



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

Sıçanlarda Asebutalolün P-glikoprotein ile Modüle Edildiği *In Situ* Absorpsiyon Çalışması

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ABSTRACT

Objectives: Acebutolol HCl (ABL) is a selective β -adrenergic receptor blocking agent that 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 (P-gp)] 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 was conducted in healthy male Wistar albino 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 ± 0.045 h⁻¹. In the third group k_{ap} increased 3-fold (1.37 ± 0.031 h⁻¹); however, the second group showed a statistically insignificant change in k_{ap} (0.39 ± 0.076 h⁻¹).

Conclusion: The results revealed that VER at a concentration of 400 μ g/mL has a pronounced effect on the absorption kinetics of ABL (increased k_{ap}). This could be linked to the inhibition of P-gp, which is considered a contributing factor in low bioavailability of ABL.

Key words: Acebutolol HCl, verapamil HCl, P-glycoprotein, intestinal perfusion technique, absorption

ÖZ

Amaç: Acebutolol HCl (ABL), düşük biyoyararlanımı (%30-50) olmasına rağmen oral yoldan uygulanan seçici β -adrenerjik reseptör bloke edici ajandır. Bu çalışmanın amacı verapamil HCl'nin (VER) [P-glikoprotein (P-gp) inhibitörü olarak] ABL'nin absorpsiyon hızı sabitindeki (k_{ap}) değişimleri karşılaştırarak ABL'nin intestinal emilimine etkisini değerlendirmektir.

Gereç ve Yöntemler: ABL'nin absorpsiyon fazını incelemek için sağlıklı Wistar albino erkek sıçanlarda *in situ* bağırsak perfüzyon tekniği uygulanmıştır. On sekiz sıçan üç gruba ayrılmıştır. Birinci grup (kontrol grubu) sadece ABL ile (260 μ g/mL) perfüze edilmiştir. İkinci ve üçüncü gruplar, farklı konsantrasyonlarda (sırasıyla 200 ve 400 μ g/mL) VER ile birlikte ABL (260 μ g/mL) ile perfüze edilmiştir. Analiz basit, hızlı ve onaylanmış bir spektroskopik yöntem kullanılarak yapılmıştır.

Bulgular: Absorpsiyon çalışması birinci gruptaki ABL k_{ap} 'nin $0,47 \pm 0,045$ saat⁻¹ olduğunu gösterdi. Üçüncü grupta k_{ap} 'nin 3 kat ($1,37 \pm 0,031$ saat⁻¹) arttığı belirlenmiş; ancak ikinci grupta istatistiksel olarak anlamlı olmayan bir değişiklik ($0,39 \pm 0,076$ saat⁻¹) görülmüştür.

Sonuç: Bulgular, 400 μ g/mL konsantrasyondaki VER'nin, ABL'nin (artırılmış k_{ap}) absorpsiyon kinetiği üzerinde belirgin bir etkiye sahip olduğunu ortaya koymuştur. Bu etkinin, ABL'nin düşük biyoyararlanımına yol açan bir faktör olan P-gp'nin inhibisyonu ile bağlantılı olabileceği söylenebilir.

Anahtar kelimeler: Acebutolol HCl, verapamil HCl, P-glikoprotein, bağırsak perfüzyon tekniği, absorpsiyon

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Received: 25.08.2019, Accepted: 21.11.2019

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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 drug 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 its large surface area. The rate and extent of drug absorption across the intestinal membrane are dependent on many drug and patient factors.¹ 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 the absorbing surface (efflux and influx protein transporters); vascularity; pH; gastrointestinal motility; presence of other substance such as foods, fluids, or drugs; and physiological characteristics of the patient such as malabsorption syndrome.^{2,3} Drug transporters as one of the main factors affecting intestinal absorption have become increasingly evident in influencing orally administered drugs.^{4,5}

P-glycoprotein (P-gp), a multidrug resistant protein 1, is one of the ATP binding cassette superfamily. This protein is found in many tissues including the intestine, liver, kidney, brain, testis, placenta, and lung and is also expressed in many cancer cells.⁶ Its physiological role is to protect some tissues such as the brain from harmful substances. In the intestine P-gp plays an important role in drug absorption by returning the drug to the intestinal lumen. In addition, P-gp mediates drug-drug and food-drug interactions due to its broad specificity, which could affect the safety and efficacy of its substrate.^{7,8} Induction or inhibition of P-gp leads to drug interactions in humans.⁹ 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 agent.¹² The oral bioavailability of ABL is approximately 30-50% as it undergoes significant first-pass metabolism.¹³ There is also evidence that ABL is a substrate for P-gp that plays a role as an absorption barrier.¹⁴ Verapamil HCl (VER) is a calcium channel blocking agent and a competitive inhibitor of intestinal P-gp and is used as a tool for studying the effect of P-gp inhibition on the absorption and bioavailability of many drugs and significant changes in the absorption kinetics have been observed.¹⁵⁻¹⁷ The aim of the present work was to study the effect of VER at different concentrations on the absorption of ABL using *in situ* intestinal perfusion on anesthetized rats as it is based on the disappearance of the drug in the luminal fluid.

