Rat and Rabbit Plasma Distribution of Free and Chylomicron-Associated BIRT 377, a Novel Small Molecule Antagonist of LFA-1-Mediated Cell Adhesion

Kishor M. Wasan,[1,5] Manisha Ramaswamy,[1] Lorilynne Holtorf,[1] Andrew A. Jayaraj,[2] and David J. Hauss[3,4]

Received August 23, 2000; accepted December 22, 2000

Purpose. The objectives of this study are to determine the plasma distribution of free and chylomicron-associated BIRT 377 within rats and rabbits.

Methods. For the rat studies free and chylomicron-associated BIRT 377 was incubated in plasma from CD 1 non-fasted rats for 60 minutes at 37°C. Following incubation the plasma was separated into its lipoprotein and lipoprotein-deficient plasma (LPDP) fractions by three different methods and analyzed for BIRT 377 content by HPLC. For the rabbit studies New Zealand fasted white rabbits (3 kg; n = 4) were administered an intravenous dose of free BIRT 377 (1 mg/kg). Following administration, serial blood samples were obtained and the plasma was analyzed for BIRT 377. The plasma collected at the 0.083-h time point was separated into each of its lipoprotein fractions and analyzed for BIRT 377.

Results. 37.8 ± 1.2% of the original drug amount incubated in rat plasma was recovered within the lipoprotein-rich fraction. 41.5 ± 0.4% of the original chylomicron-associated drug concentration incubated was recovered within the lipoprotein-rich fraction. The percentage of drug recovered within the TRL fraction was significantly greater following the incubation of chylomicron-associated BIRT 377 compared to free BIRT 377. In addition, BIRT 377 apparently follows a two-compartment pharmacokinetic model following single intravenous dose administration to rabbits.

Conclusions. These findings suggest that plasma lipoprotein binding of BIRT 377 is evident and may be a factor in evaluating the pharmacological fate of this drug when administered to patients that exhibit changes in their plasma lipoprotein lipid.

KEY WORDS: lipid-based drug delivery; lipoproteins; hydrophobic drugs; chylomicrons.

INTRODUCTION

The plasma lipoprotein distribution of potential drug candidates is not commonly studied. For some hydrophobic drug candidates, attainment of similar plasma free drug levels has not been associated with uniform production of pharmacological activity in different animal species (1). It is well known that plasma lipoprotein profiles vary considerably between different animal species (1,2). In addition, disease states can significantly influence plasma lipoprotein profiles, resulting in altered therapeutic outcomes. Current research has shown that the association of drug compounds with lipoproteins can significantly influence not only the pharmacological and pharmacokinetic properties of the drug, but the relative toxicity as well. Elucidation of drug distribution among plasma lipoproteins is expected to yield valuable insight into factors governing the pharmacological activity and potential toxicity of the drug (3–9).

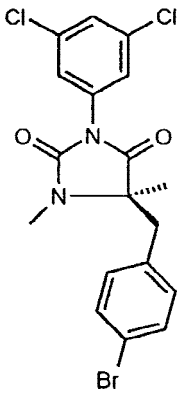
Amphotericin B (AmpB), halofantrine (HF) and cyclosporine (CSA) are examples of hydrophobic drug compounds with different physical chemical properties (i.e., MW, log P, aqueous solubility etc.) that associate to serum lipoproteins *in vivo* and *in vitro* (1,3,8,10–13). In addition there is growing evidence that supports the hypothesis that modifications in serum lipid levels (14–19) may alter the renal toxicity of AmpB (20–26) and CSA (3), and the plasma distribution of HF (8).

Recently Kelly and co-workers have reported the discovery and characterization of (R)-5-(4-bromobenzyl)-3-(3,5-dichlorophenyl)-1,5-dimethylimidazolidine-2,4-dione (BIRT 377) (Figure 1), an orally bioavailable small molecule that interacts with LFA-1 by noncovalent binding to the CD11a chain and prevents LFA-1 from binding to its ligand, ICAM-1 (27). LFA-1 (CD18, CD11a) is a cell-adhesion molecule that mediates critical immunological processes. These investigators further reported that BIRT 377 inhibits lymphocyte activity in both *in vitro* and *in vivo,* in assays that require LFA-1-mediated cell adhesion (27). The mechanism(s) by which this compound is orally bioavailable remains unknown. Preliminary work by Hauss *et al.* (unpublished data) have suggests that a relatively high concentration of BIRT 377 (~10 μg/ml; approximately 25–30% of the oral dose given) was found in mesenteric lymph associated with chylomicrons following oral administration of BIRT 377 to male rats and could serve as a pathway for increased systemic bioavailability of BIRT 377 by avoiding first pass metabolism. However, to date, very little is known about the plasma distribution and biological fate of chylomicron-associated drug upon entry into the bloodstream. Thus, one of the objectives of the proposed study was to determine if free or chylomicron-associated BIRT 377 found in whole mesenteric lymph associates with plasma lipoproteins upon *in vitro* incubation with CD-1 male rat plasma (non fasted).

Although the information gathered from this initial experiment would provide insight into understanding the behavior of chylomicron-associated drug in plasma, the non-chylomicron associated BIRT 377 distribution data generated from these studies using rat plasma cannot be extrapolated to what may be observed in humans because the behavior and relative proportions of lipoproteins in rats are very different from humans [i.e. high density lipoproteins (HDL) in rats are the major carrier of cholesterol while low density lipoproteins (LDL) are the major carrier of cholesterol in humans (2)]. Therefore, subsequent studies will determine (a) the *in vivo* plasma lipoprotein distribution of BIRT 377 when administered to normolipidemic rabbits [where the behavior of lipoproteins are similar to humans (2)], (b) the *in vitro* plasma lipoprotein distribution of BIRT 377 when incubated in rabbit

[1] Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, British Columbia, Canada.
[2] Department of Bioassay, Biogen Inc., Cambridge, Massachusetts.
[3] Drug Metabolism & Pharmacokinetics, Boehringer Ingelheim Pharmaceuticals Inc., Indianapolis, Indiana.
[4] Current address: Eli Lilly, Indianapolis, Indiana.
[5] To whom correspondence should be addressed.

0724-8741/01/0400-0510$19.50/0



BIRT 377

MW = 442.15

Solubility at pH 2.0 <0.05 μg/mL

Solubility at pH 7.4 < 0.05 μg/mL

Log P = 4.08

Fig. 1. Chemical Structure of BIRT 377.

plasma and (c) the percentage of BIRT 377 within the lipoprotein-deficient plasma fraction which is unbound (i.e., BIRT 377 free fraction). Since BIRT 377 is a small hydrophobic compound (log $P = 4.08$) with similar physical chemical characteristics as HF (8), our working hypothesis was that a significant proportion of the drug would be recovered in the lipoprotein fraction and elevations, primarily lipoproteins rich in cholesterol (LDL) and triglycerides (chylomicrons and very-low density lipoproteins), would increase the percentage of drug recovered in these lipoprotein fractions respectively. In addition, we further hypothesized that chylomicron-associated BIRT 377 would primarily be recovered in the triglyceride-rich lipoprotein fraction. Finally, since the disposition of BIRT 377 is unknown the pharmacokinetics of BIRT 377 in this relevant animal model (rabbit) will be determined following single intravenous dose administration.

