In Situ Absorption Study of Acebutolol by Modulating P-glycoprotein with Verapamil in Rats

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ABSTRACT: Acebutolol HCl (ABL) is a selective β -adrenergic receptor blocking agent which is preferably administered by the oral route despite its low bioavailability (30-50 %). The purpose of this study was to evaluate the effect of verapamil HCl (VER) (as P-glycoprotein inhibitor) on the intestinal absorption of ABL by comparing the changes in the absorption rate constant (k_{ap}) of ABL.

Materials and Methods: In situ intestinal perfusion technique was conducted in healthy Wistar albino male rats to study the absorption phase of ABL. Eighteen rats were divided into three groups. The first group (the control group) was perfused with ABL alone (260 μ g/mL). The second and third groups were perfused with ABL (260 μ g/mL) in combination with VER at different concentrations (200 and 400 μ g/mL, respectively). The analysis was performed using a simple, rapid and validated spectroscopic method.

Results: The absorption study showed that k_{ap} of ABL in the first group was $0.47 \pm 0.045 \text{ h}^{-1}$. In the third group k_{ap} increased 3 folds ($1.37 \pm 0.031 \text{ h}^{-1}$), however, the second group showed statistically insignificant change in k_{ap} (0.39 ± 0.076).

Conclusion: Results revealed that VER at concentration 400 μ g/mL has a pronounced effect on the absorption kinetic of ABL (increased k_{ap}) which could be linked to the inhibition of P-gp that considered as a contributed factor of low bioavailability of ABL.

Keywords: Acebutolol HCl, Verapamil HCl, P-glycoprotein, Intestinal perfusion technique, Absorption.

Özet: Acebutolol HCl (ABL), seçici β -adrenerjik reseptör bloke edici ajandır. Düşük biyoyararlanıma rağmen (% 30-50), oral yoldan tatbik edilmesi tercih edilmektedir. Bu çalışmanın amacı verapamil HCl (VER) 'nin (P-glikoprotein inhibitörü olarak) ABL'nin emilim hızı sabitindeki (k_{ap}) değişimleri karşılaştırarak ABL'nin intestinal emilimine etkisini değerlendirmektir.

Gereç ve Yöntem: ABL'nin emilim fazını incelemek için sağlıklı Wistar albino erkek sıçanlarda in situ bağırsak perfüzyon tekniği uygulandı. 18 sıçan üç gruba ayrıldı. İlk grup (kontrol grubu) sadece ABL ile (260 μ g / mL) perfüze edildi. İkinci ve üçüncü gruplar, farklı konsantrasyonlarda

(sırasıyla 200 ve 400 µg / mL) VER ile birlikte ABL (260 µg / mL) ile perfüze edildi. Analiz basit, hızlı ve onaylanmış bir spektroskopik yöntem kullanılarak yapıldı.

Bulgular: Absorpsiyon çalışması birinci gruptaki ABL k_{ap} 'ının $0,47 \pm 0,045$ saat⁻¹ olduğunu gösterdi. Üçüncü grupta k_{ap} 3 kat arttı (1.37 ± 0.031 saat⁻¹), ancak ikinci grup kapta istatistiksel olarak anlamsız bir değişiklik gösterdi (0.39 ± 0.076).

Sonuç: Bulgular, 400 ug / mL konsantrasyondaki VER'nin, ABL'nin (arttırılmış k_{ap}) emilim kinetiği üzerinde belirgin bir etkiye sahip olduğunu ortaya koydu. Bu etkinin sebebi, ABL'nin düşük biyoyararlanım faktörü olarak kabul edilen P-gp'nin inhibisyonu ile bağlantılı olabilecek.

Anahtar Kelimeler: Acebutolol HCl, Verapamil HCl, P-glikoprotein, Bağırsak perfüzyon tekniği, Emilim.

