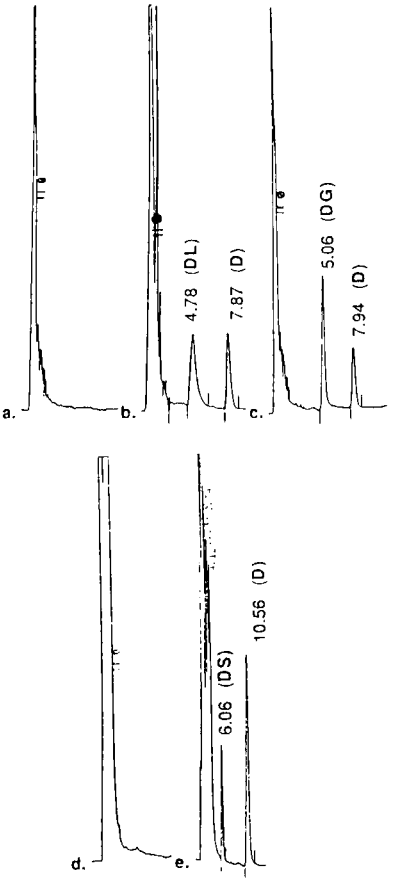

FIGURE 3
HPLC chromatograms of extracts from human blood plasma; (a) blank, (b) **DL** and **D**, (c) **DG** and **D**, (d) blank, (e) **DS** and **D**.

Rat Intestinal Absorption: Rat portal vein concentrations (mmoles/mL) of **D** following intraduodenal bolus administration of 8 and 31 mmoles of **DL** are shown in FIGURE 4. With the intraduodenal bolus, gastric influence was eliminated as a variable of unknown effect. No **DL** was detected in the rat portal blood, but dose proportionality was demonstrated at both doses. Since the absorption process was not saturated, solubility limited absorption kinetics were not evident, and all **DL** was hydrolyzed to **D** prior to reaching the portal circulation.

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TABLE 1
Comparison of In-vivo and In-vitro metabolic disposition of three danazol aminoacid ester prodrugs.

	DANAZOL PRODRUG		
Type of Study	Lysinate	Glycinate	Sarcosinate
Rat Intraduodenal Bolus	**D** present, no ester in portal plasma	**D**:**DG** ratio 2:1 in portal plasma	**D** present, no ester in portal plasma
Rat Liver Perfusion	No **D** in systemic plasma - No hydrolysis	Both **D** and **DG** in hepatic and systemic plasma	No **DS** in hepatic or systemic plasma; complete hydrolysis
Rat Intestinal Fluid	Rapid hydrolysis	No hydrolysis	Slow hydrolysis
Human Gastric Fluid	No hydrolysis	No hydrolysis	Study not done
Human Intestinal Fluid	Rapid hydrolysis	No hydrolysis	Slow hydrolysis
Porcine Liver Esterase	No hydrolysis	Very rapid hydrolysis	Very rapid hydrolysis
Porcine Pancreatin	Very rapid hydrolysis	Very slow hydrolysis	Very slow hydrolysis
Rat Plasma	No hydrolysis	Rapid hydrolysis	Rapid hydrolysis
Human Plasma	No hydrolysis	No hydrolysis	No hydrolysis
Dog Plasma	Slow hydrolysis	No hydrolysis	No hydrolysis

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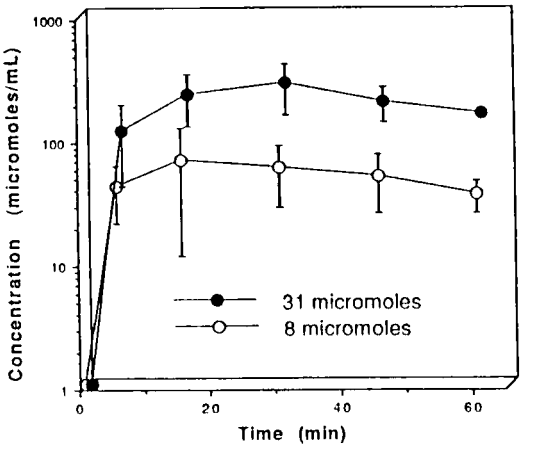

FIGURE 4

Plots of the portal plasma concentrations of **D** (mmole/mL) versus time (min), following intraduodenal bolus dosing of **DL** at 31 mmoles (n=4) and 8 mmoles (n=5) in rats ranging in weight from 256 g to 477 g. The vertical bars represent the standard deviations.

