



The Effect of P-Glycoprotein on Propranolol Absorption Using In Situ Rats Single-Pass Intestinal Perfusion

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ABSTRACT

In order to study the effect of P-glycoprotein (P-gp) on absorption phase of the anti-hypertensive agent propranolol HCL (PLH), in situ rat Single-Pass Intestinal Perfusion (SPIP) technique was applied. Rats were divided into three groups. The first group was perfused with PLH only (50 µg/ml). The second and third groups were perfused with PLH in combination with verapamil hydrochloride (VLH), a P-gp inhibitor, at a concentration of 400 and 2000 µg/ml, respectively. The samples of the perfused solutions were collected in certain intervals. The analysis was performed using a simple, rapid and validated spectroscopic method. The results demonstrated that the mean absorption rate constant (Ka) in first, second and third groups was $0.18 \pm 0.042 \text{ hr}^{-1}$, $0.43 \pm 0.05 \text{ hr}^{-1}$ and $0.42 \pm 0.042 \text{ hr}^{-1}$, respectively. The increased value of Ka (2.4 folds) upon co-perfusion with verapamil was statistically significant ($p = 0.0$). A five-fold increase in verapamil concentration (from 400 to 2000 µg/ml) had not influenced Ka of PLH ($P = 0.065$). The results indicated that P-glycoprotein affected the intestinal absorption of PLH, which can explain its lower bioavailability after oral administration. It can explain also the possible drug-drug interactions of PLH with P-gp substrates. Moreover, development of a combination dosage form of PLH and VLH can be advantageous; decreasing PLH dose and hence the side effects.

Keywords: Propranolol, P-glycoprotein, Single-Pass Intestinal Perfusion, Verapamil.

INTRODUCTION

Propranolol, (1-(isopropylamino)-3-(1-naphthoxy)-2-propanol) is a nonselective beta-adrenergic blocker that interacts with β_1 and β_2 receptors of the autonomic nervous system with equal affinity. It lacks intrinsic sympathomimetic activity (negative inotropic effect) and does not block α -adrenergic receptors¹.

Propranolol is a highly lipophilic substance and is almost completely absorbed following oral administration. However, most of the drug is metabolized in the liver during its first passage through the portal circulation; on average, about 25% reach the systemic circulation^{2,3}.

Oral bioavailability of drugs depends on numerous factors affecting intestinal absorption such as intestinal secretion, and intestinal and hepatic metabolism, which can lead to nonlinear dependency on concentration⁴⁻⁷.

Intestinal secretion is mediated by P-glycoprotein (P-gp), a very well investigated efflux pump. P-gp is energy dependent transporter protein involved in effluxing a number of drugs such as beta-blockers⁸⁻¹⁰. P-gp is present over the gastrointestinal tract, which impedes pharmacokinetic parameter of many drugs and ultimately affects bioavailability and failure of therapy by peroral route¹¹⁻¹⁵. The active excretion can be inhibited by a number of drugs that are substrates of the corresponding efflux transporters (P-gp), such as vinblastine, nifedipine, and particularly verapamil^{16,17}.

PLH is a poorly water soluble drug known substrate of p-gp¹⁸. An enhancement of propranolol solubility and increased apical to basolateral apparent permeability

coefficient were achieved using a dendrimer approach in caco-2 cell monolayers¹⁹. In addition to that, PLH effective permeability in different regions of rat intestine was evaluated and the site of absorption was determined using in situ Single-Pass Intestinal Perfusion (SPIP). Colon was the main site of absorption, where the highest Peff was recorded²⁰.

The aim of the present study was to study PLH absorption process and to investigate the implication of P-gp by means of inhibitory experiments using in-situ SPIP though the whole rat intestine and verapamil.

MATERIALS AND METHODS

Materials

Propranolol hydrochloride and verapamil hydrochloride standards were purchased from Sigma-Aldrich (Germany). Normal saline (0.9% w/v) was obtained from pharmaceutical solution industry (kingdom of Saudi Arabia) and Thiopental sodium (500 mg vial) was obtained from Egyptian INT. Pharmaceutical Industry (Egypt).

Instrumentation

SHIMADZU (UV-1601) Spectrophotometer and 1 cm quartz cells were used. Centrifugation was made with Kokusan (H-103N) Series Centrifuge. A hot plate (P/Selecta) was required.