MATERIALS AND METHODS

BIRT 377

BIRT 377 MW 442.15 (Figure 1) is a hydrophobic small molecule antagonist of LFA-1-mediated cell adhesion with a log P of 4.08 and an aqueous solubility of <0.05 ug/mL at pH 2 and pH 7.4 (27). BIRT 377 was reconstituted and diluted with HPLC grade methanol. To achieve a plasma concentration of 1000 ng of BIRT 377 per ml of rat plasma, 26.5 μl of a BIRT 377 solution (113.2 μg/ml of methanol) was incubated in 3000 μl of rat plasma. For the *in vivo* rabbit study only, BIRT 377 (1 mg/ml) was dissolved into a 70% PEG 400 solution. Preliminary studies have shown that drug solvents such as PEG 400, propylene glycol, DMSO (10%) and ethanol (<10%) do not alter the plasma lipoprotein lipid profile (unpublished data).

Free and chylomicron-associated BIRT 377 contained in mesenteric lymph fluid was assayed for BIRT 377 concentration by HPLC. It was determined that chylomicron-associated BIRT 377 contained 16.8 μg of BIRT 377 per ml of lymph fluid. To achieve a plasma concentration of 1000 ng of BIRT 377 per ml of rat plasma, 178.2 μl of chylomicron-associated BIRT 377 was incubated in 3000 μl of rat plasma. The triglyceride concentration of this chylomicron-associated BIRT 377 suspension was 16.8 mg/ml. The cholesterol and protein concentration of this suspension was 2.7 and 2.67 mg/ml respectively.

Plasma Lipoprotein Separation

Density Gradient Ultracentrifugation

The plasma was separated into its HDL, LDL, triglyceride rich-lipoprotein (TRL, which includes very low-density lipoproteins and chylomicrons) and lipoprotein deficient lipoprotein plasma (LPDP; which includes albumin and α-1 glycoprotein) fractions by step-gradient ultracentrifugation as previously described (28).

To assure that the distribution of free and chylomicron-associated BIRT 377 found in each of these fractions was a result of its association with each lipoprotein or lipoprotein deficient fraction and not a result of the density of the formulation, the density of the free BIRT 377 and chylomicron-associated BIRT 377 formulation incubated in LPDP fraction for 60 minutes at 37°C was determined by ultracentrifugation. The majority of free and chylomicron-associated BIRT 377 (>95%) was found in the density range of 1.21-1.25 g/ml suggesting that the BIRT 377 distribution within the ultracentrifuge tubes following incubation in rat plasma was not a function of formulation density.

Affinity Chromatography

Lipoproteins were separated into the HDL and LDL/TRL fractions by the LDL-Direct cholesterol chromatographic column (Isolab) as previously described (11).

Sequential Phosphotungstic Acid (PTA) Precipitation

Plasma was separated into its TRL/LDL, HDL and LPDP fractions by sequential precipitation as previously described (29). The amount of BIRT 377 associated with the supernatant (LPDP) and pellet (HDL) was determined by high pressure liquid chromatography (HPLC) as described below. All drug incubations were done at 37°C.

Analytical Analysis

HPLC Analysis of BIRT 377

The concentration of BIRT 377 in each lipoprotein and lipoprotein deficient sample was determined by the following HPLC methodology. Sample preparation and drug extraction from each total plasma, lipoprotein and lipoprotein deficient plasma fraction follows: An aliquot of 0.5 ml of sample was used for analysis. To this sample 5 ml of hexane (for total plasma, TRL and LDL samples only) was added and vortexed rapidly for 2 minutes. The supernatant was transferred to set of silicated test tubes. This extraction was repeated again with another 5 ml of hexane. Then 1 ml of 1M acetic acid was added to the supernatant and vortexed for 1 minute. For HDL and LPDP fractions tert-butyl methyl ether was used instead of hexane. This substitution improved extraction ef-

ficiency. The supernatant from this addition was transferred to another set of tubes and evaporated to dryness under nitrogen gas. The remaining residue was reconstituted with 0.5 ml of mobile phase (acetonitrile: water 60:40 v/v) and 100 μl was injected onto a Beckman 4.6 × 25 cm, ODS 5μ column (flow rate = 2 ml/min; wavelength = 220 nm; retention time = 9.5–9.8 min).

The limit of quantitation in all total plasma, lipoprotein and lipoprotein-deficient plasma fraction samples of varying lipid profiles (assuming a signal to noise ratio of 4:1) for BIRT 377 was determined to be 0.019 μg/ml (19 ng/ml) with intra-day coefficient of variation of 6-18%. The validation procedure was as follows: BIRT 377 at concentrations of 0.019, 0.039 and 0.078 μg/ml were reconstituted in each lipoprotein and lipoprotein-deficient fraction and the drug was extracted from each of these samples using the same extraction procedure as described above. Six replicate samples of each concentration on days 1, 2, and 5 were injected to determine inter-day and intra-day accuracy and precision. These external calibration curves, which were run on the same day as the samples themselves, were used to quanitate the percentage of BIRT 377 recovered in each fraction.

Lipid and Protein Analysis

Total plasma triglycerides (TG), cholesterol, and protein concentrations was determined by enzymatic assays purchased from Sigma Diagnostics (St. Louis Mo.) as previously described (8). The strategy for determining the percentage of BIRT 377 that is free and not bound to either lipoproteins or plasma proteins (albumin and α-1 glycoprotein) was as follows: plasma containing BIRT 377 was separated into its lipoprotein (HDL, LDL and TRL) and LPDP by density gradient ultracentrifugation. The LPDP fraction was removed and 1 ml was placed in the upper chamber of the ultrafuge filter column (Centrifree Micropartition Column; Amicon Inc., Beverly MA). This column is designed to separate free from protein-bound microsolute. The sample was then centrifuged in a J2-21 centrifuge using a JA-17 rotor (Beckman Instruments Inc.) at room temperature at 10,000 g for 30 minutes. The resulting filtrate obtained was measured for protein and BIRT 377. Greater than 99% of the protein was removed by this filter (data not shown) and the BIRT 377 measured represents free (unbound) drug. Non specific binding of BIRT 377 to the filter was <2%.

Rabbit Model

The operative technique for chronic catheter insertion was modified from that of Walsh and coworkers to include a heparin lock device as previously described (23,30).

EXPERIMENTAL DESIGN

***In Vitro* Rat Plasma Studies**

To determine the plasma lipoprotein distribution of free and chylomicron-associated BIRT 377, BIRT 377 dissolved in methanol and chylomicron-associated BIRT 377 contained in mesenteric lymph fluid (1000 ng/ml) was incubated for 60 minutes at 37°C in non fasted CD 1 rat plasma. Following incubation the plasma was separated into its HDL, LDL, TRL and LPDP fractions by density gradient ultracentrifugation, affinity chromatography, or sequential PTA precipitation. Each of these fractions was analyzed for BIRT 377 content by an HPLC technique as described above. The final volume of methanol used to reconstitute free BIRT 377 was less than 5% of the total plasma volume and did not alter lipoprotein concentration or composition (data not shown).

***In Vitro* and *In Vivo* Rabbit Studies**

In Vivo *Plasma Distribution and Pharmacokinetic Studies*

Normolipidemic New Zealand fasted white rabbits (3 kg; n = 4) were administered an intravenous dose of BIRT 377 (1 mg/kg) through the jugular vein. Following administration serial blood samples were obtained and stored with 0.25% (w/v) EDTA in centrifuge tubes prior to and 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 36 and 48 hours after the injection. Rabbits were housed in individualized metabolism cages. Plasma samples were stored at 4°C prior to analysis to prevent any redistribution of drug. Plasma (0.5 ml) from each of the serial blood samples were obtained and analyzed for BIRT 377 by HPLC. The plasma collected at the 0.083 h time point was separated into each of its lipoprotein (HDL, LDL, TRL) and LPDP fractions by density gradient ultracentrifugation. Confirmation of BIRT 377 lipoprotein binding was determined by affinity chromatography (data not shown). Absolute BIRT 377 lipoprotein binding was assessed on the basis of total lipoprotein-lipid and protein mass (Figure 3).

