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International Journal of Pharmaceutics 132 (1996) 63–69

international journal of pharmaceutics

Evidence of competitive inhibition for the intestinal absorption of baclofen by phenylalanine

E. Cejudo-Ferragud, A. Nácher, A. Polache, T. Cercós-Fortea, M. Merino, V.G. Casabó*

Department of Pharmaceutics, Faculty of Pharmacy, University of Valencia, Valencia, Spain

Received 26 May 1995; revised 18 September 1995; accepted 25 September 1995

Abstract

Previous studies showed that the absorption of the antispastic drug baclofen, in the rat middle intestine, is inhibited by β-alanine, γ-aminobutyric acid (GABA) and leucine. It was concluded that baclofen intestinal transport was mediated, at least in part, by the β-, γ- and α-amino acid carriers. We therefore focused our next studies on the analysis of the possible inhibition of drug absorption by an aromatic α-amino acid model compound, phenylalanine. An in situ study in the rat small intestine was undertaken in order to evaluate the effect of phenylalanine on baclofen absorption and to establish the inhibition model. Assays using isotonic perfusion solutions of 0.5 mM baclofen with initial phenylalanine concentrations ranging from 0 to 100 mM are reported. The results show that the absorption rate pseudoconstants of the drug decrease as the phenylalanine concentration increases, with a limiting value of 0.33 h^{-1} ($\pm$ 0.06). Complete competitive inhibition in the presence of a second component could define the interaction phenomena between these substances, since higher concentrations of phenylalanine do not completely abolish baclofen absorption. We have completed the studies using phenylalanine and GABA together as inhibitors of drug absorption. An isotonic perfusion solution of 0.5 mM baclofen in the presence of 100 mM phenylalanine and 100 mM GABA was perfused. Under these conditions the absorption rate pseudoconstant of baclofen decreases until 0.16 h^{-1} ($\pm$ 0.06).

Keywords: Phenylalanine; γ-Aminobutyric acid; Baclofen; Intestinal absorption; Michaelis–Menten kinetics; Competitive inhibition

1. Introduction

Previous studies carried out in our laboratory showed that baclofen (β-*p*-chlorophenyl acid), a widely used drug, is absorbed in the rat small intestine through a specialized transport mechanism (Merino et al., 1989a).

Further absorption studies using β-alanine (Polache et al., 1991) and GABA (Nácher et al., 1994) as inhibitors of baclofen intestinal transport, have demonstrated that the drug uses, at least in part, the transport system for β-amino acids in the middle fraction of the rat small intestine.

* Corresponding author.

SSDI 0378-5173(95)04342-8

The interaction between the drug and these amino acids would be described as partial competitive inhibition or complete competitive inhibition with residual absorption since both hypothesis are in agreement with the results (Nácher et al., 1994).

Since high concentrations of the amino acids tested did not completely abolish the absorption of baclofen, we continued the experiments in order to find another different carrier system which could be responsible for the residual absorption. We started the studies with leucine (Cercós-Fortea et al., 1995), an α-amino acid. In this paper we report experiments with phenylalanine, another α-amino acid structurally related to baclofen.

Phenylalanine transport has been studied in several tissues using different techniques: human intestinal epithelial cell line (Caco-2) (Hidalgo and Borchardt, 1990), isolated rat erythrocytes (Pico et al., 1992), fish intestinal brush border vesicles (Reshkin and Ahearn, 1991), rabbit isolated oxyntic glands (Sobrevía et al., 1992), rat nasal mucosa (Tengamnuay et al., 1991), and bovine renal epithelial cell line (NBL-1) (Doyle and McGivan, 1992). All the authors reach the same conclusion: phenylalanine transport is a specialized mechanism mediated by a carrier whose characteristics depend on the tissue and the technique used in the experiments.

Thus, we have considered the possibility that baclofen absorption could be mediated in part by phenylalanine carrier. A similar situation occurs with L-α-methyldopa. It has been demonstrated that the carrier system associated with phenylalanine is responsible for the absorption of this antihypertensive drug (Hu and Borchardt, 1990).