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INTRODUCTION

Drug absorption is a key part of most pharmacokinetic processes and it represents the first step that can greatly influence drugs bioavailability. Oral administration is the most common and preferable route of administration. The major site of absorption of orally administered drugs is the small intestine due to the large surface area. The rate and extent of drug absorption across the intestinal membrane are dependent on many drug and patient factors¹. Drug related factors involve physicochemical properties of the drug (molecular size, lipid solubility, degree of ionization and chemical nature) and dosage characteristics (dosage form, formulation, and concentration of drug entering the intestine). Patient related factors include the structure of absorbing surface (efflux and influx protein transporters), vascularity, pH, GI motility, presence of other substance as foods, fluids or drugs, and physiological characteristic of the patient as malabsorption syndrome^{2,3}. Drug transporters as one of the main factors affecting intestinal absorption become increasingly evident in influencing orally administered drugs^{4,5}.

P-glycoprotein (P-gp), a multidrug resistant protein 1 (MDR1), is one of the ATP binding cassette (ABC) superfamily. This protein is found in many tissues including intestine, liver, kidney, brain, testis, placenta, lung and also expressed in many cancer cells⁶. Its physiological role is to protect some tissues such as brain from harmful substances. In intestine P-gp plays an important role in drug absorption by returning the drug back into the intestinal lumen. In addition, P-gp mediates drug-drug and food-drug interaction due to its broad specificity which could affect safety and efficacy of its substrate^{7,8}. Induction or inhibition of P-gp leads to drug interaction in humans⁹. Previous kinetic studies emphasized the importance of using P-gp inhibitors to evaluate the effect of P-gp on the absorption and bioavailability of many drugs^{10,11}. Acebutolol HCl (ABL) is a cardioselective β_1 adrenoceptor blocking agent¹². Oral bioavailability of ABL is approximately 30-50% as it undergoes significant first-pass metabolism¹³. Also there

is evidence that ACH is a substrate for P-gp which plays a role as an absorption barrier¹⁴. VER is a calcium channel blocking agent, is a competitive inhibitor of intestinal P-gp and used as a tool for studying the effect of P-gp inhibition on the absorption and bioavailability of many drugs and a significant changes in the absorption kinetic have been observed¹⁵⁻¹⁷. The aim of this work was to study the effect of VER at different concentrations on the absorption of ABL using in situ intestinal perfusion technique on anesthetized rats as it is based on the disappearance of the drug in the luminal fluid.

MATERIALS AND METHODS

1.1. Materials and Instruments

Acebutolol HCl and Verapamil HCl standards were purchased from Sigma-Aldrich Company. Normal saline (0.9% w/v) was obtained from B. Braun Melsungen AG (Germany). Thiopental sodium (500 mg vial) was obtained from Rotex medica (Germany). Shimadzu UV-spectrophotometer (UV-1601) was used. Centrifugation was made with Kokusan (H-103N) Series Centrifuge. A hotplate (J.P. Selecta) was required.

1.2. Animals and Study Design

Eighteen healthy Wistar albino male rats (weighted: 250-300 g) were purchased from Center of Experimental Animals, Harlan Laboratories (Israel). Animals were housed 4 per cage in an air-conditioned room under constant temperature ($22 \pm 2^\circ\text{C}$) with free access to food and drinking water¹⁰. Rats were maintained on a 12 h light-dark cycle¹⁸. The normal life conditions for the animals were kept based on the International Animal Ethics Committee.

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 use of laboratory animals¹⁹. An *in situ* intestinal perfusion procedures were performed in rats according to the methods described previously²⁰⁻²². Rats had been fasted for 12-18 h before experiment with free access to water (Add Lipitum) then they were anaesthetized by administration of an intraperitoneal thiopental (50 mg/kg). Anaesthetized rats were placed on the fixing plate under a heating lamp keeping normal body temperature (37°C) of the rats during all experiments. The surgical procedure was initialized by a midline abdominal incision of approximately 10 cm to expose small intestine and then two L-shaped cannula were inserted carefully through small narrow open at the initiate of duodenum and end of ileum. The cannulas were secured by ligation with silk suture and the biliary duct also was legated then the small intestine was returned to abdominal cavity to maintain its integrity. The intestinal lumen was rinsed using a syringe containing normal saline (37°C) that pumped slowly through the gut via the inlet duodenum cannula and out the ileal cannula until the effluent solution was free of feces and clear. After cleaning the intestine the remaining perfusion solution was expelled from the intestine by air pumped via syringe, and 10 mL of drug solution was immediately introduced into the small intestine segment by the syringe.