Glycine ester and **D** portal plasma concentrations, following intraduodenal bolus dosing of **DG** at 1.8, 6.1, and 12.2 mmoles base are plotted in FIGURE 5. The concentration ratios between the ester and **D** were approximately 1:2 in the portal circulation. There were no detectable levels of **DG** or **D** in the systemic plasma at any dose level. Therefore in contrast to **DL**, the glycinate is more stable to hydrolysis prior to portal system circulation but is completely hydrolyzed by the liver. (See Rat Liver Perfusions).

Rat portal vein plasma concentrations of **D** (nmoles/mL) following intraduodenal bolus administration of 2 and 20 mmoles of **DS** are shown in FIGURE 6. **DS** was not detected in the rat portal blood in any of the six rats over the 1 hour experiment. All **DS** was completely hydrolyzed to **D** prior to reaching the portal circulation. Peak portal blood concentrations of **D** occurred by 10 minutes at both doses (2 and 20 mmoles). A rapid decline followed the low dose administration, but the higher portal blood levels were sustained after the high dose was given (FIGURE 6). Thus dose proportionality and sustained delivery of **D** to the portal circulation were demonstrated when **DS** was administered intraduodenally.

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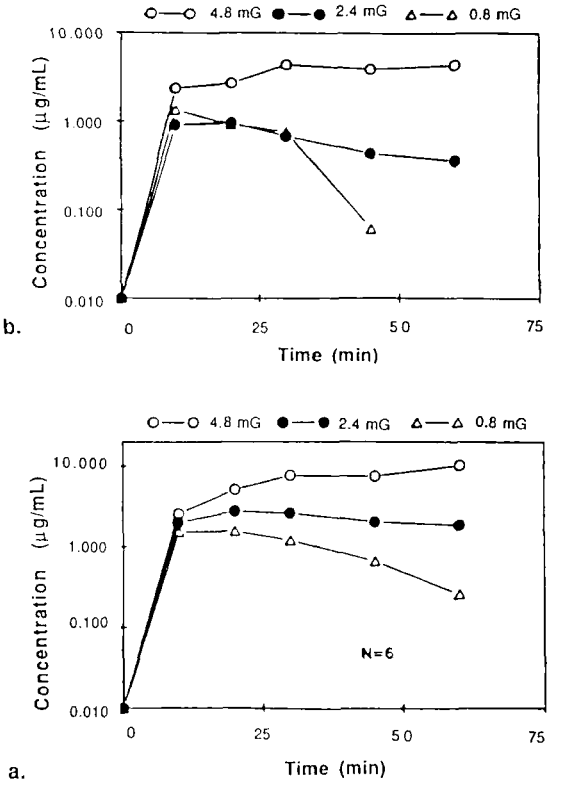

FIGURE 5

Plots of the rat portal vein plasma concentrations of danazol (**D**) (a) and glycine ester (**DG**) (b), versus time (min) following intraduodenal bolus dosing of **DG** at 1.8, 6.1 and 12.2 mmoles. The data points overlap the standard deviations. n=6.

Rat Liver Perfusions: Since **DG** was detected in the portal blood, liver perfusions were conducted to assess the extent of hepatic activity on intact prodrug. **DL** and **DS** were also tested. A plot of systemic plasma concentrations of danazol lysinate versus time following intraportal infusion of **DL** (2.2 nmoles/mL at 0.1 mL/min for 60 min) is shown in FIGURE 7. No **D** was found in the systemic plasma. The apparent half-life estimated from the slope of the systemic plasma curve of **DL**, following cessation of portal infusion, was less than 10 minutes, indicative of rapid distribution and/or elimination of **DL**.