MATERIALS AND METHODS

Materials and instruments

ABL and VER 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 Rotexmedica (Germany). A Shimadzu ultraviolet (UV)-spectrophotometer (UV-1601) was used.

Centrifugation was performed with a Kokusan (H-103N) series centrifuge. A hotplate (J.P. Selecta) was also required.

Animals and study design

Eighteen healthy Wistar albino male rats (weight: 250-300 g) were purchased from the Center of Experimental Animals, Harlan Laboratories (Israel). The 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 water.¹⁰ The rats were subjected to a 12-h light-dark cycle.¹⁸ The normal life conditions for the animals were based on guidelines of the International Animal Ethics Committee.

Approval for the study was obtained from the Helsinki Committee (Gaza, Palestine). All experiments with rats were conducted according to the Canadian Guide for the Use of Laboratory Animals.¹⁹ *In situ* intestinal perfusion procedures were performed in rats according to the methods described previously.²⁰⁻²² The rats had been fasted for 12-18 h before the experiment with *ad libitum* access to water. Then they were anesthetized by intraperitoneal administration of thiopental (50 mg/kg). Anesthetized rats were placed on the fixing plate under a heating lamp maintaining their normal body temperature (37°C) during all experiments. The surgical procedure was initialized by a midline abdominal incision of approximately 10 cm to expose the small intestine and then two L-shaped cannulas were inserted carefully through the small narrow opening at the beginning of the duodenum and the end of the ileum. The cannulas were secured by ligation with silk sutures and the biliary duct also was ligated. Then the small intestine was returned to the abdominal cavity to maintain its integrity. The intestinal lumen was rinsed using a syringe containing normal saline (37°C) that was 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 the intestine was cleaned the remaining perfusion solution was expelled from the intestine by air pumping via a syringe and 10 mL of drug solution was immediately introduced into the small intestine segment by the syringe.

In the first group 10 mL of solution containing ABL alone (concentration 260 $\mu\text{g}/\text{mL}$) in normal saline (0.9% w/v) was perfused into the small intestine segment of six rats. The second and third groups of rats were perfused with 10 mL of solution containing ABL (260 $\mu\text{g}/\text{mL}$) in combination with VER HCl (200 and 400 $\mu\text{g}/\text{mL}$, 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 disturbance of the circulatory system and dryness of the intestine. Next, 300 μL of perfused samples was collected from both sides alternatively every 5 min for a total of 30 min. The collected samples were transferred into 2 mL Eppendorf tubes, centrifuged at 5000 rpm for 10 min, and then 200 μL of the supernatant was 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 a blank and then the concentration of each sample was determined using a calibration curve to determine the k_{ap} of ABL.

Analytical procedures

The determination of ABL in intestinal luminal fluid was performed using a spectrophotometric method that 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 follows: standard stock solution of ACH 200 µg/mL was prepared by dissolving 50 mg of standard sample (ABL powder) with normal saline solution in a 250 mL volumetric flask. Intestinal luminal fluid (blank) was collected from the rats by intestinal perfusion after administration of 10 mL of normal saline without drugs. From stock solution 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 mL were transferred into a 5 mL volumetric flask and diluted with intestinal luminal fluid (blank) collected previously to produce a series of ABL concentrations, 4, 8, 16, 32, 64, and 128 µg/mL, respectively. Next, 300 µL of diluted solutions was centrifuged at 5000 rpm for 10 min. Absorbance was measured against the blank at 320 nm. The calibration curve was constructed by plotting absorbance against ABL concentration.

Pharmacokinetic analysis

The 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 postperfusion at time t, $\ln C_0$: Initial perfused drug concentration preperfusion, and t: time of sampling)

Statistical analysis

The data obtained were treated and analyzed using Statistical Package for the Social Sciences (SPSS) version 16²⁴ (One-Way ANOVA and Bonferroni tests were applied in this study). The results were assumed to be statistically significant for a p value <0.05.

RESULTS

Analytical procedure

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

The calibration curve was repeated 5 times. The calculated regression lines 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 amounts of ABL spiked with luminal intestinal fluid blank (Table 1).

The accuracy was checked at 3 different concentrations of ACH in intestinal fluid (8, 32, and 128 µg/mL) and the results of the recovery were in the range 99.8%-102.5%, which reveals 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 interday and intraday precision results show low relative standard deviation (<2%), which indicates good precision (Table 2).

