Evaluating Barriers to Bioavailability *in Vivo*: Validation of a Technique for Separately Assessing Gastrointestinal Absorption and Hepatic Extraction

Laura Letendre,[1,2] Mark Scott,[1] Glenn Dobson,[1] Ismael Hidalgo,[1] and Bruce Aungst[1]

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Purpose. The purpose of this study was to develop and validate a method for separately evaluating the roles of gastrointestinal absorption and hepatic extraction as barriers to oral bioavailability (BA). The method was validated using five reference compounds known to have different absorption and hepatic extraction properties. Dose-dependence was also investigated for one reference compound.

Methods. Five reference compounds, amoxicillin, antipyrine, atenolol, propranolol, and testosterone, were administered as a cassette intravenouly (IV), via the hepatoportal vein (IPV), intraduodenally (ID), and intracolonically (IC) to male Sprague-Dawley rats. Blood samples were taken at nine time points, and the compounds were extracted from plasma using solid phase extraction. Plasma concentrations of each compound were determined using Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS). Pharmacokinetic parameters including bioavailability were calculated for each compound for each route of administration.

Results. Testosterone BA was less than 10% by ID, IC, and IPV routes, due to high hepatic extraction, consistent with its high systemic clearance (63 ml $\cdot$ min^{-1} $\cdot$ kg^{-1}) and short terminal plasma half-life (23 min). The IPV BA of amoxicillin was 95% ± 6% indicating the absence of hepatic extraction in the rat, but with an ID BA of approximately 39% suggesting incomplete GI absorption to be the main barrier to bioavailability. Absorption was poor from the colon, demonstrating site-dependence consistent with literature reports of site-dependent absorption. Low oral BA of propranolol was due in part to first-pass hepatic extraction (IPV BA of 36%). The IPV BA of propranolol was dose-dependent, most likely due to saturation of the P450 enzymes. Atenolol was incompletely bioavailable due to incomplete intestinal absorption, with no contribution of hepatic first-pass metabolism. Antipyrine was highly bioavailable by all routes.

Conclusions. This *in vivo* rat model is demonstrated to be useful for identifying and quantifying the causes of incomplete bioavailabilty. It separately evaluates intestinal absorption, hepatic extraction, and site-dependent absorption. Concentration-dependence of saturable processes can also be examined.

KEY WORDS: absorption; bioavailability; hepatic extraction.

INTRODUCTION

The oral route of administration is preferred for the majority of new drug candidates. Greatest control of plasma concentrations and pharmacologic and toxic effects is attained when oral bioavailability is maximized. Therefore, a common goal in new drug discovery and development is to maximize oral bioavailability. This requires a thorough understanding of what constitutes the barriers to bioavailability for a test compound or series.

Incomplete oral bioavailability could be due to poor intestinal absorption caused by poor solubility, poor permeability, or secretory transport, degradation or metabolism by the intestinal membrane or within the gastrointestinal lumen, or presystemic hepatic extraction. Various *in vitro* techniques are available for evaluating the potential role of each of these. But *in vitro* vs. *in vivo* correlations are not always known. It could be very beneficial to evaluate the barriers to bioavailability in an *in vivo* model where all of the factors mentioned above are operative in addition to applying *in vitro* methods. In this report, we demonstrate the utility of an *in vivo* rat model that is useful for separately evaluating gastrointestinal and hepatic contributions to incomplete oral bioavailability.

Several techniques have been described for studying the barriers to bioavailability *in vivo*. One of these uses dosing by various routes such as IV, oral, and intraportal, and comparing systemic AUC values to identify the fraction of the dose passing through various organs. This was used, for example, to assess the extrahepatic and hepatic metabolism of phenol in rats (1), and to examine gut vs. liver first-pass effects for salicylamide (2). Alternative techniques may involve sampling the portal or mesenteric circulation after oral dosing. Though various techniques have been described in the literature, there have not been studies comparing results of various reference compounds in one of these models. We chose to use the technique of dosing via intestinal, portal, and IV sites, and to examine this model for reference compounds having diverse pharmacokinetic properties. Antipyrine was selected as a well-absorbed reference compound. Atenolol was selected as an incompletely absorbed, nonmetabolized reference compound. Propranolol and testosterone were selected to represent compounds known to undergo significant presystemic metabolism. Amoxicillin was studied as a compound known to have site-dependent absorption because of the involvement of absorptive active transport predominantly localized in the upper small intestine.