In the first group 10 mL solution containing ABL alone at concentration ($260\text{ }\mu\text{g/mL}$) in normal saline (0.9% w/v) was perfused into small intestine segment of six rats. The second and third groups of rats were perfused with 10 mL solution containing ABL ($260\text{ }\mu\text{g/mL}$) in combination with verapamil HCl (200, 400 $\mu\text{g/mL}$) respectively. The surgical area was covered with a wet cotton pad, and drops of normal saline (37°C) were added to the cotton to prevent disturbing the

circulatory system and dryness of intestine. 300 μ L of perfused samples were collected from both sides alternatively each 5 minutes for a total of 30 minutes. The collected samples were transferred into 2 mL Eppendorf tubes, centrifuged at 5000 rpm for 10 minutes then 200 μ L of the supernatant were transferred and diluted to 3 mL with normal saline to be analyzed by UV spectrophotometer on the same day. The absorbance was measured at 320 nm against blank and then the concentration of each sample was determined using calibration curve to determine k_{ap} of ABL.

1.3. Analytical procedures

The determination of ABL in intestinal luminal fluid performed using spectrophotometric method which was validated for linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy according to ICH guidelines (23). For quantitative analysis of ABL, a calibration curve was constructed as following: standard stock solution of ACH 200 μ g/mL was prepared by dissolving 50 mg of standard sample (ABL powder) with normal saline solution in 250 mL volumetric flask. Intestinal luminal fluid (blank) was collected from rats by intestinal perfusion technique after administration of 10 mL normal saline without drugs. From stock solution 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mL were transferred into 5 mL volumetric flask and diluted with intestinal luminal fluid (blank) collected previously to produce a series of ABL concentration 4, 8, 16, 32, 64 and 128 μ g/mL respectively. 300 μ L of diluted solutions were centrifuged at 5000 rpm for 10 min. Absorbance was measured against blank at 320 nm. The calibration curve was constructed by plotting the absorbance against ABL concentration.

1.4. Pharmacokinetic Analysis

Intestinal absorption of ABL was evaluated using its apparent first-order rate constant k_{ap} calculated according to the following equation:

$$\ln C_t = \ln C_0 - k_{ap} \cdot t$$

($\ln C_t$: Intestinal luminal drug concentration collect post perfusion at time t; $\ln C_0$: initial perfused drug concentration preperfusion and t: time of sampling).

1.5. Statistical Analysis

The obtained data were treated and analyzed by using Statistical Package of Social Science (SPSS) program version 16 (24). The obtained data were treated and analyzed by using Statistical Package of Social Science (SPSS) program version 16 (24). (One way ANOVA and Bonferroni tests were applied in this study). Results were assumed to be statistically significant for a P-value < 0.05 .

RESULTS

a. Analytical Procedure

The analysis was performed by UV-spectrophotometric assay of ABL in intestinal luminal fluid collected during intestinal perfusion technique. No spectral interference was identified during determination of ABL in the presence of verapamil HCl and intestinal luminal fluid at the selected wavelength 320 nm.

The calibration curve was repeated 5 times. The calculated regression lines parameters showed a linear relationship between the absorbance and the concentrations of ABL in the range of 4-200

µg/ml. LOD and LOQ were determined by an empirical method consisted of analyzing series of solutions containing decreased amount of ABL spiked with luminal intestinal fluid blank (table 1).

Table 1: analytical parameters of spectroscopic method

Regression Equation	R ²	SD _a	SD _b	LOD (µg/mL)	LOQ (µg/mL)
Y = 0.007 X + 0.003	0.999	4.5X10 ⁻⁵	1.4X10 ⁻³	0.670	1.938

R²: Correlation coefficient, SD_a: Standard deviation of slope of regression line, SD_b: Standard deviation of intercept of regression line,

The accuracy was checked at 3 different concentrations of ACH in intestinal fluid (8, 32, 128 µg/mL) and the results of the recovery was in the range between 99.8% - 102.5% which reveal good accuracy of the developed method with low standard deviation. Intraday and interday precision were evaluated by triplicate analysis of ACH solution at 3 different concentration levels for 3 consecutive days. Both inter-day and intra-day precision results show low RSD (<2%) which indicates good precision (table 2).