For all studies, cholesterol, triglyceride, protein and BIRT 377 concentrations were quantified in each fraction as above. Pharmacokinetics was done by multicompartmental analysis as described by Shargel and Yu (30). WINNONLIN was the pharmacokinetic package used for analysis.

In Vitro *Plasma Distribution*

To determine the *in vitro* rabbit plasma distribution of free BIRT 377, BIRT 377 at a physiological concentration (800 ng/ml) was incubated in rabbit plasma for 0.083 h (5 minutes) at 37°C. This concentration was chosen to reflect the peak concentrations determined in the pharmacokinetic studies (Table V). Following incubation the plasma fractions were separated into their lipoprotein, and plasma lipoprotein deficient (LPDP) fractions by step-gradient ultracentrifugation and each of these fractions analyzed for BIRT 377 content by HPLC. External calibration curves in each lipoprotein and lipoprotein-deficient plasma fraction were used to quantify the amount of BIRT 377 recovered within each fraction. Furthermore, to assure that the plasma distribution of BIRT 377 was not a function of the drug's formulation or due to drug redistribution during ultracentrifugation, BIRT 377 was incubated in lipoprotein-deficient rabbit plasma for 5 minutes at 37°C and formulation density determined. In addition, to confirm our findings BIRT 377 lipoprotein binding was determined by affinity chromatography.

STATISTICAL ANALYSIS

In vitro distribution among rat plasma lipoproteins of BIRT 377, added as either free drug in methanolic solution or associated with chylomicrons in mesenteric lymph fluid was compared by analysis of variance (INSTAT 2; GraphPad). Critical differences were assessed by Newman-Keuls post hoc

tests (32). A difference was considered significant if the probability of chance explaining the results was reduced to less than 5% ($P < 0.05$). All data were expressed as a mean ± standard deviation.

RESULTS AND DISCUSSION

In Vitro Rat Plasma Studies

Total plasma and lipoprotein cholesterol (esterified and unesterified), triglyceride and protein concentrations were determined in non fasted CD I rat plasma and non fasted CD 1 rat plasma which had been supplemented with chylomicrons (2.98 mg TG / 3 ml of plasma). Rat plasma which had been supplemented with chylomicrons had significantly higher TRL cholesterol, TRL triglyceride, LDL triglyceride, total plasma triglyceride, and TRL protein concentrations than non fasted rat plasma (Table I). This is not surprising because triglyceride from TRL can spontaneously transfer through diffusion to LDL without the facilitation of lipid transfer proteins (25). Furthemore, the increase in triglyceride in the LDL fraction may be due to the presence of chylomicrons in this fraction.

Incubation of free BIRT 377 resulted in 37.8 ± 1.2% of the original drug amount incubated recovered within the lipoprotein-rich fraction (TRL, HDL, and LDL fractions combined) when separating lipoproteins by density gradient ultracentrifugation (Table II). Incubation of chylomicron-associated BIRT 377 resulted in 41.5 ± 0.4% of the original drug concentration incubated recovered within the lipoprotein-rich fraction when separating lipoproteins by density gradient ultracentrifugation (Table II). In addition, the percentage of drug recovered within the TRL fraction was significantly greater following the incubation of chylomicron-associated BIRT 377 compared to free BIRT 377 when separating lipoproteins by density gradient ultracentrifugation (12.5 ± 1.0% vs. 18.9 ± 0.5%, $P<0.05$; Table II). These values were confirmed when lipoprotein separation was done by sequential PTA precipitation and affinity chromatography (data not shown). Multiple lipoprotein separation techniques were employed in this study to demonstrate that lipoprotein binding of free and chylomicron-associated BIRT 377 was not a function of the lipoprotein separation technique used.

Furthermore, the amount of BIRT 377 recovered in each lipoprotein fraction was normalized to the amount of cholesterol, triglyceride, protein, and total lipid (cholesterol + triglyceride) recovered within each lipoprotein fraction (Figures 2A–D). In addition, the amount of BIRT 377 recovered in the lipoprotein-deficient plasma fraction was normalized to the amount of protein within the lipoprotein-deficient plasma (Figure 2C). The amount of BIRT 377 per total lipid (cholesterol+triglyceride) (Figure 2D) and triglyceride (Figure 2B) recovered within the TRL and LDL fractions was significantly less following the incubation of chylomicron-associated BIRT 377 compared to free BIRT 377. The amount of BIRT 377 per protein recovered within the lipoprotein-deficient fraction was significantly less following the incubation of chylomicron-associated BIRT 377 compared to free BIRT 377 (Figure 2C). No significant differences were observed when normalizing for the amount of cholesterol within each lipoprotein fraction (Figure 2A). These findings suggest that BIRT 377 distribution is not dependent on lipoprotein cholesterol content when it is presented to the plasma as free or chylomicron-associated drug.

Taken together, the data reported in these studies support the finding that a significant percentage of BIRT 377 associates with plasma lipoproteins (primarily TRL and HDL fractions) on incubation in non fasted rat plasma. Furthermore, eventhough the incubation of chylomicron-associated BIRT 377 in plasma results in the redistribution of drug from chylomicrons to the other lipoprotein and lipoprotein-deficient fractions, more drug is still retained within the TRL fraction than following the incubation of free BIRT 377. This may be due to the increase lipid and protein load within the non fasted rat plasma when exogenous chylomicrons are added.

In Vitro and *In Vivo* Rabbit Plasma Studies

Total and lipoprotein plasma cholesterol (esterified and unesterified), triglyceride and protein concentrations within plasma *in vitro* versus *in vivo* were determined in fasted New Zealand white female rabbits. Total, LDL and HDL cholesterol levels were significantly lower within *in vitro* rabbit plasma than *in vivo* rabbit plasma (Table III). In addition, LDL triglyceride and protein, HDL protein and total plasma pro-

Table I. Total and Lipoprotein Plasma Cholesterol (Esterified + Unesterified), Triglyceride and Protein Concentrations Within Plasma from Non-Fasted CD 1 Rats

Rat profile	Triglyceride rich lipoproteins mg/dl	Low density lipoproteins mg/dl	High density lipoproteins mg/dl	Total plasma mg/dl
Cholesterol (esterified + unesterified)				
No Chylomicrons Added	6.8 ± 0.8	20.5 ± 4.9	39.7 ± 10.3	67.0 ± 4.8
Chylomicrons Added**	8.8 ± 1.1*	22.2 ± 1.5	33.7 ± 3.8	64.7 ± 1.3
Triglyceride				
No Chylomicrons Added	72.0 ± 3.1	12.2 ± 2.6	22.3 ± 8.6	106.6 ± 4.7
Chylomicrons Added**	185.2 ± 7.8*	22.6 ± 3.0*	20.8 ± 1.5	228.7 ± 4.7*
Protein				
No Chylomicrons Added	10.2 ± 0.7	21.6 ± 7.1	288.2 ± 100.2	1958 ± 51
Chylomicrons Added**	23.0 ± 1.4*	28.8 ± 5.2	258.7 ± 29.8	2016 ± 24

Note: Data is expressed as mean ± standard deviation (n = 3); *$p < 0.05$ vs no chylomicrons added; **178 μl = 2.98 mg Triglyceride of purified rat chylomirons was added to non fasted rat plasma; Triglyceride rich lipoproteins include chylomicrons and very low density lipoproteins.