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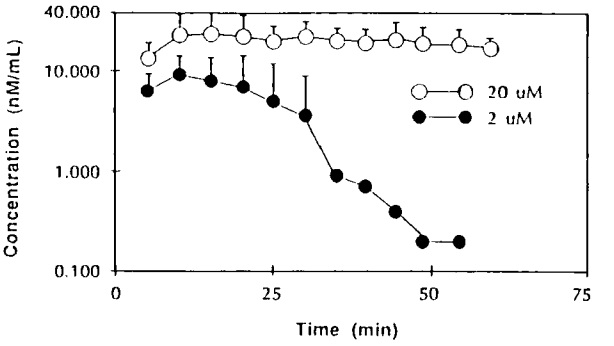

FIGURE 6

Semilogarithmic plots of portal vein concentrations of **D** (nmoles/mL) versus time (min) following intraduodenal bolus dosing of **DS** at 2 and 20 mmoles. The vertical bars represent the standard deviations. n=6.

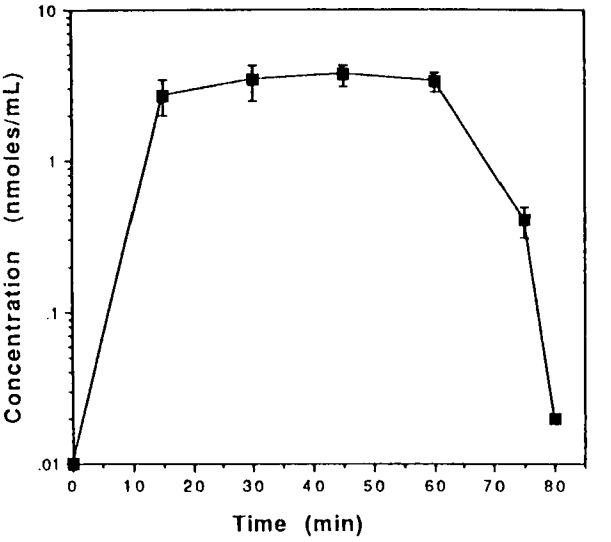

FIGURE 7

Plot of systemic plasma **DL** concentrations (mmoles/mL) versus time (min) following intraportal infusion of **DL** (2.2 nmoles/mL, 0.1 mL/min for 60 min). No **D** was detected (n=4). The vertical bars represent the standard deviations.

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In contrast to the marked lack of metabolism of **DL** by the liver, hydrolysis of **DG** occured intraportally in the rat as shown at two dose levels (6 and 30 mmoles), followed by measurement of the ester and **D** levels in the hepatic vein. Plots of **DG** and **D** concentrations in the hepatic veinous plasma versus time, following intraportal infusion of **DG** are shown in FIGURE 8. Significant bioconversion upon first pass at the 0.1 mL/min infusion rates for 30 min (2 and 10 mmoles/mL concentration) was observed. The disappearance of **D** from the hepatic veinous plasma was apparently first order following cessation of infusion at 30 minutes (FIGURE 8).

When **DS** was infused into the rat portal vein (6 mmoles: 0.1 mL/min x 2 mmoles/mL x 30 min), only **D** was detected in the hepatic and systemic plasmas. **D** concentrations increased during the 30 minute perfusion of **DS** and decline by first order elimination afterward (approximately a 9 minute half-life; FIGURE 9). **DS** was shown to be rapidly and completely hydrolyzed by the rat liver to **D** which was then quickly eliminated. The degree of aminoacid ester hydrolysis appears to be related to the polarity and aqueous solubility of the **D**-ester. For these three **D**-esters, as the polarity increases from N-methylglycine (sarcosine) to glycine to lysine, the degree of hydrolysis by the rat liver changes from complete bioconversion to no hydrolysis.

Hydrolysis of Esters in Rat, Dog, and Human Blood Plasma: No enzymatic hydrolysis of **DL** was found in rat or human blood plasma, but slow hydrolysis occurred in dog plasma. Plots of **DG** and **DS** conversion to **D** in rat plasma, with an approximate half-life of 1 hour and 74 minutes respectively, are shown in FIGURE 10. This demonstrated that **D** observed in hepatic veinous plasma following intraportal infusion of **DG** (FIGURE 8) was mainly due to liver bioconversion and not mediated by plasma esterases.