2. Materials and methods

2.1. Absorption studies

The in situ rat gut preparation (Doluisio et al., 1969), modified as previously reported (Merino et al., 1989a; Sánchez-Picó et al., 1989), was used for absorption tests. Male Wistar rats weighing 250–300 g, fasted for 20 h but with free access to water, were anaesthetized 1 h before the experiment by an intraperitoneal injection of urethane (25%, w/v).

Perfusion was performed in the middle fraction of the small intestine, measured 30 cm from the pylorus. After rinsing with physiological saline solution (25 ml) in order to eliminate faecal residues and debris, 5 ml of a 0.5 mM isotonized baclofen solution, in the presence of phenylalanine (0, 5, 10, 25, 50 or 100 mM) and buffered to pH 7.6 (Sánchez-Picó et al., 1984), was perfused at 37°C. Samples of the remaining drug in the intestinal lumen were taken every 5 min for a total time of 30 min. Seven animals per set were employed, and the average concentrations were used for kinetic calculations.

In order to check if the simultaneous presence of α- and β-amino acids abolish baclofen intestinal absorption, a 0.5 mM baclofen solution added with 100 mM phenylalanine and 100 mM GABA was perfused. Seven animals were employed for kinetic calculations.

Water reabsorption was evaluated separately for each animal. This process obeys apparent zero-order kinetics (Martín-Villodre et al., 1986; Sánchez-Picó et al., 1989). In order to characterize the zero-order equation in each experiment, the volume remaining at 30 min was measured. After taking the last sample, this volume was assessed by separating the intestinal fraction and withdrawing the residual liquid. The remaining volume at 0 min was evaluated, using five animals, as previously reported (Martín-Villodre et al., 1986).

With the mean remaining value at 0 min ($n = 5$) and the individual value at 30 min, a straight line was obtained. Thus, theoretical volumes at each sampling time can be obtained and the remaining drug concentrations properly corrected.

2.2. Analytical procedures

Intestinal samples were assayed for baclofen content by high-performance liquid chromatography (HPLC), according to a previously reported procedure (Polache et al., 1991).

In brief, a 150 × 4.6 mm Spherisorb S-5 ODS-2 analytical column (Phase Separations, Ltd., Queensferry) in conjunction with a C-130 B precolumn (Teknokroma C-18) were used. The mo-

bile phase was a mixture of acetonitrile and aqueous phosphate buffer 0.05 M (pH 3), 16:84 (v/v) and a flow rate of 1.0 ml min^{-1} was used. Intestinal samples were centrifuged at 3000 rpm for 10 min, and 20 μl of the supernatant were injected into the chromatograph. Because of the simplicity of the procedure, no internal standard was needed.

2.3. Fitting of models to data and statistical procedures

2.3.1. Baclofen absorption rate measurements

To quantify the baclofen intestinal absorption, the classical first-order kinetic equation was fitted to data:

$$A = A_0 \, e^{-k_{ap}t} \tag{1}$$

Here, A represents the concentration of baclofen remaining in the luminal solution at each sampling time, t, corrected for water reabsorption, A_0 is the calculated intercept at zero time, which is always lower than 100% due to the membrane uptake, and k_{ap} is the apparent absorption rate constant of the drug. In order to prevent membrane adsorption effects, only the samples obtained between 5 and 30 min were used for calculations, i.e. the zero time sample was not used for regression (Doluisio et al., 1969; Martín-Villodre et al., 1986; Merino et al., 1989a).

Both parameters (A_0 and k_{ap}) were then calculated according to a non-linear regression least-squares procedure using the PCNONLIN 3.0 program. The resulting k_{ap} values were statistically compared in order to detect inhibition phenomena in absorption using a one-way ANOVA test.

The absorption rate pseudoconstants of baclofen significantly decrease as the initial phenylalanine concentration in the perfusion fluid increases, so the existence of a common carrier-mediated transport can be reasonably inferred. In this case, the fitting of the inhibition equations to data is strongly recommended.