Table II. Distribution of Free and Chylomicron-Associated BIRT 377 at 1000 ng/ml Within Plasma from Non-Fasted CD 1 Male Rats Following Incubation for 60 Minutes at 37°C

Compounds	TRL fraction %[a]	HDL fraction %	LDL fraction %	LPDP fraction %	Percent recovery %
Free BIRT 377	12.5 ± 1.0	17.4 ± 3.7	7.9 ± 0.7	62.2 ± 3.3	101 ± 4.5
Chylomicron-associated BIRT 377	18.9 ± 0.5*	15.8 ± 1.4	6.8 ± 0.6	58.5 ± 1.2	99.7 ± 8.0

Note: Data expressed as mean ± standard; n = 3; [a]percent of initial BIRT 377 amount; LPDP, lipoprotein-deficient plasma; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TRL, triglyceride rich lipoprotein fraction which consists of very low-density lipoproteins and chylomicrons; *$p < 0.05$ vs. Free BIRT 377. Following incubation plasma samples were assayed by high pressure liquid chromatography for drug in each of the lipoprotein and lipoprotein-deficient plasma fractions. Plasma was separated into its lipoprotein and lipoprotein-deficient fractions by density gradient ultracentrifugation.

tein levels were significantly lower within *in vitro* rabbit plasma than *in vivo* rabbit plasma (Table III). These findings are not surprising since rabbits are very sensitive to changes in dietary cholesterol intake (23) and thus are a very atherogenic animal (2,23,30). In this study different rabbits were used in the *in vitro* and *in vivo* studies. To compare these studies the percentage of BIRT 377 recovered in each lipoprotein fraction was corrected for their cholesterol content respectively (Figure 3).

The plasma distribution of BIRT 377 5 minutes following

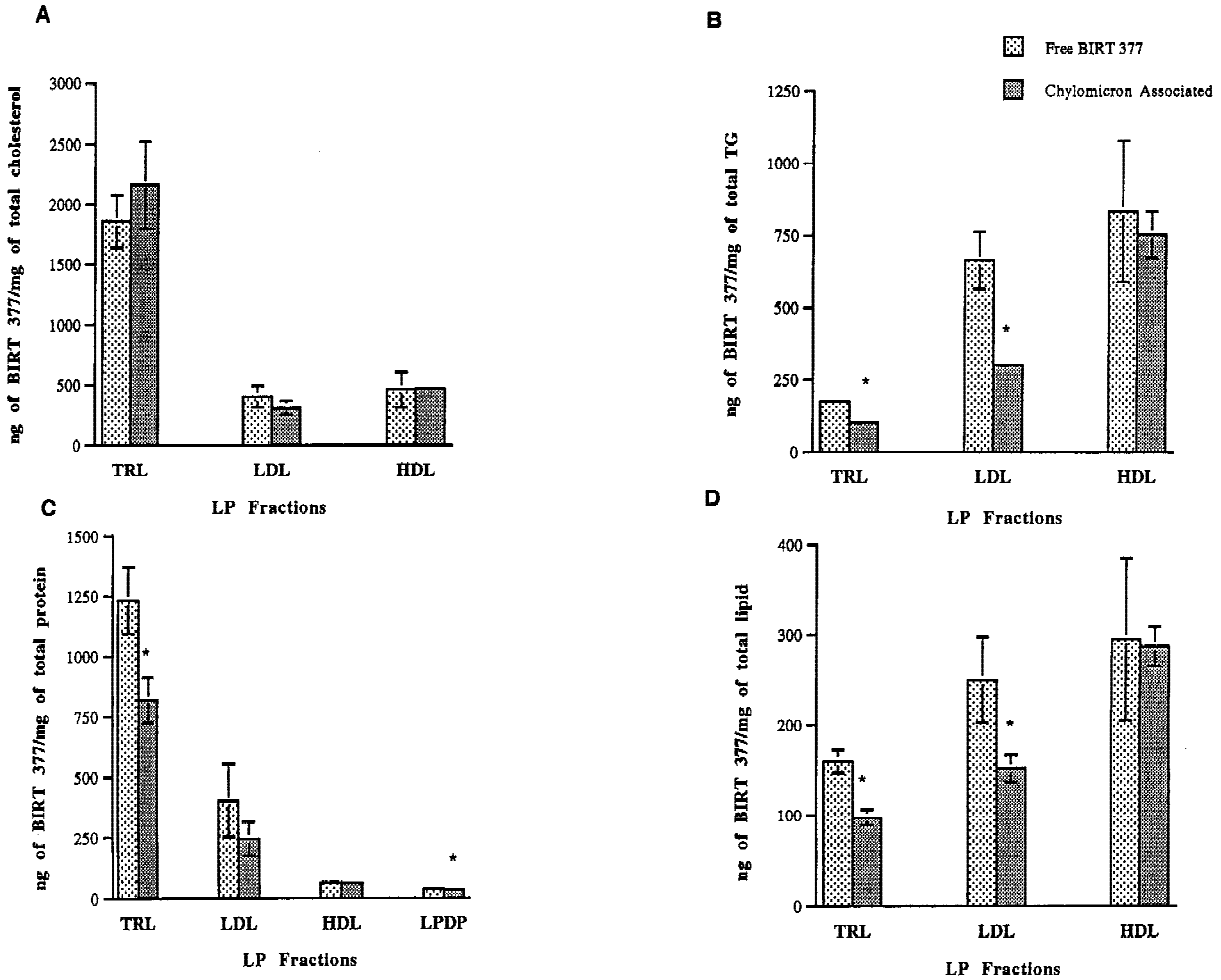


Fig. 2. The amount of BIRT 377 per the amount of [A] total cholesterol, [B] total triglyceride (TG), [C] total protein, and [D] total lipid (cholesterol+ TG) recovered within the triglyceride-rich lipoprotein (TRL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) fractions following the incubation of free and chylomicron-associated BIRT 377 at 1000 ng/ml for 60 minutes at 37°C *in vitro* within rat plasma. Data was expressed as mean ± standard deviation (n = 3); *$P < 0.05$ vs. Free BIRT 377. The plasma was separated into their individual lipoprotein and lipoprotein deficient subclasses by density gradient ultracentrifugation. Note standard deviations on some bar graphs are so small they do not shown up on the figures.

Table III. Total and Lipoprotein Plasma Cholesterol (Esterified + Unesterified), Triglyceride and Protein Concentrations Within Plasma from Fasted New Zealand White Female Rabbits (3.5–4.0 kg)

	Triglyceride rich lipoproteins mg/dl	Low density lipoproteins mg/dl	High density lipoproteins mg/dl	Total mg/dl
Cholesterol (esterified + unesterified)				
In vivo (n = 4)	10.7 ± 5.6	16.1 ± 2.9	28.8 ± 3.7	55.5 ± 9.3
In vitro (n = 6)	10.5 ± 0.5	4.3 ± 1.6*	5.1 ± 0.5*	19.9 ± 1.4*
Triglyceride				
In vivo (n = 4)	9.9 ± 3.2	9.0 ± 3.2	7.7 ± 7.0	26.6 ± 10.2
In vitro (n = 6)	10.8 ± 0.8	4.8 ± 0.3*	7.1 ± 0.6	22.7 ± 1.1
Protein				
In vivo (n = 4)	7.6 ± 0.7	15.5 ± 7.0	704 ± 29	3449 ± 321
In vitro (n = 6)	7.8 ± 0.6	7.5 ± 0.9*	41 ± 2.5*	2564 ± 131*

Note: Data is expressed as mean ± standard deviation; *$p < 0.05$ vs. *in vivo.*

intravenous administration [*in vivo*] or incubation in rabbit plasma [*in vitro*] for 5 minutes at 37°C was determined (Table IV). The percentage of BIRT 377 recovered in the TRL fraction is significantly greater and in the LDL fraction is significantly lower within *in vitro* rabbit plasma than *in vivo* rabbit plasma.