Animals

Fifteen adult Wistar albino male rats (weighted: 250-300 g, aged: 7-9 weeks) were obtained from Cairo University (Cairo, Egypt). Animals were housed three per



polypropylene cage, at relative humidity of 60 %. An approval for study conduction was obtained from Helsinki Committee (Gaza, Palestine). All experiments with rats were conducted according to the Canadian guide for the care and use of laboratory animals²¹.

Preparation of perfusion solutions

PLH (50 µg/ml):

25 mg PLH were accurately weighed and dissolved in 50 ml volumetric flask with normal saline (0.5 mg/ml). From the solution 10 ml were transferred in 100 ml volumetric flask and diluted with normal saline.

PLH (50 µg/ml) in combination with VLH (400 µg/ml):

25 mg PLH and 200 mg VLH were accurately weighed and dissolved in 50 ml volumetric flask. From the solution 10 ml were transferred in 100 ml volumetric flask and diluted with normal saline.

PLH (50 µg/ml) in combination with VLH (2000 µg/ml):

25 mg and 1.000 g VHL were accurately weighed and dissolved in 50 ml volumetric flask with normal saline. From the solution 10 ml were transferred in 100 ml volumetric flask and diluted with normal saline.

In situ SPIP

Preparation of animals:

In situ rat small intestine was prepared according to the traditional SPIP procedure²². Small intestine was exposed by a midline abdominal incision, and two L-shaped glass cannulae were inserted through small slits at the duodenal and ileal ends. Biliary duct was previously ligated by silk suture. Care was taken to handle the small intestine gently and to reduce surgery to a minimum in order to maintain an intact blood supply. The cannulae were secured by ligation with silk suture, and the intestine was returned to the abdominal cavity to aid in maintaining its integrity, and then was covered with cotton pad soaked with normal saline to prevent dryness of intestine. Four-centimeter segments of Tygon tubing were attached to the exposed ends of both cannulae, and a 30-ml hypodermic syringe fitted with a three way stopcock and containing perfusion fluid warmed to 37°C was attached to the duodenal cannula. As a means of clearing the gut, perfusion fluid was then passed slowly through it and out the ileal cannula and discarded until the effluent solution was clear. The remaining perfusion solution was carefully expelled from the intestine by means of air pumped through from the syringe, and 10 ml of drug solution was immediately introduced into the intestine by means of the syringe. The stopwatch was started, and the ileal cannula was connected to another syringe fitted with a three-way stopcock. This arrangement enabled the operator to pump the lumen solution into either the ileal or the duodenal syringe, remove a 200 µl aliquot, and return the remaining solution to the intestine within 10-15 sec. To assure uniform drug solution concentrations throughout the gut

segment, aliquots were removed from the two syringes alternately. When the experiment was completed, the animal was euthanized with a cardiac injection of saturated solution of KCl.

Absorption studies of PLH:

A 10 ml of PLH solution at a concentration (50 µg/ml) was perfused into small intestine segment of five rats (the first group). The second and third rat groups were perfused with 10 ml solution containing PLH (50 µg/ml) in combination with VLH (400 µg/ml) and (2000 µg/ml), respectively. 200 µl of luminal intestinal fluid samples were collected from duodenal and ileal sides alternately at different time intervals 5, 10, 15, 20, 25, and 30 min. Samples were diluted to 3 ml with normal saline and centrifuged at 5000 rpm for 5 min. The supernatant was separated and kept at room temperature until being analyzed. Absorption was measured at 319 nm against blank²³.

Construction of calibration curve

50 mg PLH were accurately weighed and dissolved in 50 ml volumetric flask with normal saline (stock solution 1mg/ml). From the stock solution the following volumes 0.5, 1.0, 1.5, 2.0 and 2.5 ml were transferred into 5 ml volumetric flask and diluted with intestinal luminal fluid (blank solution), to produce a series of PLH concentrations (spiked samples) 10, 20, 30, 40, and 50 µg/ml. 200 µl of each working solution were diluted to 3 ml with normal saline and centrifuged at 5000 rpm for 5 min. The supernatant was separated and absorption was measured at 319 nm against blank²³. The calibration curve was constructed by plotting absorption against PLH concentration.

Data analysis

Data analysis was performed by Statistical Package of Social Sciences SPSS version 13²⁴.