The inhibitory effect of phenylalanine on baclofen absorption was detected and evaluated using a general computer procedure based on the application of the following equations: Eq. (2) represents complete competitive inhibition and Eq. (3) represents complete competitive inhibition with a residual absorption component

$$-\frac{dA}{dt} = \frac{V_m}{K_m(1 + (I/K_i)) + A} A \tag{2}$$

$$-\frac{dA}{dt} = \frac{V_m A}{K_m(1 + (I/K_i)) + A} + KA \tag{3}$$

Here, dA/dt is the absorption rate of baclofen, V_m the maximal absorption rate, K_m the Michaelis–Menten constant for baclofen, K_i the inhibitor Michaelis–Menten constant and K could be the first-order rate constant, k_a, if the second component is passive diffusion, or the expression $V_{m2}/(K_{m2} + A)$ when the second component represents another, non-inhibited carrier system. I and A are the inhibitor and drug concentrations remaining in the intestinal lumen. Since the baclofen concentration in the perfusate is always low and virtually does not change as a result of the effect of the inhibitor, A in the denominator can be ignored when Eqs. (2) and (3) are fitted to data.

By rearranging Eqs. (2) and (3) and bearing in mind its analogy with Eq. (1), in its differential form (i.e. $-dA/dt = k_{ap}A$), one can write:

$$\frac{-dA/dt}{A} = k_{ap} = \frac{V_m}{K_m(1 + (I/K_i))} \tag{4}$$

$$\frac{-dA/dt}{A} = k_{ap} = \frac{V_m}{K_m(1 + (I/K_i))} + K \tag{5}$$

Therefore, in Eq. (5), K represents an apparent first-order constant (k_a) or a second non-inhibited carrier system (V_{m2}/K_{m2}).

The PCNONLIN program was used to fit Eqs. (4) and (5) to the data (Simplex algorithm, using the same weight for all the data). Calculations were carried out using as dependent variable the first-order rate pseudoconstants of baclofen found in the presence of each starting phenylalanine concentration, k_{ap} (average values), and as independent variable the initial phenylalanine concentration, I.

The Akaike information criterion, called AIC (Akaike, 1986), correlation coefficients between predicted and experimental values, r, and sum of squares of residuals, SS, were used to assess the goodness of the fits.

Table 1
Luminal disappearance of baclofen. Average concentrations of baclofen (μM) ± SD, relative to initial concentration perfused of 500 μM, remaining in luminal fluid at each phenylalanine starting concentration (A_i, mM)

Sampling time (min)	Remaining concentrations of baclofen (μM) in jejunal fluid for each starting phenylalanine concentration (A_i, mM)					
	$A_i = 0$	$A_i = 5$	$A_i = 10$	$A_i = 25$	$A_i = 50$	$A_i = 100$
5	405 (± 13)	428 (± 20)	420 (± 32)	418 (± 16)	418 (± 12)	420 (± 10)
10	375 (± 16)	405 (± 19)	400 (± 31)	404 (± 15)	406 (± 13)	410 (± 09)
15	345 (± 21)	382 (± 19)	379 (± 26)	390 (± 15)	394 (± 14)	397 (± 09)
20	316 (± 23)	357 (± 18)	358 (± 28)	379 (± 17)	386 (± 15)	388 (± 11)
25	289 (± 27)	332 (± 16)	338 (± 30)	364 (± 20)	374 (± 16)	377 (± 13)
30	265 (± 29)	314 (± 17)	319 (± 28)	350 (± 22)	362 (± 21)	367 (± 12)
k_{ap} (h^{-1})	1.03 (± 0.23)	0.76 (± 0.12)	0.67 (± 0.08)	0.42(± 0.11)	0.34 (± 0.13)	0.33 (± 0.06)
r	0.999	0.999	0.999	0.999	0.998	0.999

3. Results

Average concentrations of baclofen remaining in the intestinal samples after perfusion of the drug solutions (0.5 mM) in the presence of variable concentrations of phenylalanine, already corrected for water reabsorption (A values, means of seven animals), are given in Table 1. In Table 2 the average concentrations of baclofen remaining in the intestinal samples after perfusion of the drug solutions (0.5 mM) in the presence of a mixture of phenylalanine and GABA (100 mM concentration of each) are reported. The absorption rate pseudoconstants, k_{ap}, calculated according to Eq. (1), are also given, as well as the correlation coefficients found for apparent first-order kinetics.

In Table 3, the V_m/K_m, K_i and K values and r, SS and AIC found after fitting Eqs. (4) and (5) to the data are given.