When the amount of BIRT 377 recovered in each lipoprotein fraction was normalized to the amount of [A] total

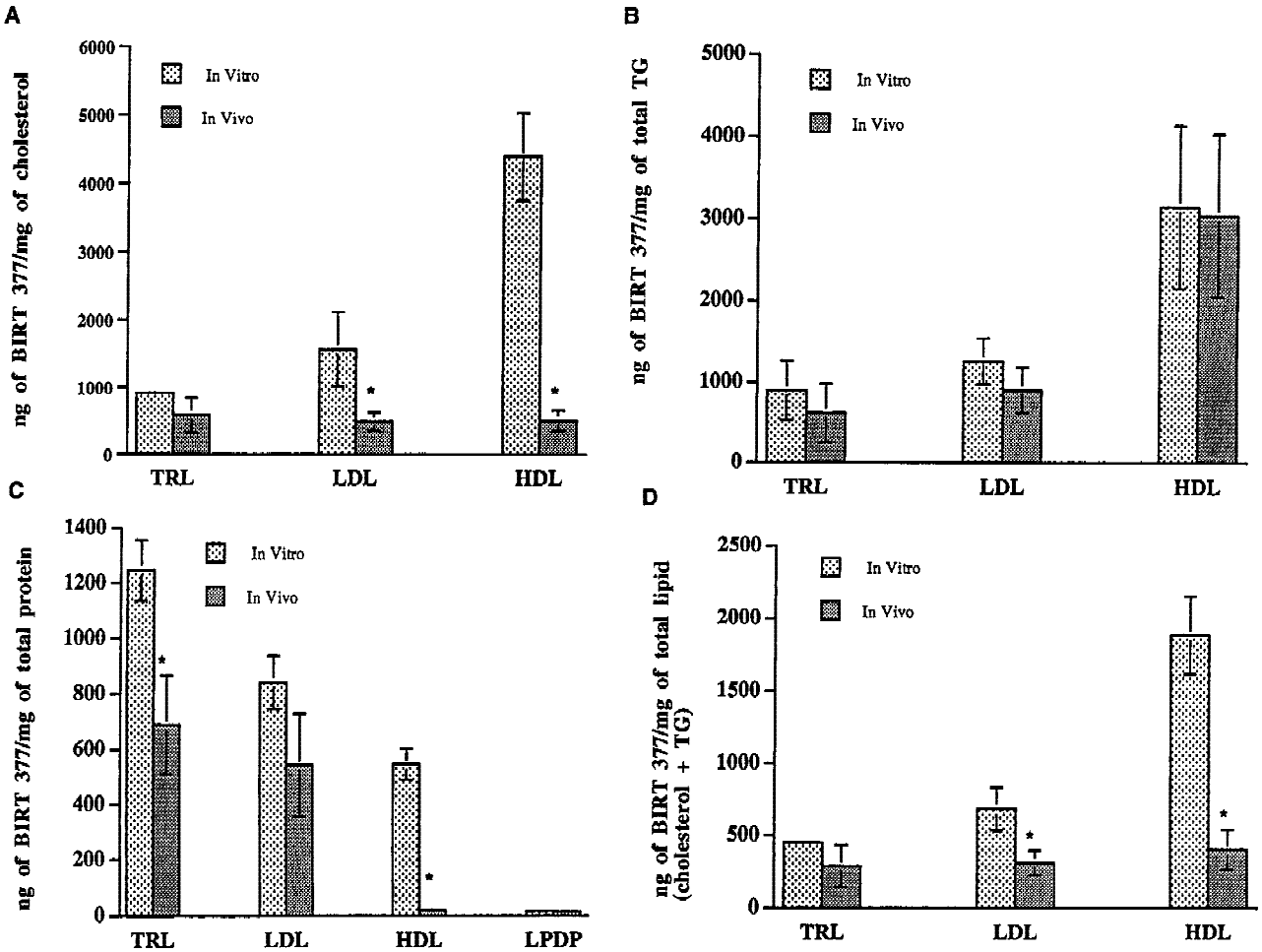


Fig. 3. The amount of BIRT 377 per the amount of [A] total cholesterol, [B] total triglyceride (TG), [C] total protein, and [D] total lipid (cholesterol+ TG) recovered within the triglyceride-rich lipoprotein (TRL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) fractions following the incubation of BIRT 377 at 800 ng/ml for 5 minutes at 37°C *in vitro* and *in vivo* within rabbit plasma . Data was expressed as mean ± standard deviation (n = 6 *in vitro* ; n = 4 *in vivo*); *$P < 0.05$ vs. *In vitro*. The plasma was separated into their individual lipoprotein and lipoprotein deficient subclasses by density gradient ultracentrifugation. Note standard deviations on some bar graphs are so small they do not shown up on the figures. LPDP represents the lipoprotein-deficient plasma fraction.

Table IV. Distribution of BIRT 377 Within Plasma from Fasted Rabbits After 5 Minutes of iv Administration (1 mg/kg) [*In Vivo*] or Following Incubation in Plasma [*In Vitro*] for 5 Minutes at 37°C

	TRL fraction %[a]	HDL fraction %	LDL fraction %	LPDP fraction %		Percent recovery %
				free	bound	
In vivo (n = 4)	8.1 ± 2.7	21.1 ± 5.1	11.4 ± 2.3	8.2 ± 3.1	45.3 ± 3.6	94.1 ± 7.8
In vitro (n = 6)	12.1 ± 0.6*	27.9 ± 2.6	7.6 ± 1.1*	7.5 ± 1.5	42.8 ± 7.4	97.9 ± 8.4

Note: Data is expressed as mean ± standard deviation; [a]percent of initial BIRT 377 amount; LPDP, lipoprotein-deficient plasma; unbound, percentage of BIRT 377 that is free; bound, percentage of BIRT 377 that is bound to plasma proteins (albumin and alpha-1-glycoprotein); HDL, high-density lipoprotein; LDL, low-density lipoprotein; TRL, Triglyceride rich lipoprotein fraction which consists of very low-density lipoproteins and chylomicrons; *$p < 0.05$ vs. *in vivo.* Plasma samples were assayed by high pressure liquid chromatography for drug in each of the lipoprotein and lipoprotein-deficient plasma fractions. Plasma lipoproteins were separated by density gradient ultracentrifugation.

cholesterol, [B] total triglyceride, [C] total protein, and [D] total lipid (cholesterol & triglyceride) recovered within the triglyceride-rich lipoprotein (TRL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) fractions (Figure 3) and the amount of BIRT 377 recovered in the lipoprotein-deficient plasma (LPDP) fraction was normalized to the amount of protein within the LPDP fraction (Figure 3C) the following was observed. The amount of BIRT 377 per cholesterol recovered within the LDL and HDL fractions were significantly lower within *in vivo* rabbit plasma than *in vitro* rabbit plasma (Figure 3A). The amount of BIRT 377 per protein recovered within the TRL and HDL fractions were significantly lower within *in vivo* rabbit plasma than *in vitro* rabbit plasma (Figure 3C). The amount of BIRT 377 per total lipid recovered within the LDL and HDL fractions were significantly lower within *in vivo* rabbit plasma than *in vitro* rabbit plasma (Figure 3D). No differences in the amount of BIRT 377 recovered in each lipoprotein fraction when normalized to the amount of triglycerides (Figure 3B). *$P < 0.05$ versus *in vitro*.