MATERIALS AND METHODS

Study Design

Groups of three rats were dosed intravenously, intraportally, intraduodenally, and intracolonically via indwelling cannulae. Each animal was dosed with a cassette of the five reference compounds, antipyrine, atenolol, amoxicillin, testosterone, and HCl salt of propranolol at 1 mg/kg for each compound. We compare the bioavailabilities of these test compounds following administration of a bolus dose via each route. Experiments to determine the concentration dependence of propranolol were also performed on a second set of animals dosed with the same cassette but increasing the propranolol concentration to 10 mg/kg. Blood samples were taken at specified times, and plasma was separated and analyzed. The area under the plasma concentration vs. time curve (AUC) was then determined for each route of administration.

Animal Dosing

Surgically modified male Sprague-Dawley rats were obtained from Hilltop Lab Animals (Scottdale, PA, USA). Rats

[1] Absorption Systems, Exton, Pennsylvania 19341, USA.
[2] To whom correspondence should be addressed. (email: laura.letendre@avantixlabs.com)

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were housed for at least 24 h prior to being used in the study and were not anesthetized during the experiment. The animals weighed between 320 and 390 g and were fasted for approximately 16 h prior to dosing. Each rat had a jugular vein cannula for blood sample collection and a second cannula for drug administration.

The dosing solution was 10% dimethylacetamide (DMA), 40% PEG400, and 50% water, containing 0.5 mg/ml of each compound. At the time of dosing, the dosing vehicle was a clear solution. The dosing volume was 2 ml/kg. To examine the effect of concentration on the bioavailability of propranolol, a solution was administered with 0.5 mg/ml of each compound in the cassette but with 5 mg/ml of propranolol in 10% dimethyl acetamide (DMA), 40% PEG400, and 50% water. Dosing solutions were used within 24 h and were analyzed and found to be stable for this time period. Only amoxicillin was somewhat unstable in the DMA/PEG/saline formulation, showing 80% of the nominal concentration when assayed post-dosing. The effect of the dosing vehicle on the pharmacokinetic parameters was examined by dosing animals intravenously and intraduodenally using an aqueous solution with each compound at 1 mg/kg. Testosterone was excluded from this cassette due to its aqueous insolubility. For each of the four compounds evaluated, the IV pharmacokinetic parameters and the hepatic extraction ratios were not related to the dosing vehicle used. The reported IV pharmacokinetic parameters for amoxicillin, antipyrine, atenolol, and propranolol represent composite results of both dosing vehicles.

Animals were dosed via an indwelling jugular vein, portal vein, colonic, or duodenal cannula. Blood samples were taken from a jugular vein cannula at the following time points: 0 (pre-dose), 2, 5, 15, 30, 60, 120, 240, 360, and 480 min for intravenous and intra-portal doses; 0 (pre-dose), 5, 15, 30, 60, 120, 240, 360, and 480 min for oral and intraduodenal doses. The blood samples were withdrawn and placed into tubes containing 30 μl of a solution of 500 U per ml of heparin in saline, and centrifuged at 13,000 rpm for 10 min. Approximately 250 μl of plasma was then removed and dispensed into appropriately labeled polypropylene tubes. The plasma samples were stored in a –80°C freezer pending analysis.