Fig. 1 shows a graph plotted according to complete competitive inhibition in the presence of a second component (Eq. (5)).

4. Discussion

4.1. Biological methods

Baclofen absorption studies were developed in the rat small intestine because of its high degree of specialization, and, particularly, in its middle fraction, where an absorption window for the drug has been characterized (Merino et al., 1989a).

The in situ perfusion technique is considered to be the method of choice for inhibition studies since intestinal manipulation is minimal and normal blood supply is maintained, thus leading to perfect sink conditions throughout the absorption test. On the other hand, the aqueous diffusion layer remains practically unchanged, as occurs in vivo.

Moreover, the technique has been tested in our laboratory to characterize carrier-mediated absorption kinetics with quite acceptable results (Merino et al., 1989a,b; Sánchez-Picó et al., 1989) and has proved useful in providing guidance on human bioavailability trials (Garrigues et al., 1991).

4.2. Baclofen carrier characterization

Absorption rate pseudoconstants for baclofen show a reduction as phenylalanine concentration in the perfusion solution is increased.

One-way ANOVA test showed the existence of significant differences, and the Scheffé test showed that the absorption rate pseudoconstant of baclofen in solution is significantly different from that found in the presence of 5 mM phenylalanine. Therefore, it can be concluded that both substances share some carrier for their absorption process. Thus, the α-amino acid seems to act as a clear inhibitor of baclofen absorption.

Table 2
Luminal disappearance of baclofen. Average concentrations of baclofen (μM) $\pm$ SD, relative to initial concentration perfused of 500 μM, remaining in luminal fluid at each phenylalanine and GABA starting concentration (A_i, mM)

Sampling time (min)	Remaining concentrations of baclofen (μM) in jejunal fluid for each starting phenylalanine and GABA concentration (A_i, mM)
	$A_i = 100$
5	417 ($\pm$ 15)
10	413 ($\pm$ 16)
15	408 ($\pm$ 15)
20	402 ($\pm$ 17)
25	397 ($\pm$ 18)
30	391 ($\pm$ 19)
k_{ap} (h^{-1})	0.16 ($\pm$ 0.06)
r	0.997

In order to investigate the nature of the interaction, Eqs. (4) and (5), representative of inhibition models, were fitted to the available results, as a function of the initial inhibitor concentration, I. Results, shown in Table 3, indicate that complete competitive inhibition with a residual absorption component is statistically better as an inhibition pathway.

Since previous results showed that baclofen absorption obeys Michaelis–Menten kinetics (Merino et al., 1989a), the residual absorption component should not be identified only as a passive one, and the existence of another carrier system can be suggested. This latter assumption is consistent with the results found in former studies (Nácher et al., 1994). In Table 4, the parameters found after fitting Eq. (5) to the results obtained for baclofen absorption in the presence of increasing concentrations of phenylalanine and GABA are shown.

As can be seen, the K value found after fitting Eq. (5) to the available results ($K = 0.22 \pm 0.04$ h^{-1}) resembles that found in the absorption test for baclofen in the presence of GABA ($V_m/K_m = 0.27 \pm 0.02$ h^{-1}) (Nácher et al., 1994). Thus, it seems that baclofen requires at least two carrier systems to be absorbed: that of the β-amino acids (which is also shared by the γ-amino acids), and that of the neutral α-amino acids. In order to corroborate this latter assumption and to elucidate if the baclofen absorption rate is effectively cancelled in the presence of high concentrations of different amino acids, a mixed solution of baclofen (0.5 mM) with phenylalanine and GABA (100 mM each) was perfused in rats. As shown in Table 2, the baclofen absorption rate pseudoconstant was significantly reduced relative to that found in the presence of phenylalanine (100 mM) and in the presence of GABA (100 mM): $k_{ap} = 0.33 \pm 0.06$ h^{-1} ($p < 3.45 \times 10^{-4}$) and $k_{ap} = 0.65 \pm 0.01$ h^{-1} ($p < 1.14 \times 10^{-10}$), respectively (Nácher et al., 1994). Notwithstanding, the value of this pseudoconstant was significantly different from zero. This implies that some residual absorption exists, probably passive in nature. These results are consistent with other previous studies that demonstrate the existence of some colonic absorption for the drug, as small as 0.13 ± 0.07 h^{-1} (Merino et al., 1989a); as currently assumed, colon absorption is always passive.