When the correlation between the percentage of BIRT 377 recovered in the TRL, LDL, and HDL fractions from fasted white female rabbit in plasma [this plasma was obtained 5 minutes following an intravenous bolus of BIRT 377 at a dose of 1 mg/kg] and the proportional distribution of cholesterol and protein within individual lipoprotein fractions and the total cholesterol (TC) / total protein (TP) and total triglyceride (TG) / TP ratios within different lipoprotein fractions was determined the following correlations were observed. A proportional increase in lipoprotein cholesterol ($r = 0.75$) and protein ($r = 0.83$) levels in all lipoprotein fractions resulted in a proportional increase of BIRT 377 recovered in those lipoprotein fractions. Furthermore as the TC/TP ($r = -0.66$) and TG/TP ($r = -0.78$) ratios increased within different lipoprotein fractions the percentage of BIRT 377 recovered in these fractions decreased.

These findings suggest that proportion of protein and cholesterol within all lipoproteins influences the percentage of BIRT 377 recovered in this fraction. The compositional proportional makeup of lipid and protein also influences this distribution. Future studies under controlled *in vitro* conditions need to be done in order to determine which component(s) of each lipoprotein dictate BIRT 377 association. These data further suggest that a significant percentage of BIRT 377 associates with plasma lipoproteins on administration to rabbits and incubation in fasted rabbit plasma (Table IV). In addition, differences in the percentage of drug recovered in the individual lipoprotein subclasses were observed following incubation *in vitro* versus *in vivo* within rabbit plasma (Table IV and Figure 3). Although differences in the *in vitro* versus the *in vivo* rabbit plasma distribution of BIRT 377 are observed when the amount of BIRT 377 recovered in each lipoprotein fraction is normalized to total cholesterol, protein and lipid (Figures 3A, 3C, 3D), no differences are observed when normalized to total triglyceride (Figure 3B). These findings suggest that BIRT 377 distribution may be influenced by TG levels. However, other factors such as available lipoprotein surface area, particle number, size and surface charge may also influence the partitioning of BIRT 377 into various lipoproteins. Studies to investigate these factors is warranted.

Plasma Distribution of BIRT 377: Rat vs. Rabbit Plasma

Figure 4 reports the amount of BIRT 377 recovered in each lipoprotein fraction normalized to the amount of total cholesterol, total triglyceride, total protein, and total lipid (cholesterol & triglyceride) recovered within each lipoprotein fraction following the distribution of BIRT 377 (800 ng/ml) for 5 minutes at 37°C within rat and rabbit plasma. In addition, the amount of BIRT 377 recovered in the LPDP fraction was normalized to the amount of protein within the LPDP fraction (Figure 4C).

The amount of BIRT 377 per cholesterol recovered within the TRL fraction was significantly lower and greater in the LDL and HDL fractions within rabbit plasma than rat plasma (Figure 4A). The amount of BIRT 377 per triglyceride recovered within the TRL, LDL and HDL fractions were significantly greater within rabbit plasma than rat plasma (Figure 4B). The amount of BIRT 377 per protein recovered within the LDL and HDL fractions were significantly greater within rabbit plasma than rat plasma (Figure 4C). The amount of BIRT 377 per total lipid recovered within the lipoprotein fractions were significantly greater within rabbit plasma than rat plasma (Figure 4D). Taken together, these findings suggest that the plasma distribution of BIRT 377 is different when incubated in rat versus rabbit plasma. These differences appear to be a result of the distinct plasma lipoprotein lipid levels found in these two species. Specifically, within rabbit plasma the data suggests that TG levels may be an important determinant of BIRT 377 lipoprotein distribution. As plasma and TRL TG levels increase a greater per-