Sample Preparation and Analysis

Test compounds were extracted from plasma via solid phase extraction. A 200-μl aliquot of plasma was combined with 200 μl of 1% phosphoric acid spiked with 100 ng/ml of warfarin, used as an internal standard. The solution was mixed for 1 min at 950 rpm. After mixing, 1 ml of water was added to the solution and further mixed for five minutes at 700 rpm (Brinkmann Thermomixer from VWR International). A Waters Oasis HLB 30 mg extraction plate was equilibrated with 1 ml of methanol and 1 ml of water. A 1-ml aliquot of the plasma mixture was added to the extraction plate and vacuumed through. The extraction column was washed with 400 μl of water. After washing, the samples were eluted with 500 μl of 50% acetonitrile and 50% methanol. The eluates were evaporated to dryness. The residue was reconstituted with 100 μl of 10% ammonium formate buffer and 90% water, mixing for 5 min at 950 rpm prior to being analyzed by Liquid Chromatography Mass Spectrometry (LC/MS).

Samples were analyzed for the concentration of each drug using tandem mass spectrometry to simultaneously monitor each compound of the five-compound cassette as well as the internal standard, warfarin. Samples were analyzed on either an API 4000 mass spectrometer with a Perkin Elmer autosampler or an API 3000 mass spectrometer with a CTC Analytics autosampler system (Applied Biosystems, Foster, CA). Multiple reaction monitoring was used with the following transitions $365.8 \rightarrow 207.8$ (amoxicillin), $188.0 \rightarrow 77.0$ (antipyrine), $267.0 \rightarrow 145.0$ (atenolol), $260.0 \rightarrow 116.0$ (propranolol), $289.0 \rightarrow 97.0$ (testosterone), and $309.2 \rightarrow 251.0$ (warfarin). A 10-μl aliquot of sample was injected onto a reverse phase Keystone Hypersil BDS C18 30 × 2.1 mm i.d., 3 μm column (Thermo Electon Corporation, North America). An ammonium formate buffer was used for the mobile phase and was prepared by adjusting the pH of 25 mM aqueous sodium hydroxide to pH 3.5 with formic acid. Aqueous mobile phase consisted of 10% buffer and 90% water. Organic mobile phase consisted of 10% buffer and 90% acetonitrile. Compounds were eluted with a gradient created by ramping the organic mobile phase from 0% to 100% in 3 min at 300 μl/min followed by a 1 min organic hold and 1 min column re-equilibration. Retention times ranged from 1.5 to 2.9 min. No interfering peaks were observed in blank plasma samples. Figure 1 shows a typical chromatogram.

Standard curves were fit with a quadratic function weighted by the concentration for all compounds except for amoxicillin, which was fit with a linear function and weighted by the concentration. Reproducibility of the method was tested by injecting six replicates of drug extracted from spiked plasma at three concentrations (0.1, 10, and 1000 ng/ml for antipyrine, atenolol, propranolol, and testosterone; 1, 10, and 1000 ng/ml for amoxicillin). Relative Standard Deviations (RSDs) of the six replicates were less than 11% in all cases. Concentrations of quality control samples were between 88% and 106% of their nominal value. The analytical method provided good accuracy and precision for quantitation of samples between 0.1 ng/ml (1 ng/ml for amoxicillin) (LLOQ) and 1000 ng/ml (ULOQ). Recovery of all compounds from plasma was greater than 70% with no apparent concentration dependence.

Pharmacokinetic Analysis

Pharmacokinetic analysis was performed on the plasma concentration vs. time profile of each compound in the cassette for each rat and on the average plasma concentration for all three rats in the group. The data were subjected to noncompartmental analysis using the pharmacokinetic program WinNonLin v. 3.1 (Pharsight Co. Mountain View, CA, USA). Intravenous AUCs were used as a reference for bioavailability calculations and were determined up to 8 h using linear trapezoidal analysis. Disposition characteristics of each drug, including volume of distribution (V_{ss}), half-life ($T_{½}$), and clearance (CL_s), were also derived from the plasma concentration vs. time data following intravenous administration. The terminal plasma half-life was calculated by linear regression of the terminal portion of the plasma concentration vs. time curve. Systemic clearance was calculated by dividing the dose by the AUC, and the volume of distribution was calculated by multiplying the mean residence time extrapolated to infinity by the clearance.