Thus, in any case, the contribution of the passive process to global baclofen absorption can be considered as minor; carrier-mediated absorption is, by far, most important and lucrative. Therefore, it becomes relevant to elucidate the interactions which can arise between the drug and the natural diet components.

4.3. Practical implications

From the clinical viewpoint, the interactions influencing the efficacy and safety of drug treatment should always be investigated. Its importance can be decisive in severe pathologies and whenever toxic implications are involved.

For the orally administered drugs, nutrient–drug interactions are considered whenever they can influence bioavailability, and, therefore, efficacy.

From the results obtained in the present study, it can be deduced that the practical implications concerning the interaction between phenylalanine and baclofen may be clinically important since the inhibitor effect is high (67%), as occurred with

Table 3
Parameter values (± SD) obtained after fitting the inhibition model equations to the data. Statistical figure, SS, AIC and r obtained for each fit are also shown

Parameter	Complete competitive inhibition	Complete competitive inhibition + second component
V_m/K_m (h^{-1})	(0.98 ± 0.07)	(0.81 ± 0.05)
K (h^{-1})		(0.22 ± 0.04)
K_i (mM)	(23.78 ± 6.31)	(10.07 ± 2.25)
SS	0.0286	0.0041
AIC	−17.327	−26.936
$r>$	0.971	0.995

leucine (72%) (Cercós-Fortea et al., 1995). Both amino acids are essential, present in normal food (cereals, meats and many others). This inhibitory effect can be reinforced by other nonessential but usual dietary amino acids (β and γ), of which β-alanine causes 52% and GABA 30% inhibition of baclofen absorption (Polache et al., 1991; Nácher et al., 1994). Thus, the intestinal absorption of the drug at the doses currently used can be significantly reduced in the presence of these food components resulting from protein digestion in the gut.

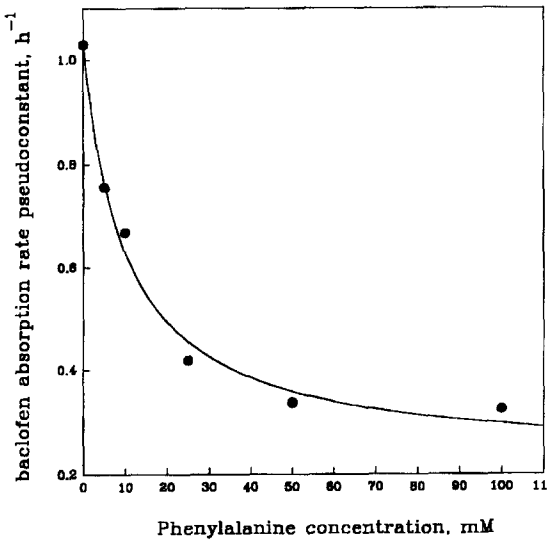

Fig. 1. Plot obtained from the fit of complete competitive inhibition in the presence of a second component equation to the data (baclofen absorption rate pseudoconstant at each phenylalanine concentration in perfusion fluid). Parameter values are shown in Table 3.

Table 4
Parameter values (± SD) found after fitting Eq. (5) to the data obtained for baclofen absorption in the presence of increasing concentrations of phenylalanine and GABA. Statistical figure, AIC and r obtained for each fit are also shown

Parameter	Baclofen + phenylalanine	Baclofen + GABA
V_m/K_m (h^{-1})	0.81 ± 0.05	0.27 ± 0.02
K (h^{-1})	0.22 ± 0.04	0.65 ± 0.02
K_i (mM)	10.07 ± 2.25	5.80 ± 1.56
AIC	−26.936	−36.982
$r>$	0.995	0.991

Summarizing, it is the authors' opinion that oral administration of baclofen in the presence of a meal should be avoided. The drug should be administered some time before meals or together with controlled diets free from potentially interacting amino acids. Otherwise, therapeutic baclofen levels may not be reached.

Acknowledgements

We are grateful to Professor J.M. Plá-Delfina for the kind review and useful discussion of the manuscript.

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