Table 2: Intraday and Interday precision of the spectroscopic method:

ABL Conc. (µg/mL)	Intraday precision (n = 3)			Interday precision (n = 3)		
	Mean	SD	%RSD	Mean	SD	%RSD
8	7.98	0.08	1.00	7.95	0.06	0.75
32	32.57	0.60	1.84	32.31	0.62	1.92
128	131.17	1.63	1.24	131.15	0.12	0.09

ABL:

HCl, SD: Standard Deviation, %RSD: Relative Standard Deviation

Acebutolol

For stability study, ABL in intestinal luminal fluid was established over a period of 6 hours at room temperature and no significant change in concentrations was noticed. The validation parameters confirm that the method is appropriate and suitable for quantitative determination of ABL in intestinal luminal fluid.

b. Acebutolol HCl absorption studies

The allometric dose of ABL for absorption study was calculated according to the following equation: human dose/human weight = animal dose/animal weight²⁵. The absorption rate constants obtained for ABL in rat intestine were measured from intestinal sampling which based on disappearance of drug from intestinal lumen. The means of ln remnant concentrations of ABL obtained experimentally from the three groups were assembled in Table 3 and Figure 1 to show the difference of ABL absorption behavior among three groups.

Table 3: The mean of ln remnant concentrations of all data obtained experimentally for the three groups:

Time (min)	ABL (260 µg/mL) alone ^a	ABL/Verapamil HCl (260/200 µg/mL) ^a	ABL/Verapamil HCl (260/400 µg/mL) ^a
0	5.4864	5.5407	5.5452
5	5.3884	5.4692	5.3493
10	5.3290	5.4138	5.1967
15	5.3076	5.3585	5.1699
20	5.2934	5.3400	4.9122
25	5.2596	5.3150	4.8328
30	5.2053	5.2572	4.8185

ABL: Acebutolol HCl: Mean of ln remnant concentrations

Figure 1: Graphical representation of the fit of the apparent First-order equation to the obtained mean data (remaining luminal concentrations of 260 µg/mL acebutololHCl♦, acebutolol HCl with 200 µg/mL of verapamil HCl● and acebutolol HCl with 400 µg/mL of verapamil HCl▲).

In situ intestinal perfusion model assumed that drug concentrations in the enterocyte and the intestinal lumen were in dynamic equilibrium after 5 min. Therefore, only samples obtained between 5–30 min, at which ABL concentration in the enterocyte were assumed to be proportional to the ABL concentrations in the intestinal lumen, were used for calculation of k_{ap} ²². This is due to the effect of membrane uptake, enterocyte loading and other factors which resulting in lower predicted initial concentration (the intercept of the regression line at time zero) than actual initial concentration (concentration of non-perfused sample at time zero)^{21,26}. The gradual decrease of ABL concentration in the intestinal lumen indicates that ABL follows first-order kinetic and the dose used does not cause saturation of the transporter (table 3, figure 1). The mean of absorption rate constants k_{ap} of three groups shown in table 4.

Table 4: calculated parameters of ABL

	ABL (260 µg/mL) alone	ABL/Verapamil HCl (260/200 µg/mL)	ABL/Verapamil HCl (260/400 µg/mL)
k_a (hr ⁻¹)	0.47 ± 0.045	0.39 ± 0.076	1.37 ± 0.031
% A ₀	99.06 ± 0.22	97.66 ± 0.32	98.23 ± 0.06
R	0.98 ± 0.0095	0.96 ± 0.0514	0.97 ± 0.0031

ABL: Acebutolol HCl, **ka**: Absorption rate constant, **%A₀**: Estimated inclination of the absorption line, **R**: Correlation coefficient.

The determination of k_{ap} using the control group was a necessary step to gain insight into ABL absorption process because k_{ap} value for the same drug is not constant as other pharmacokinetic parameters but many factors could affect it. Therefore, this group gives k_{ap} value under the same conditions of all rats used in this study and with the same dose of ABL which can be compared with those values obtained in the presence of P-gp inhibitor.

c. Statistical analysis of data

The homogeneity between rats within a group was statistically evaluated and results demonstrated low inter-individual variation among rats (P-value > 0.05, table 5).