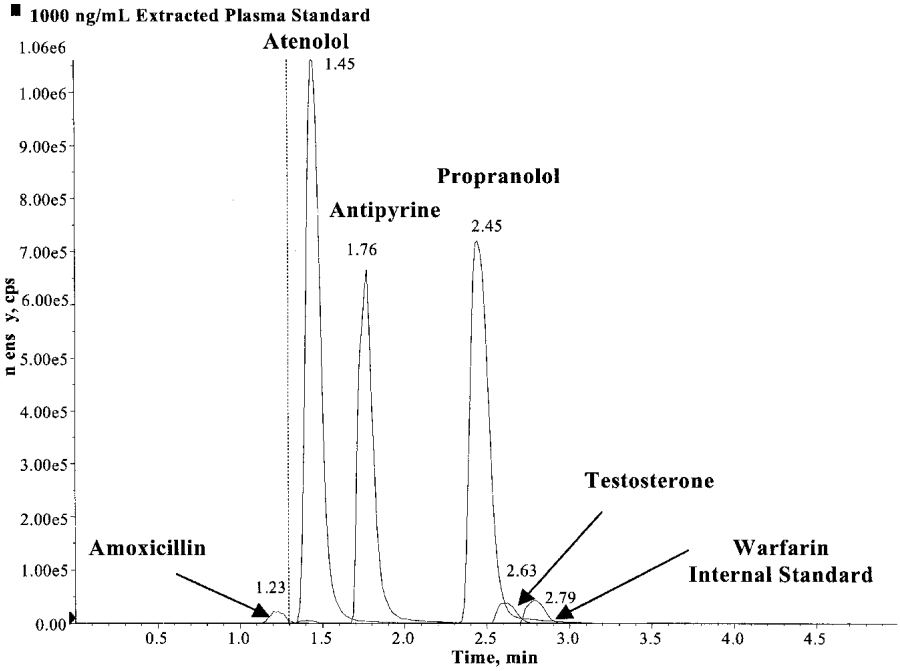

Fig. 1. Typical chromatogram for the five reference compounds plus the internal standard.

RESULTS

A cassette containing antipyrine, atenolol, amoxicillin, propranolol, and testosterone was dosed intravenously, intraduodenally, intracolonically, and intraportally to nonanesthetized rats. A method was developed enabling analysis of all five compounds simultaneously. Pharmacokinetic parameters after IV dosing are given in Table I. The bioavailabilities of each compound in the cassette for each route of administration are reported in Table II. Relative AUCs for each compound and route of administration are illustrated in Fig. 2. These results are compared with pharmacokinetics reported in the literature, to examine whether results are consistent with the known gastrointestinal absorption and hepatic extraction characteristics of these compounds. Pharmacokinetic parameters listed in Table I were also separately determined following individual dosing of each compound. These results, not presented here, were within 90% confidence interval of the pooled pharmacokinetic parameters given in Table I suggesting that the parameters were not affected by the presence of multiple compounds in the cassette.

Antipyrine had a terminal plasma half-life of 103 min. The systemic clearance was 8.9 $ml \cdot min^{-1} \cdot kg^{-1}$, much less than the hepatic blood flow in rats (3). The volume of distribution was 1.2 L/kg, indicating little tissue penetration. Antipyrine disposition kinetics agree well with those reported in literature for rats. A study by Svensson *et al.* (4) reported systemic clearance values between 5 and 13 $ml \cdot min^{-1} \cdot kg^{-1}$ with half-lives between 43 and 138 min. The volume of distribution ranged from 0.75 to 1 L/kg. Similar results were obtained in BN/BiRij rats (5) ($T_{1/2}$ = 112 min, CL_s = 6 $ml \cdot min^{-1} \cdot kg^{-1}$, and V_d = 1.0 L/kg for 3-month-old rats) and Wistar rats (6) ($T_{1/2}$ = 90 to 114 min, CL_s = 5.7 to 6.8 $ml \cdot min^{-1} \cdot kg^{-1}$, and V_d = 0.9 L/kg). Results from the current study show that antipyrine was completely absorbed after intraduodenal and intraportal vein dosing. These results are consistent with its known complete oral bioavailability (7). Colonic absorption was 53.7%, which could be due to lower permeability in the colon or a shorter time available for absorption. Antipyrine is therefore characterized by this assay as a well-absorbed compound with a low hepatic extraction ratio.