The ranking of these three esters by hydrolytic reactivity in rat plasma is the same as for the rat liver.

In dog plasma, no change in **DS** or **DG** concentrations were seen after 2 hours, but a slow bioconversion was shown for **DL** with an approximate half life of 90 minutes. Species difference was also demonstrated using human plasma where none of the esters were changed over a 3 hour period.

Hydrolysis of the Esters in Porcine Esterase and Porcine Pancreatin: In porcine pancreatin **DG** and **DS** were not hydrolyzed but **DL** had a half life of about 2 minutes, forming fine oily droplets of danazol, with an average

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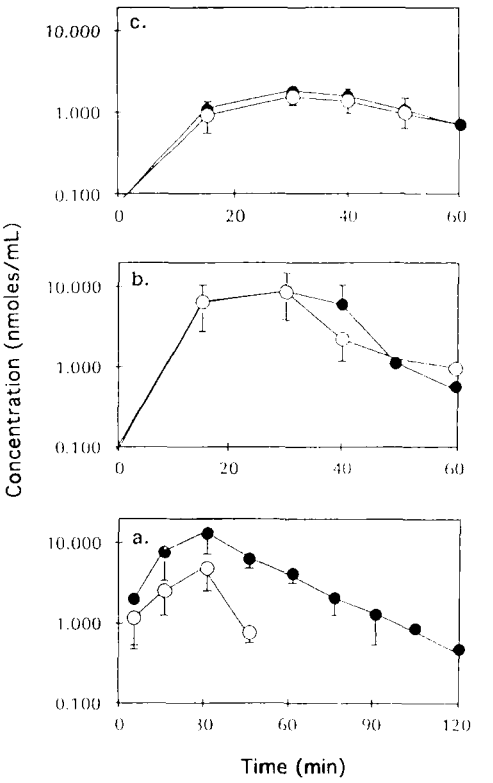

FIGURE 8

Plots of rat blood plasma profiles of **DG** (O) and **D** (O) following intraportal vein infusions of **DG** at 0.1 mL/min for 30 min; (a) **DG** and **D** concentrations (nmole/mL) observed in hepatic vein (post-liver) plasma after infusion of **DG** (10 mmoles); (b) **DG** and **D** concentrations measured in the hepatic venous plasma after infusion of **DG** (2 mmoles); (c) levels of **D** and **DG** in systemic plasma under the same conditions as (b). n=5.

particle size of 2.5 mm in a narrow distribution (FIGURE 11). The oily droplets were identified as danazol by retention time during HPLC analysis. This is a significant finding since liquids have greater bioavailability and **D** is a high melting compound (225°C). The formation of oily droplets in biological fluids has been previously reported (10). No hydrolysis occurred in the buffer control.

In porcine esterase, **DS** was the most rapidly hydrolyzed prodrug (half life of less than 1 minute) followed by **DG** (half life of about 1.2

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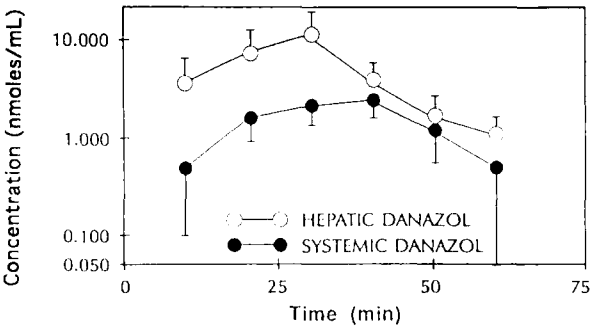

FIGURE 9
Semilogarithmic plots of hepatic and systemic danazol concentrations (nmoles/mL) following hepatic portal vein infusion of **DS** (6 mmoles) in rats. The vertical bars represent the standard deviations. n=4

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minutes; FIGURE 12). No appreciable conversion of **DL** to **D** occurred for up to 60 minutes. Ranking of the prodrugs by hydrolytic rates in porcine esterase is in agreement with that seen for rat liver metabolism (FIGURES 7, 8 and 9).