RESULTS AND DISCUSSION

Analytical procedure

The analysis was performed by direct spectrophotometric assay of collected intestinal fluid samples. The selected wavelength was 319 nm, where no interferences from intestinal components or VLH were recorded. The validation parameters of the method were carried out according to ICH guidelines by determining the following parameters: linearity, range, limit of detection, limit of quantification, and intra- and inter-day precision and accuracy²⁵.

Linearity and range

The calibration curve was obtained with 5 concentrations of spiked PLH samples in the range of 10 -50 µg/ml, mean of five replicate. The linearity was evaluated by linear regression analysis by least square regression method. The data of regression line were evaluated by statistical analysis (Table 1).



Table 1: Analytical parameters of spectroscopic method

Range (µg/ml)	Regression equation	R	Sa	Sb
10-50	$Y = 0.003X + 0.1504$	0.999	0.0015	0.0024

R: Correlation coefficient, Sa: Standard deviation of slope of regression line, Sb: Standard deviation of intercept of regression line.

Limit of detection (LOD) and quantification (LOQ)

LOD and LOQ were determined by an empirical method consisted of analyzing series of solutions containing decreased amount of PLH spiked with luminal intestinal fluid blank. The LOD was defined as the lowest concentration on the calibration curve that presented a relative standard deviation RSD did not exceed 10% and LOQ was defined as the lowest concentration that presented a RSD that did not exceed 20%. The LOD and LOQ were 1 and 5 µg/ml, respectively.

Intra-day and inter-day precision and accuracy

Assay of precision and accuracy was assessed by analyzing three PLH spiked samples at concentrations (10, 20, 40 µg/ml) in six replicate on one day for intra-day precision and once daily for six days for inter-day precision. The RSD was less than 2% indicating a precise analytical method. The accuracy was checked at three concentration levels (10, 20, 40 µg/ml) and relative errors were found to be less than 2% (Table 2).

Table 2: intra-day and inter-day precision and accuracy of the spectroscopic method.

PLH conc. (µg/ml)	Intra-day (n=6)			Inter-day (n=6)		
	Mean	%RE	%RSD	Mean	%RE	%RSD
10	10.33	0.207	2.0	9.83	0.193	1.96
20	19.99	0.376	1.88	20.11	0.360	1.79
40	40.1	0.620	1.55	39.99	0.790	1.98

RE: Relative error, RSD: Relative standard deviation

Table 3: Stability of PLH in spiked samples at room temperature

PLH conc. (µg/ml)	Stability % (8 hrs.)
10	96.12%
30	97.09%
50	96.31%

Stability of PLH spiked samples

Three sets of PLH spiked samples (10, 30, 50 µg/ml) were prepared and kept at room temperature. One set was analyzed immediately and taken as standard 100%. Two sets were stored at room temperature for 8 hrs. , then were analyzed. The results were evaluated by comparing these measurements with those of standard and expressed as percentage deviation (Table 3). PLH was stable up to 8 hours which enable accurate analysis of

spiked samples without need for special storage conditions or prior extraction steps.

Absorption studies of PLH

In the present study, PLH was perfused into the whole rat intestine by SPIP technique, because no differences in absorption through intestinal segments were reported²⁰. The gradual decrease of remnant PLH concentrations with time, indicated that PLH absorption followed first-order kinetic (Figure 1). The absorption rate constant was calculated according to the following equation:

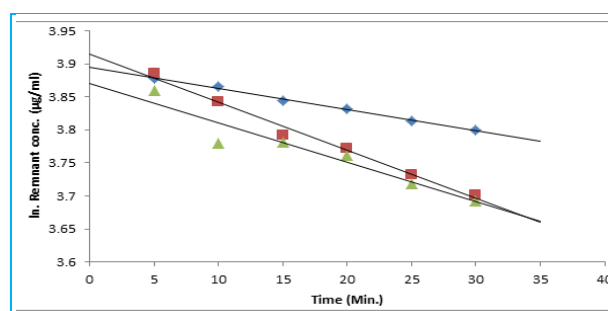
$\ln C_t = \ln C_o - K_a.t$, where C_t : Concentration of drug after t time of perfusion, C_o : Initial drug concentration, k_a : Absorption rate constant and t: Time.