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

Acebutolol HCl absorption

The allometric dose of ABL for the absorption study was calculated according to the following equation: human dose/human weight=animal dose/animal weight.²⁵ The absorption rate constants obtained for ABL in rat intestine were measured from intestinal sampling, which was based on disappearance of drug from the intestinal lumen. The means of \ln remnant concentrations of ABL obtained experimentally from the three groups are collected in Table 3 and Figure 1 to show the differences in ABL absorption behavior among the three groups. The *in situ* intestinal perfusion model assumed that drug concentrations in the enterocytes and the intestinal lumen were

Table 1. Analytical parameters of the spectroscopic method

Regression equation	R ²	SD _a	SD _b	LOD (µg/mL)	LOQ (µg/mL)
Y=0.007X+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, LOD: Limit of detection, LOQ: Limit of quantification

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: Acebutolol HCl, SD: Standard deviation, % RSD: Relative standard deviation

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, ^a: Mean of \ln remnant concentrations

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

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

Statistical analysis of data

The homogeneity between rats within a group was statistically evaluated and the results demonstrated low interindividual variation among rats (p value >0.05 , Table 5).

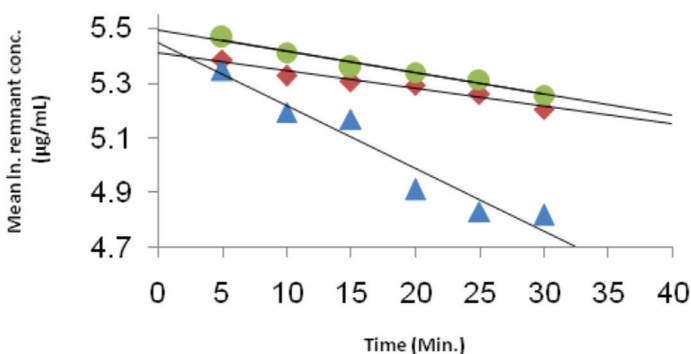


Figure 1. Graphical representation of the fit of the apparent first-order equation to the obtained mean data (remaining luminal concentrations of 260 µg/mL acebutolol HCl♦, acebutolol HCl with 200 µg/mL verapamil HCl●, and acebutolol HCl with 400 µg/mL verapamil HCl▲)

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 (h^{-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, k_a : Absorption rate constant, % A_0 : Estimated inclination of the absorption line, R: Correlation coefficient

Table 5. One-Way ANOVA 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 the k_{ap} value increased 3-fold from 0.47±0.045 h^{-1} to 1.37±0.031 h^{-1} (Table 4). Statistical analysis using the Bonferroni test showed a p value <0.001 (Table 6). In contrast, no significant effect of VER, at a concentration of 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 ABL obtained was 0.39±0.076 h^{-1} in the presence of VER (200 µg/mL), which is not significantly different from the k_{ap} value obtained for the control group, 0.47±0.045 h^{-1} ($p=0.146$, Table 6).

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

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	$<0.001^*$

*Statistically significant (p value ≤ 0.05), ABL: Acebutolol HCl

Despite the fact that anesthesia as used in this technique may decrease blood flow and intestinal motility, which may decrease both passive and active transport and affect the estimation of drug absorption, it has been reported that barbiturates have the least effect on intestinal permeability in rats.²⁷ Therefore, in the present study thiopental 50 µg/kg, which is a barbiturate, was used as the anesthetic drug in all experiments.

The oral drug bioavailability is directly related to the drug absorption and metabolism in the gut wall. In the case of ABL, intestinal metabolism was not observed.²⁸ The present study confirmed clearly the role of P-gp in 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 administration.¹⁴ On the other hand, the obtained results revealed that VER at a concentration of 400 µg/mL is almost sufficient to saturate P-gp efflux transporter, which was

reflected in enhancement of ABL absorption. Other studies showed that an increase in the concentration of VER up to 5-fold did not significantly affect the absorption rate constant of P-gp substrate due to saturation of P-gp transporter.¹¹ Furthermore, a lower VER concentration (200 µg/mL) did not significantly affect the absorption rate constant of ABL, which indicates that VER 200 µg/mL was not sufficient to saturate P-gp efflux transporter or to affect the absorption of ABL. A similar effect of VER at the higher dose level was manifested with other β-blockers such as salbutamol,²⁹ labetalol,³⁰ and propranolol.¹¹ In addition, 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 enterocytes by P-gp efflux pump as confirmed by the inhibition study performed with VER, which indicated that P-gp is a critical factor that participates in low oral bioavailability of ABL. The absorption rate constant (k_{ap}) of ABL was increased 3-fold in the presence of VER 400 µg/mL. In contrast, no effect of lower VER concentration (200 µg/mL) was seen on the k_{ap} of ABL.

ACKNOWLEDGMENTS

I wish to express my deepest gratitude thanks to all staff in the Faculty of Pharmacy at Al-Azhar University-Gaza for their kind support and help and many thanks to Mr. Mohamed Abu Affash, the director of Medical Relief Society-Gaza, for providing me the facilities to perform analysis in his laboratory.

Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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