Hydrolysis in Rat Intestinal Fluids: **DL** was rapidly hydrolyzed in rat intestinal fluid with a half life of 5.2 minutes, whereas **DS** was slowly hydrolyzed (about 10% in 2 hours) and **DG** concentrations remained unchanged. After the **DS** intraduodenal bolus in the rat, high **D** levels resulted in the portal blood but no **DS** was present. Since enzymatic activity in the rat intestinal fluid was low, hydrolysis of **DS** must have occurred in the mucosal cells. For **DL**, ester hydrolysis could occur both in intestinal fluid and in mucosal cells which resulted in the detection of no ester in the portal circulation. Only partial hydrolysis of **DG** was seen prior to reaching the portal circulation. Since rat intestinal fluid had very little effect, most of the hydrolysis probably occurred in the mucosa.

Physical characteristics of the prodrug-intestinal fluid mixtures are different as shown in the photomicrographs (FIGURE 13). Droplets were formed with **DL** and **DS** which were 1 to 5 microns in diameter. Particles (2 to 5 microns) that were determined to be amorphous (by microscopy using cross polarized light), and many large aggregates were formed with **DG**. These physical characteristics are conducive to rapid dissolution and

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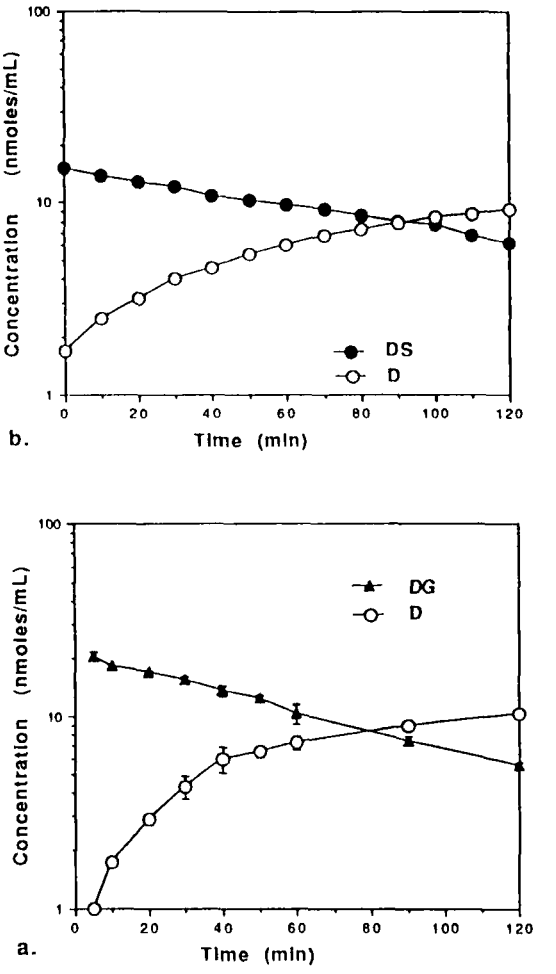

FIGURE 10
Concentrations of **DG** and **D** (a) and **DS** and **D** (b) in 3 mL of rat plasma at 37°C versus time. The vertical bars represent standard deviations. n=4.

rapid absorption. Oily droplet formation in human gastric and intestinal fluids was previously reported (10).

Hydrolysis in Human Gastro-Intestinal (GI) Fluids: The test results for enzymatic activity of the human intestinal fluids are shown in TABLE 2.

No substantial differences were seen between fasted and "fed" conditions for pancreatic lipase or amylase activity in the intestinal fluid of the six volunteers.

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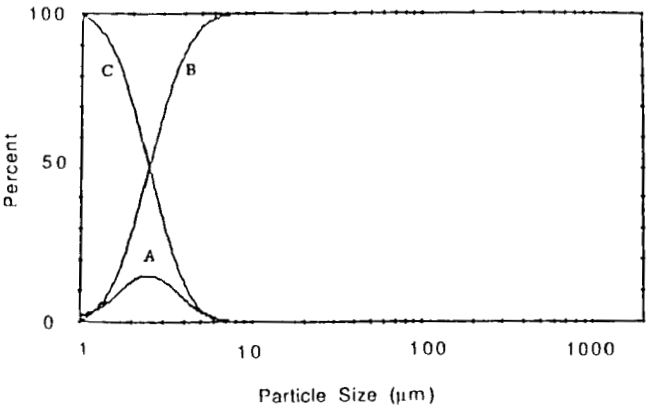

FIGURE 11

Particle size distribution of oily droplets formed from in-vitro hydrolysis of **DL** in the presence of porcine pancreatin (pH 7.2, 37°C). The estimated average particle size was 2.5 mm. Curve A is the frequency distribution in each particle size range. Curves B and C are percent of the particles under and over the indicated particle size respectively. n=3.