Average plasma concentration-time profiles of amoxicillin for each route of administration are shown in Fig. 3. The terminal plasma half-life, volume of distribution, and systemic clearance determined following intravenous dosing were 92 min, 0.7 L/kg, and 25 $ml \cdot min^{-1} \cdot kg^{-1}$, respectively. The volume of distribution was approximately equal to the total body water of the rat (3) suggesting that the compound was not selectively taken up by the tissues. Clearance was much lower

Table I. Pharmacokinetic Parameters of Reference Compounds in Rats Following IV Bolus Administration

Reference compound	AUC (min · μg/ml)	$T_{1/2}$ (min)	Clearance ($ml \cdot min^{-1} \cdot kg^{-1}$)	Volume of distribution (L/kg)
Antipyrine	107 ± 22	103 ± 4	9 ± 2	1.2 ± 0.2
Amoxicillin	40 ± 2.7	92 ± 26	25 ± 2	0.7 ± 0.1
Atenolol	18 ± 1.5	106 ± 2	53 ± 6	4.6 ± 0.4
Propranolol	13 ± 2.0	93 ± 12	78 ± 11	2.2 ± 0.5
Testosterone	15 ± 1.8	23 ± 1.0	63 ± 7	2.1 ± 0.5

Table II. Bioavailability of Reference Compounds After Intraportal Vein (IPV), Intraduodenal (ID), or Intracolonic (IC) Dosing*

Reference compound	Concentration dosed (mg/kg)	IPV	ID	IC
Antipyrine	1	132 (25)	105.9 (16)	23.4 (5.8)
Amoxicillin	1	95.4 (6.1)	38.8 (11)	2.4 (1.9)
Atenolol	1	101.6 (4.8)	11.0 (1.7)	3.3 (1.6)
Propranolol	1	36.3 (23)	8.7 (3.3)	13.8 (6.9)
Propranolol	10	109 (8.5)	26 (11.2)	17 (3.8)
Testosterone	1	7.4 (1.6)	5.9 (1.7)	7.6 (5.2)

* Mean (SD).

than hepatic blood flow of the rat (55.2 $ml \cdot min^{-1} \cdot kg^{-1}$). Bioavailability after intraportal dosing was 95% ± 6%, indicating that amoxicillin does not undergo significant first-pass hepatic extraction.

Amoxicillin is a β-lactam antibiotic for which the main barrier to oral bioavailability is site-dependent intestinal absorption. Intestinal absorption of amoxicillin in both rats (8,9) and humans (10,11) occurs by both passive diffusion and an active transport mechanism. The reported active transport occurs via an oligopeptide transporter (PepT1) concentrated in the upper small intestine (12). As a consequence, in rats the intestinal permeability was saturable and could be competitively inhibited, whereas colonic permeability was low and exclusively involved passive diffusion (9).

In this study, intraduodenal bioavailability of amoxicillin was 39 ± 11%. The antibiotic was rapidly absorbed with the C_{max} of 307 ng/ml occurring 15 min post-dosing, which is consistent with an absorption window primarily in the upper regions of the small intestine. Bioavailability following intracolonic dosing was 2.4% ± 1.9%, more than ten times lower than that following intraduodenal dosing, which is consistent with the decreased surface area and the lack of active transporters in this region.

The disposition characteristics of atenolol following an intravenous dose were also similar to those reported previously in literature. The terminal plasma half-life, volume of distribution and clearance were 106 min, 4.6 L/kg, and 53 $ml \cdot min^{-1} \cdot kg^{-1}$, respectively. Studies in Sprague-Dawley rats have reported half-lives in the range of 107 to 132 min (13). Systemic clearance value of 23.6 $ml \cdot min^{-1} \cdot kg^{-1}$ (Sprague-Dawley rats) and 23 to 39 $ml \cdot min^{-1} \cdot kg^{-1}$ (Wistar rats) were reported by Mehvar *et al.* (13) and Belpaire *et al.* (14), respectively. Atenolol oral bioavailability in humans was reported as 56% (15). It has been reported that 0–15% of an administered dose is metabolized in humans (16).