The mean absorption rate constants k_a were 0.18 ± 0.042 , 0.432 ± 0.05 and $0.42 \pm 0.042 \text{ hr}^{-1}$ for the first, second and third groups, respectively (Table 4). The absorption rate constant was increased by 2.4 folds upon co-perfusion with verapamil, a p-gp inhibitor (Figure 1). A five folds increase of verapamil concentration had not influenced absorption rate constant, indicating that efflux protein (p-gp) were saturated.

Table 4: Calculated parameter of PLH

	First group	Second group	Third group
$K_a (\text{hr}^{-1})$	0.18 ± 0.0424	0.432 ± 0.05	0.42 ± 0.042
%A ⁰	98.20 ± 0.716	99.27 ± 3.42	98.52 ± 2.87
R	0.976 ± 0.024	0.952 ± 0.02	0.958 ± 0.02

k_a : Absorption rate constant, %A⁰: Estimated inclination of the rectal absorption line, R: Correlation coefficient, Rat groups: first group perfused with PLH (50 µg/ml), second and third groups perfused with PLH in combination with VLH (400 and 2000 µg/ml), respectively.

**Figure 1:** Graphical representation Plot of the fit of the apparent First-order equation to the mean data (remaining luminal concentrations of 50 µg/ml PLH ◆, co-perfusion with 400 µg/ml VLH ■, co-perfusion with 400 µg/ml VLH ▲).

Statistical analysis of data

One way ANOVA test:

The statistical test was performed to investigate the homogeneity within a group (Table 5). A low inter-individual variation among rats per group was estimated (p-value > 0.05). It was reported that, rat intestinal permeability within the age 5-30 weeks showed minimal differences²⁶.

Table 5: One way ANOVA test

Rat group	N	F-value	P-value
First group	5	1.337	0.284
Second group	5	0.022	0.999
Third group	5	0.840	0.513

Rat groups: first group perfused with PLH (50 µg/ml), second and third groups perfused with PLH in combination with VLH (400 and 2000 µg/ml), respectively.

Bonferroni Test

PLH absorption rate constant in free solution was a fundamental step for studying the PLH absorption phase and to be compared with those obtained when a specific P-gp inhibitor VLH was used. VLH was selected as a specific efflux P-gp inhibitor. Bonferroni test (Table 6) showed that there was a statistically significant difference, when PLH was perfused alone (first group) and when co-perfused with verapamil HCL (second and third groups). These results confirmed that, PLH is a substrate for rat intestinal P-gp, and a crucial role of P-gp was played in the uptake of PLH from the intestine. A five-fold increase in verapamil HCL concentration (from 400 – 2000 µg/ml) showed statistically no significant difference on PLH absorption rate (p- value > 0.05), which can be explained by p-gp saturation (Table 6).

Table 6: A multiple comparison Bonferroni test of rat groups.

Group	Group	Standard error	P-value
First group	Second group	0.00539	0.000*
	Third group	0.00539	0.000*
Second group	First group	0.00539	0.000*
	Third group	0.00539	0.065
Third group	First group	0.00539	0.000*
	Second group	0.00539	0.065

* Statistically significant (p-value < 0.05), rat groups: first group perfused with PLH (50 µg/ml), second and third groups perfused with PLH in combination with VLH (400 and 2000 µg/ml), respectively.

A similar effect of P-gp inhibitor (VLH) on salbutamol and labetalol were reported. The absorption rate constants (ka) were increased 2-3 folds^{27,9}.

A recent in situ rat SPIP study found that, PLH effective permeability coefficient increased through intestinal segments (duodenum, jejunum and ileum), which was explained by reduced P-gp distribution in the intestinal segments²⁰.

Another study used human subjects showed that, a lower C_{max} (highest concentration detected in blood after oral administration) of PLH in proximal- than in distal region was manifested; they were 58.4±23 ng/ml and 60.8±33 ng/ml, respectively. The difference in C_{max} was explained by the lower abundance of P-gp in the distal intestinal region compared with proximal region²⁸. The results of present study agreed with earlier published data,

confirming the role of P-gp in absorption of PLH. Moreover, the utility of in situ rat SPIP through the whole intestine was successfully applied for the aimed purpose of the study.

CONCLUSION

PLH absorption through intestine was studied through the whole intestine in rats using SPIP. A simple, cost effective and validated spectroscopic method was applied successfully in the study. P-gp is an important contributor for the low oral bioavailability and drug-drug interactions of PLH.

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