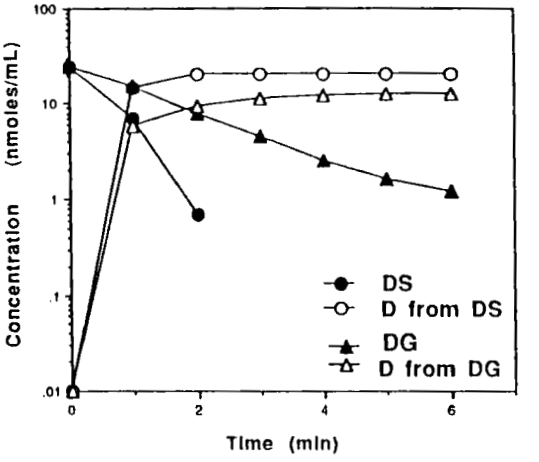

FIGURE 12

Plots of hydrolysis data of **DS**, **DG**, and generation of **D** versus time in the presence of porcine liver esterase. The data points are larger than the error bars. n=3.

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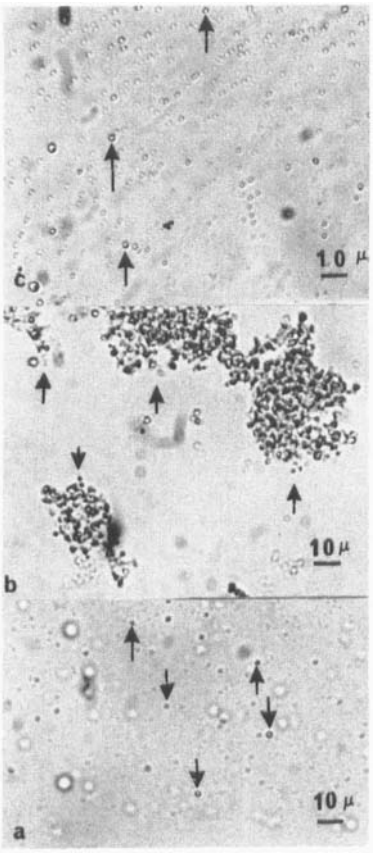

FIGURE 13
Photomicrographs (630X) of 1:1 mixtures of danazol ester solutions (1 mg/mL) and rat intestinal fluid; (a) **DS**, (b) **DG**, (c) **DL**.

TABLE 2

Pancreatic lipase and amylase activity measured in intestinal fluids of six volunteers.

	FASTED		FED	
Subject	Lipase units/mL	Amylase units/mL	Lipase units/mL	Amylase units/mL
A	576	927	49	933
B	13	1763	353	871
C	572	875	417	787
D	501	983	779	815
E	1095	859	832	783
F	1261	909	1168	781

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During the 60 minute incubation period with human gastric fluids, **DL** and **DG** concentrations remained unchanged and no **D** was detected. Therefore, these esters would be expected to pass intact through the stomach to the duodenum.

DL was rapidly hydrolyzed in intestinal fluids from both the "fed" (half-life of 22 ± 8 min) and fasted (half-life of 18 ± 9 min) states. Plots of mean concentrations are shown in FIGURE 14. Very fine oily droplets (1-2 microns) were formed in the mixtures similar to those produced by rat intestinal fluid or porcine pancreatin. **DG** concentrations did not change and no **D** was detected during the 60 minute incubation interval, in the "fed" or fasted state intestinal fluids from any of the six subjects, therefore no **DG** hydrolysis occurred. Slow ester hydrolysis occured for **DS** in intestinal fluid (about 13% over 1 hour at 37°C) in the fasted state. Hydrolysis of **DS** in intestinal fluid collected after Kinevac administration was slower, about 1% over 1 hour at 37°C. Aminoacid ester hydrolytic rates in human and rat intestinal fluids were very comparable.