The experiments reported here also indicated that first-pass hepatic extraction was not a barrier to the oral bioavailability of atenolol, as evidenced by an intra-portal vein bioavailability of 102% ± 4.8%. Although the compound is mainly excreted in the urine unchanged, the systemic clearance following intravenous dosing was 30% higher than the reported renal blood flow in the rat (36.8 $ml \cdot min^{-1} \cdot kg^{-1}$) (3) indicating the possibility of a small contribution of nonrenal clearance of the compound. This is in agreement with the findings of Mehvar *et al.* that 60% of an atenolol dose was excreted unchanged in the urine during a 24-h period (13). Intraduodenal bioavailability on the other hand was low suggesting incomplete absorption of atenolol in the gut. Intracolonic bioavailability was even lower than intraduodenal bioavailability.

Propranolol is known to be ~100% absorbed in both

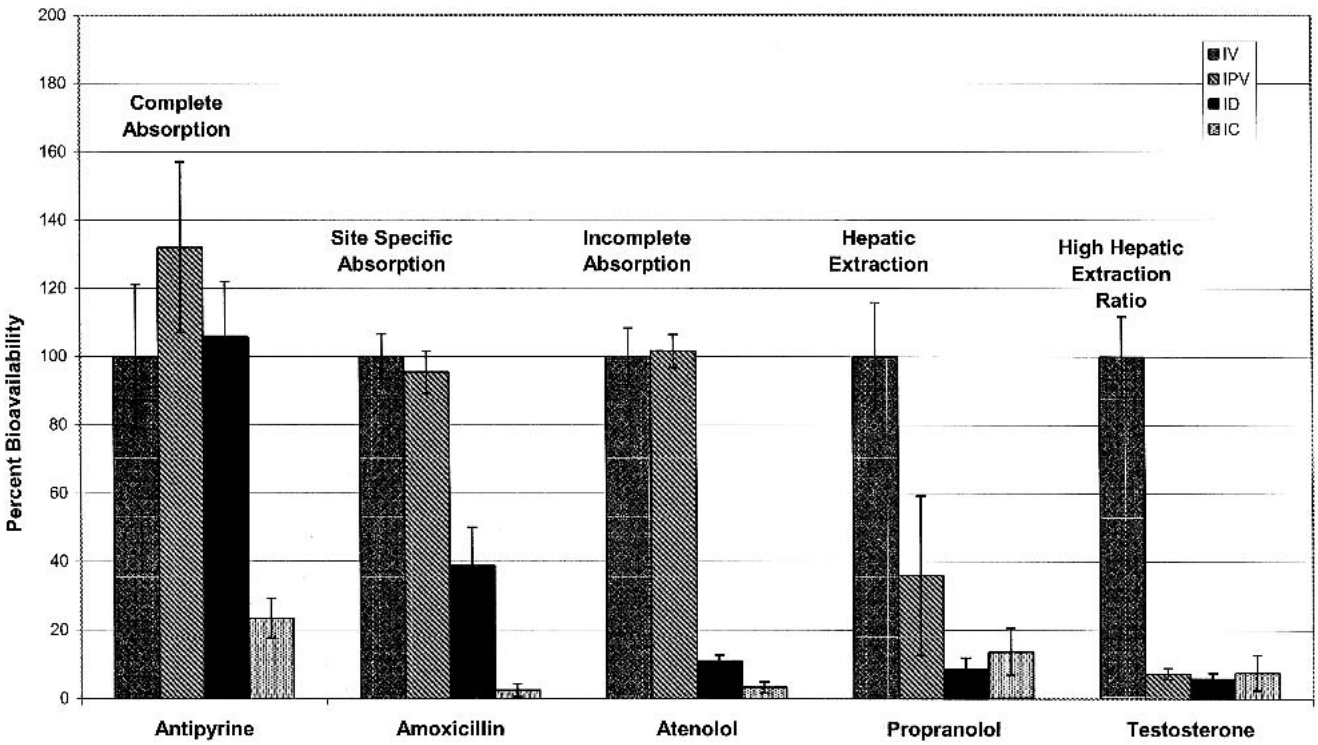

Fig. 2. Bioavailiablity of each reference compound for each route of administration. Error bars are determined by the standard deviation of the bioavailability for each group of three rats in the study.