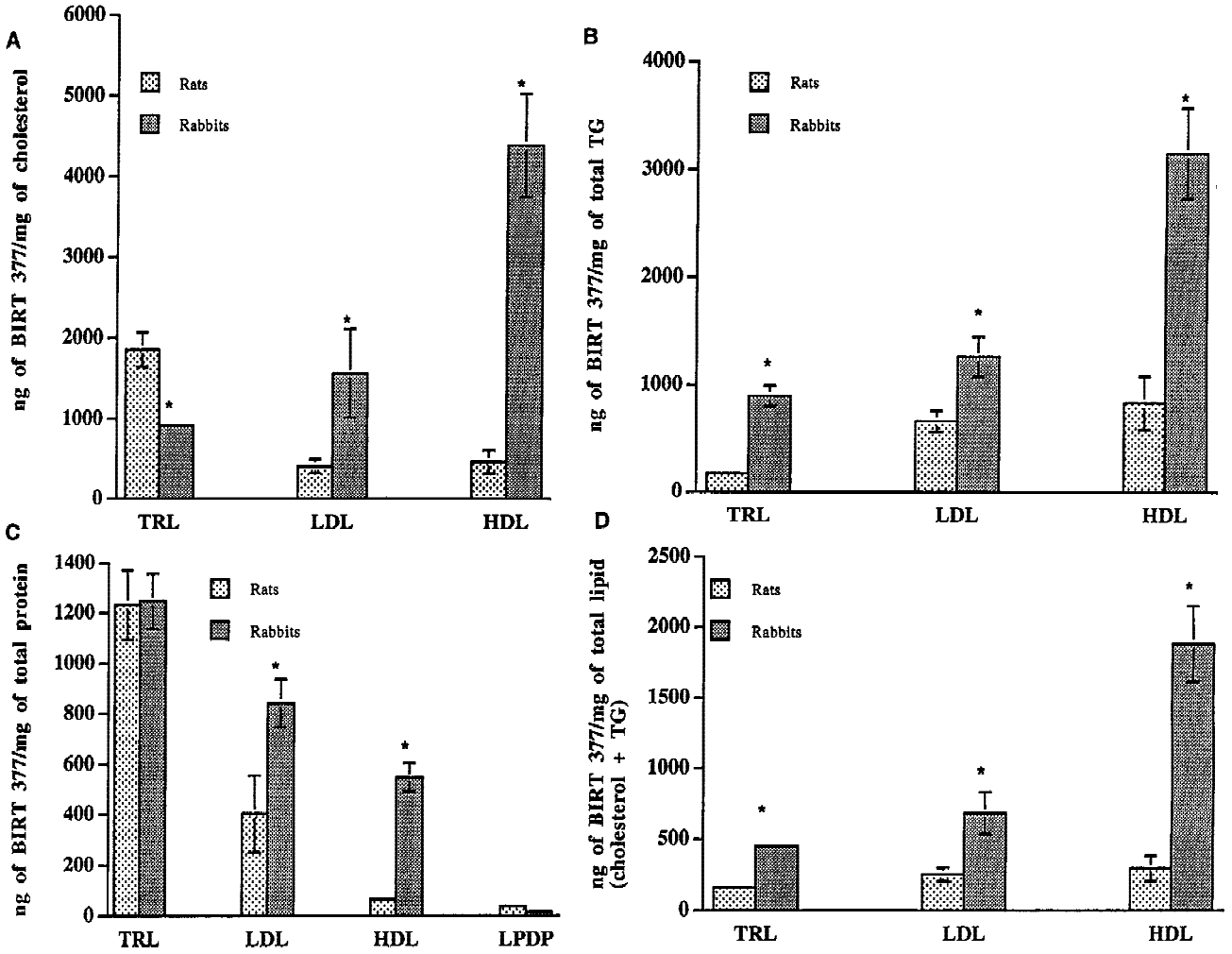


Fig. 4. The amount of BIRT 377 per the amount of [A] total cholesterol, [B] total triglyceride (TG), [C] total protein, and [D] total lipid (cholesterol+TG) recovered within the triglyceride-rich lipoprotein (TRL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) fractions following the distribution of BIRT 377 at 800 ng/ml for 5 minutes at 37°C *in vitro* within rat and rabbit plasma. Data was expressed as mean ± standard deviation (n = 3 rats and n = 6 rabbits); $^*P < 0.05$ vs. rats. The plasma was separated into their individual lipoprotein and lipoprotein deficient subclasses by density gradient ultracentrifugation. Note standard deviations on some bar graphs are so small they do not shown up on the figures. LPDP represents lipoprotein-deficient plasma fraction.

centage of BIRT 377 was recovered in the TRL fraction. Similar to our previous findings with HF (8), this may be a function of BIRT 377's high solubility in mesenteric lymph which is rich in TG. Preliminary studies in our laboratory have shown that BIRT 377 has high solubility (0.2 mg BIRT 377/ mg of triglyceride) in a short-chained triglyceride solution (Triacetin). Additional studies to investigate BIRT 377's solubility in a variety of TG-rich environments are currently being completed in our laboratory.

Plasma Pharmacokinetics of BIRT 377 in Rabbits

To determine the pharmacokinetics of BIRT 377 in a relevant animal model that can be extrapolated to humans, rabbits were used. Experimental rat models cannot be extrapolated to what may be observed in humans because the behaviour of lipoproteins in rats are very different from humans (i.e. HDLs in rats are the major carrier of cholesterol while LDLs are the major carrier of cholesterol in humans) (1,2) and the activity of a lipid transfer protein I, a protein responsible for the transfer of serum lipid and hydrophobic drugs (i.e. AmpB, HF, CSA) among different lipoprotein subfractions (1,8,13), which is measurable in humans and rabbits has minimal activity in rats (33). Table V reports the plasma concentration-time curve data and BIRT 377 pharmacokinetic parameters of the four rabbits averaged together after a single intravenous dose (1 mg/kg) through the jugular vein to New Zealand White Female Rabbits. Plasma concentration time curves for rabbits 1, 2 and 4 fit a two-compartment model for time points out to 24 hours (regression analysis $r > 0.70$). This conclusion was reached based on goodness of fit and residual sum of square estimations using the WINNONLIN program. Since, BIRT 377 exhibits significant lipoprotein distribution in rabbit plasma (Figures 4A–D) it maybe feasible to consider the drug fitting a two-compartment model, a central compartment (systemic circulation including lipoproteins) and a peripheral compartment (including tissues). However, rabbit 3 did not fit a 2-compartment model and the variability in plasma concentration between all four rabbits may be a function of differences in blood flow or plasma lipid levels. A

Table V. Plasma Concentration-Time Curves and Pharmacokinetic Parameters of BIRT 377 After a Single Intravenous Dose of BIRT 377 [1 mg/kg] to New Zealand White Female Rabbits (3.5–4.0 kg)

Time (h)	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4	Ave ± SD	Pharmacokinetic parameters (n = 4)*	
		(μg of BIRT 377/ml)					
0.083	0.806	0.823	0.483	0.621	0.683 ± 0.162	AUC, μg · h/ml	15.8 ± 11.3
0.25	0.301	0.362	0.498	0.427	0.397 ± 0.085	$t_{1/2\alpha}$, h	0.18 ± 0.14
0.5	0.296	0.383	0.308	0.355	0.336 ± 0.041	$t_{1/2\beta}$, h	72.2 ± 56.4
1	0.206	0.181	0.230	0.231	0.212 ± 0.024	MRT, h	102.8 ± 80.8
2	0.225	0.137	0.208	0.166	0.184 ± 0.040	Vss, ml/kg	5748 ± 1635
4	0.100	0.111	0.111	0.168	0.123 ± 0.031	Cl, ml/h/kg	130.8 ± 146
8	0.099	0.121	0.063	0.092	0.094 ± 0.024	Cmax, μg/ml	1.41 ± 0.88
10	0.091	0.078	0.078	0.143	0.098 ± 0.031		
24	0.076	0.034	0.262	0.193	0.141 ± 0.105		
48	0.303	0.130	0.079	0.089	0.150 ± 0.104		

Note: Data are expressed as mean ± standard deviation for pharmacokinetic parameters. Data for plasma concentration-time curves is individual time points. Abbreviations: AUC, area under the plasma BIRT 377 concentration-time curve; $t_{1/2\alpha}$ and $t_{1/2\beta}$; distribution and elimination half-lives; MRT, mean residence time; Vss, volume of distribution at steady state; CL, systemic clearance of BIRT 377; Cmax, Maximum Concentration of BIRT 377 following iv administration. *Note: Data was determined to represent a 2-compartment model and analysis was done on time points out to 24 hours. Rabbits 1,2, and 4 fit a 2-compartment model. Rabbit 3 did not fit a 2-compartment model.

larger scale study with more rabbits is required to confirm these hypotheses.

CONCLUSIONS

In conclusion these findings suggest that plasma lipoprotein binding of BIRT 377 is evident and may be a factor in evaluating the pharmacological fate of this drug when administered to patients that exhibit changes in their plasma lipoprotein lipid levels (i.e., sepsis, AIDS, bone marrow transplantation) (1). These changes in plasma lipid levels may also explain the variation in BIRT 377 plasma concentrations and pharmacokinetics (Table V) following single intravenous dose administation to rabbits. Furthermore, BIRT 377 *in vitro* plasma distribution is different when incubated in rat versus rabbit plasma. These differences appear to be a result of the distinct plasma lipoprotein lipid levels found in these two species.

ACKNOWLEDGMENTS

Funding for this project was provided by Boehringer Ingelheim Exploratory Research Fund (to KMW).