CONCLUSIONS

A tabulation of the principal findings of this research is shown in TABLE 1. Large differences in hydrolytic rates are evident between species and aminoacid moiety. The species effect is very evident with respect to plasma esterases in that the rat plasma esterases are able to rapidly hydrolyze the **DG** and **DS** which are not affected at all by dog or human plasma esterases. But little difference between species is seen for **DL**. The hydrolytic effect of porcine pancreatin and rat intestinal fluid is very similar to that of human intestinal fluid. **DL** has a great propensity for hydrolysis compared to slow or no reaction for **DG** or **DS**.

This enzyme specificity, related to the aminoacid moiety, may be a function of the epsilon amino group of the lysine molecule which in the intestinal pH range causes **DL** to be a doubly charged cation as compared to the unit charge expected of **DG** or **DS**. Aqueous solubility is directly correlated with the polarity or molecule charge which is greatest for the **DL**. However, results from the rat liver perfusion study and the pig liver esterase hydrolysis study show that the highly polar **DL** is not susceptible to enzymatic hydrolysis whereas the two less polar esters are highly susceptible. This shows the differences between esterase enzymes.

Results of the porcine pancreatin, rat and human intestinal fluid experiments are comparable and **DL** is the only prodrug which is rapidly hydrolyzed. It is apparent from the results in TABLE 1 that the bioavailability of **D** to the portal circulation in the rat after an

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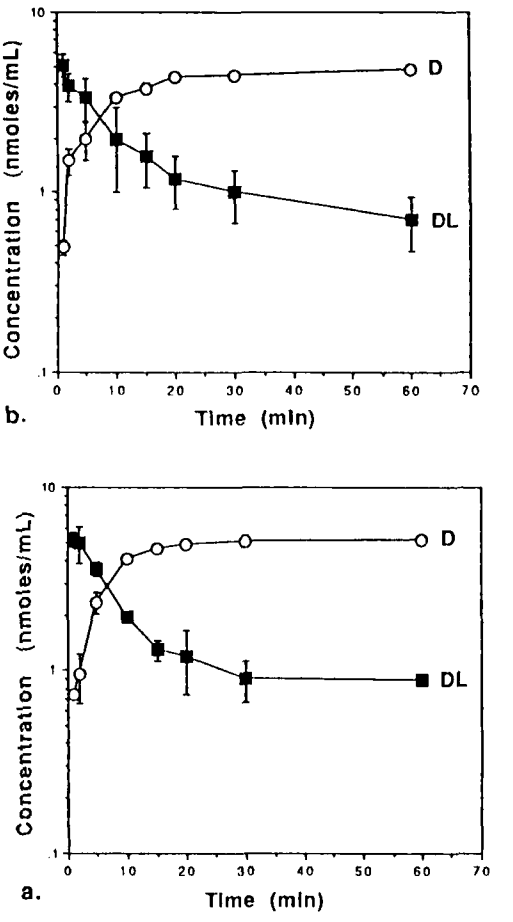

FIGURE 14
DL and **D** concentrations resulting from **DL** incubation at 37°C in human intestinal fluid of fasted (a) and "fed" (b) subjects, versus time.

intraduodenal bolus could be the result of complete hydrolysis of **DL** before absorption occurs. However, for **DS**, extensive hydrolysis probably occurs during absorption by the intestinal wall esterases. Partial hydrolysis of **DG** must take place in the intestinal wall.

This research has demonstrated that small variations in the aminoacid promoiety of **D**-esters can result in widely different delivery mechanisms for the prodrug or **D** to reach the systemic circulation. **DL** or **DS** appear to be ideal prodrugs for delivering **D** in a readily absorbed form

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with no prodrug being available in the portal circulation. Based on the data in the animal models and the limited in-vitro human experiments, a basis was established for the most probable hydrolytic profile of each ester in humans.

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