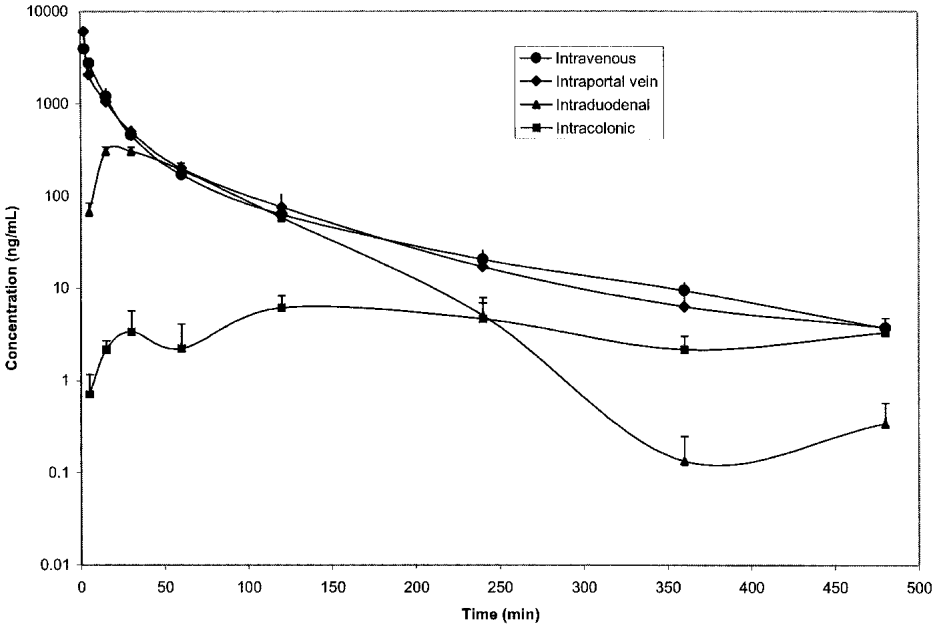

Fig. 3. Average plasma concentration vs. time curves following administration of 1 mg/kg of amoxicillin dosed to male Sprague-Dawley rats. Error bars indicate the standard deviation of the plasma concentration from the three rats in each group. Animals were dosed intravenously, intraportally, intraduodenally, and intracolonically.

human and rat with hepatic extraction presenting the main barrier to bioavailability. Oral bioavailability in humans is 26% (15). Consistent with this, the systemic clearance of propranolol in rats was high, 78.4 $ml \cdot min^{-1} \cdot kg^{-1}$. The volume of distribution was 2.2 L/kg, and the terminal plasma half-life was 93 min. These values are comparable to those published previously by Belpaire *et al.* (14) (Cl_s = 54 to 100 $ml \cdot min^{-1} \cdot kg^{-1}$, V_d = 3.7 to 6 L/kg, $T_{1/2}$ = 42 to 54 min). The bioavailability of propranolol following a 1 mg/kg intraportal vein dose was 36% ± 23%, consistent with the high first-pass hepatic extraction of the compound. Intraduodenal bioavailability was 8.7% ± 3.3%. The difference in the intraportal vein and intraduodenal bioavailability (76% difference) may initially seem to suggest poor intestinal absorption. We hypothesized that this could be due to concentration-dependent hepatic extraction, with the liver exposed to higher concentrations after portal vein dosing than after absorption from the intestines. To further test this hypothesis we administered propranolol at a higher dose (10 mg/kg) to see if saturation of hepatic extraction would result. At 10 mg/kg, intraportal propranolol was over 90% bioavailable, suggesting saturability of hepatic first-pass extraction. The intraduodenal bioavailability also increased 2.6-fold, although it was still only 23% of that following intraportal vein dosing. Hepatic extraction was not completely saturated by the intraduodenal dose of 10 mg/kg. Similar results were obtained by Hashimoto *et al.* who reported that bioavailability of an intestinal dose increased from 16.4% to 54.7% as the dose was increased from 15 mg/kg to 37.5 mg/kg (17).