REFERENCES

1. K. M. Wasan and S. M. Cassidy. Role of Plasma Lipoproteins in Modifying the Biological Activity of Hydrophobic Drugs. *J. Pharm. Sci.* **87**:411–424 (1998).
2. R. A. Davis and J. E. Vance. Structure, assembly and secretion of lipoproteins. In D. E. Vance and J. E. Vance (eds.), *Biochemistry of Lipids, Lipoproteins and Membranes*, Elsevier, New York, 1996 pp. 473–493.
3. M. Le Maire and J. P. Tillement. Role of lipoproteins and erythrocytes in the in vitro binding and distribution of cyclosporin A in the blood. *J. Pharm. Pharmacol.* **34**:715–718 (1982).
4. R. F. Aten, T. R. Kolodecik, and H. R. Behrman. Ovarian vitamin E accumulation: Evidence for a role of lipoproteins. *Endocrinology* **135**:533–539 (1994).
5. T. A. Ho Ngoc-Ta and G. Sirois. Quinidine and propanolol binding to very low and low-density lipoproteins of human plasma. *Can. J. Phys. Pharmacol.* **62**:589–595 (1984).
6. J. Oravcova, D. Sojkova, N. Fetkovska, and T. Trnovec. Factors influencing isradipine and amlodipine binding to human plasma lipoproteins. *Blood Pressure* **1(S)**:61–64 (1994).
7. Y. Ridente, J. Aubard, and J. Bolard. Absence in amphotericin B-spiked human plasma of the free monomeric drug, as detected by SERS. *FEBS Letters* **446**:283–286 (1999).
8. K. M. Wasan, M. Ramaswamy, M. P. McIntosh, C. J. H. Porter, and W. N. Charman. Differences in the lipoprotein distribution of halofantrine are regulated by lipoprotein apolar lipid and protein concentration and lipid transfer protein I activity: *In vitro* studies in normolipidemic and dyslipidemic human plasmas. *J. Pharm. Sci.* **88**:185–190 (1999).
9. E. Pike, B. Skuterud, P. Kierulf, and P. K. Lunde. Significance of lipoproteins in serum binding variations of amitriptyline, nortriptyline and quinidine. *Clin. Pharm. Ther*. **32**:599–606 (1982).
10. K. M. Wasan and J. S. Conklin. Enhanced amphotericin B nephrotoxicity in intensive care patients with elevated levels of low-density lipoprotein cholesterol. *Clin. Infect. Dis.* **24**:78–80 (1997).
11. K. M. Wasan, G. A. Brazeau, A. Keyhani, A. C. Hayman, and G. Lopez-Berestein. Role of liposome composition and temperature in distribution of amphotericin B in serum lipoproteins. *Antimicrob. Agents Chemother.* **37**:246–250 (1993).
12. J. Brajtburg, S. Elberg, J. Bolard, and G. Medoff. Interaction of plasma proteins and lipoproteins with amphotericin B. *J. Infect. Dis.* **149**:986–992 (1984).
13. K. M. Wasan, R. E. Morton, M. G. Rosenblum, and G. Lopez-Berestein. Decreased toxicity of liposomal amphotericin B due to association of amphotericin B with high density lipoproteins: Role of lipid transfer protein. *J. Pharm. Sci.* **83**:1006–1010 (1994).
14. C. Grunfeld, M. Pang, W. Doerrler, J. K. Shigenaga, P. Jensen, and K. R. Feingold. Lipids, lipoproteins, triglyceride clearance and cytokines in human immunodeficiency virus infection and the acquired immunodeficiency syndrome. *J. Clin. Endocrinology Metab*. **74**:2045–2051 (1992).
15. K R. Feingold, R. M. Krauss, M. Pang, W. Doerrler, P. Jensen, and C. Grunfeld. The hypertriglyceridemia of acquired immunodeficiency syndrome is associated with an increased prevalence of low density lipoprotein subclass pattern B. *J. Clin. Endocrinology Metab.* **76**:1423–1431 (1993).
16. S. B. Kritchevsky, T. C. Wilcosky, D. L. Morris, *et al.* Changes in plasma lipid and lipoprotein cholesterol and weight prior to the diagnosis of cancer. *Cancer Res.* **51**:3198–3203 (1991).
17. S. Umeki. Decreases in serum cholesterol levels in advanced lung cancer. *Respiration* **60**:178–181 (1993).
18. S. Vitols, G. Gahrton, M. Bjorkholm, and C. Peterson. Hypocholesterolemia in malignancy due to elevated low-density lipopro-

tein receptor activity in tumor cells: Evidence from studies in patients with leukemia. *Lancet* **5**:1150–1154 (1985).
19. F. C. Chao, B. Efron, and P. Wolf. The possible prognostic usefulness of assessing serum proteins and cholesterol in malignancy. *Cancer* **35**:1223–1229 (1975).
20. K. M. Wasan, K. Vadiei, G. Lopez-Berestein, and D. R. Luke. Pharmacokinetics, tissue distribution, and toxicity of free and liposomal amphotericin B. *J. Infect. Dis.* **161**:562–566 (1990).
21. K. M. Wasan and J. S. Conklin. Evaluation of Renal Toxicity and Antifungal Activity of Free and Liposomal Amphotericin B following a single intravenous dose to diabetic rats with systemic candidiasis. *Antimicrob. Agents Chemother.* **40**:1806–1810 (1996).
22. M. H. Koldin, G. S. Kobayashi, J. Brajtburg, and G. Medoff. Effects of elevation of serum cholesterol and administration of amphotericin B complexed to lipoproteins on amphotericin B-induced toxicity to rabbits. *Antimicrob. Agents Chemother.* **28**: 144–145 (1985).
23. K. M. Wasan, A. L. Kennedy, S. M. Cassidy, M. Ramaswamy, L. Holtorf, J. W. L. Chou, and P. H. Pritchard. Pharmacokinetics, distribution in serum lipoprotein and tissues, and renal toxicities of amphotericin B and amphotericin B lipid complex in a hypercholesterolemic rabbit model: Single-Dose Studies. *Antimicrob. Agents Chemother.* **42**:3146–3152 (1998).
24. M. Krieger. The use of amphotericin B to detect inhibitors of cellular cholesterol biosynthesis. *Anal Biochem.* **135**:383–391 (1983).
25. K. M. Wasan, M. G. Rosenblum, L. Cheung, and G. Lopez-Berestein. Influence of lipoproteins on renal cytotoxicity and antifungal activity of amphotericin B. *Antimicrob. Agents Chemother.* **38**:223–227 (1994).
26. G. Lopez-Berestein. Liposomes as carriers of antifungal drugs. *Annals New York Acad Sci.* **544**:590–597 (1988).
27. T. A. Kelly, D. D. Jeanfavre, D. W. McNeil, J. R. Woska, P. L. Reilly, E. A. Mainolfi, K. M. Kishimoto, G H. Nabozny, R. Zinter, B. J. Bormann, and R. Rothlein. Cutting edge: A small molecule antagonist of LFA-1-mediated cell adhesion. *J. Immunol.* **163**:5173–5177 (1999).
28. K. M. Wasan, S. M. Cassidy, M. Ramaswamy, A. Kennedy, F. W. Strobel, S. P. Ng, and T. Y. Lee. A comparison of step-gradient and sequential density ultracentrifugation and the use of lipoprotein deficient plasma controls in determining the plasma lipoprotein distribution of lipid-associated nystatin and cyclosporine. *Pharm. Res.* **16**:165–169 (1999).
29. M. I. Mackness and P. N. Durrington. Lipoprotein separation and analysis for clincial studies. In C. A. Converse and E. R. Skinner (eds.), *Lipoprotein Analysis: A Practical Approach*, Oxford University Press, New York, 1992 pp. 11–14.
30. T. J. Walsh, J. Bacher, and P. A. Pizzo. Chronic silastic central venous catheterization for reduction, maintenance and support of persistent granulocytopenia in rabbits. *Lab. Anim. Sci.* **38**:467–471 (1988).
31. L. Shargel and A. B. C. Yu. Multicompartment Models. In L. Shargel and A. B. C. Yu (eds.), *Applied Biopharmaceutics and Pharmacokinetics*, Appleton & Lange, Norwalk, CT, 1985 pp. 51–67.
32. J. H. Zar. Multiway Factorial Analysis of Variance. In J. H. Zar (ed.), *Biostatistical Analysis*, Prentice-Hall, Inc., Englewood Cliffs, NJ, 1984 pp. 244–252.
33. Y. C. Ha and P. J. Barter. Differences in plasma cholesteryl ester transfer activity in sixteen vertebrate species. *Comp. Biochem. Physiol.* **71**:265–269 (1982).