Table 5: One way ANOVA test for homogeneity study

Rat group	N	F-value	P-value
ABL (260 µg/mL) alone	6	1.012	0.428
ABL/Verapamil HCl (260/200 µg/mL)	6	0.198	0.961
ABL/Verapamil HCl (260/400 µg/mL)	6	0.122	0.986

ABL: Acebutolol HCl, N: sample number

DISCUSSION

The data obtained in our study revealed a significant reduction in the remnant concentrations of ABL in intestinal luminal fluids of rats in the third group and k_{ap} value increased 3-fold from $0.47 \pm 0.045 \text{ h}^{-1}$ to $1.37 \pm 0.031 \text{ h}^{-1}$ (table 4). Statistical analysis using Bonferroni test showed p value < 0.001 (table 6). On the contrary, no significant effect of verapamil HCl, at concentration 200 µg/mL, on the k_{ap} value of ABL was found. As shown in table 4, remnant concentrations of ABL in the rats' intestinal luminal fluid were not significantly decreased. The absorption rate constant of ACH obtained was $0.39 \pm 0.076 \text{ h}^{-1}$ in the presence of verapamil HCl (200 µg/mL) which is not significantly different from k_{ap} value obtained for the control group $0.47 \pm 0.045 \text{ h}^{-1}$ (p= 0.146, table 6).

Group	Group	Standard Error	P-value
ABL 260 µg/mL alone	ABL + verapamil HCl 200 µg/mL	0.3100	0.146
	ABL + verapamil HCl 400 µg/mL	0.3100	< 0.001 *
ABL + verapamil HCl 200 µg/mL	ABL 260 µg/mL alone	0.3100	0.146
	ABL + verapamil HCl 400 µg/mL	0.3100	< 0.001 *
ABL + verapamil HCl 400 µg/mL	ABL 260 µg/mL alone	0.3100	< 0.001 *
	ABL + verapamil HCl 200 µg/mL	0.3100	< 0.001 *

Table 6: A multiple comparisons Bonferroni test between the three groups

ABL: AcebutololHCl, * statistically significant (P-value ≤ 0.05)

Despite the fact that anesthesia used in this technique may decrease blood flow and intestinal motility which may decrease both passive and active transport and affecting the estimation of drug absorption, it has been reported that barbiturates have the least effect on intestinal permeability in rats²⁷. Therefore, in this study thiopental 50 µg/kg was used as anesthetic drug in all experiments which belongs to barbiturates.

The oral drug bioavailability is directly related to the drug absorption and metabolism in the gut wall. In case of ABL intestinal metabolism was not observed²⁸. The present study confirmed clearly the role of P-gp on intestinal absorption of ABL and thus may contribute to its low bioavailability. This also could explain the active secretion of ABL into the intestine after intravenous administration¹⁴. On the other hand, the obtained results revealed that verapamil HCl at concentration 400 µg/mL is almost sufficient to saturate P-gp efflux transporter which was reflected on enhancement of ABL absorption. Other studies showed that increase the concentration of verapamil HCl up to five-fold did not significantly affect the absorption rate constant of P-gp substrate due to saturation of P-gp transporter¹¹. Furthermore, lower verapamil HCl concentration (200 µg/mL) did not significantly affect the absorption rate constant of ABL which indicates that verapamil HCl 200 µg/mL was not enough to saturate P-gp efflux transporter or to affect on the absorption of ABL. Similar effect of verapamil HCl, at the higher dose level, was manifested with other β-blockers such as salbutamol²⁹, labetalol³⁰, propranolol¹¹. Furthermore, this effect was also seen with drugs other than β-blockers such as metformin and phenformin^{10,17}.

CONCLUSION

ABL is actively secreted from the enterocyte by P-gp efflux pump as confirmed by inhibition study performed with verapamil HCl which indicate that P-gp is a critical factor participates in low oral bioavailability of ABL. The absorption rate constant (k_{ap}) of ACH was increased 3 folds in the presence of verapamil HCl 400 µg/ml. In contrast, no effect of lower verapamil HCl concentration (200 µg/mL) on the k_{ap} of ABL.

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