Testosterone is readily absorbed, but highly metabolized in both rat and human. Due to the rapid presystemic metabolism of testosterone, oral administration of this androgen is not effective and alternative routes of administration have been proposed (15). Efficient hepatic extraction is consistent with the high clearance of 63.2 $ml \cdot min^{-1} \cdot kg^{-1}$ determined in this study in rats. The half-life was correspondingly short, 23 min, and the distribution volume was 2.1 L/kg. Bioavailability after intraportal dosing was 7.4% ± 1.6%. Intraduodenal and intracolonic bioavailabilities were 5.9% ± 1.7% and 7.6% ± 5.2%, respectively. Because the intraportal, intraduodenal, and intracolonic bioavailabilities are similar, intestinal absorption does not appear to contribute to the poor oral bioavailability of this compound.

DISCUSSION

This study determined the relative bioavailabilities of five compounds administered in a cassette by four routes of administration. By comparing the bioavailabilities by each route, the barriers to systemic absorption of each compound can be assessed.

The systemic bioavailabilities of three compounds investigated in this study were not limited by first-pass hepatic extraction. Antipyrine bioavailability was close to 100% following intraportal and intraduodenal administration, indicating that the compound is completely absorbed and has a low hepatic extraction ratio. Amoxicillin and atenolol also had a low hepatic extraction, with intraportal bioavailability again close to 100%. Intestinal absorption was 38.8% for amoxicillin and 11.0% for atenolol. For atenolol and antipyrine the colonic absorption was lower than absorption after duodenal dosing, which could be due to the decreased surface area of the colon or a shorter time available for absorption to occur after colonic dosing. The ratio of intraduodenal to intracolonic bioavailability for atenolol and antipyrine was 3 or less, whereas for amoxicillin the ratio was 14. This dramatic decrease in intracolonic bioavailability for amoxicillin is attributed to the site-dependent absorption of amoxicillin. The passive permeability of this water-soluble compound is quite low and the compound is absorbed primarily via active transport-

ers present mainly in the upper part of the small intestine. Further information on the absorption window for amoxicillin could be obtained by comparing the bioavailability after intrajejunal and ileal administration using a technique similar to that described here.

Two high hepatic extraction ratio compounds were also investigated, testosterone and propranolol. Extraction of testosterone in the liver is rapid and intra-portal bioavailability was 7%. Bioavailabilities following intraduodenal and intracolonic administration were not significantly different, indicating that this compound is well absorbed. Propranolol also had a low intra-portal bioavailability of 36%, and intraduodenal bioavailability of propranolol was even lower (8.7%). Although this decrease could indicate poor absorption through the intestinal mucosa, it is also likely with highly metabolized compounds that the concentration of compound through the liver is not equivalent following duodenal and portal vein dosing. In this case, further experiments confirmed that concentration-dependent hepatic extraction could have resulted in poor intraduodenal bioavailability. Saturation of the P450 enzyme system was apparent when increasing the dosing concentration, and intraportal and intraduodenal bioavailability increased significantly.

Misleading results due to saturation of the P450 enzyme system when administering a bolus dose via the portal vein can be avoided in several ways. First, two or more concentrations could be used to establish whether a concentration dependence exists, as was done in these experiments with propranolol. Alternatively, the intra-portal dose could be administered as an infusion to mimic the absorption phase. Finally, an accelerated infusion technique can be used to determine the concentration range of linear pharmacokinetics in a single experiment (18).

This model is demonstrated to be useful for identifying the causes of incomplete bioavailability for compounds with a range of pharmacokinetic parameters. It separately evaluates intestinal absorption, hepatic extraction, and site-dependent absorption. Concentration-dependence of saturable processes and the effects of dosing vehicle can also be examined.

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