Suitability of Enalapril as a Probe of the Dipeptide Transporter System: *In Vitro* and *In Vivo* Studies

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Purpose. Previous *in situ* and *in vitro* studies indicated that the intestinal absorption of enalapril is a saturable carrier-mediated process *via* the dipeptide transporter system (DTS); however, the oral absorption of enalapril has not been reported to be a saturable process *in vivo*. Our objectives were to: 1) evaluate the suitability of enalapril as a probe of the DTS, and 2) compare various experimental models as they pertain to studying the DTS.

Methods. The *in vitro* uptake of enalapril by rat intestinal rings and permeability across Caco-2 cells were studied as a function of concentration and in the presence of compounds that are known substrates of the DTS. The effect of enalapril on the uptake of [^{3}H]-glycyl-L-proline (gly-L-pro) by Caco-2 cells was also examined. *In vivo* studies were conducted in rats (1 to 50 mg/kg) and dogs (0.06 to 6 mg/kg) to evaluate the oral absorption of enalapril over a wide dose range.

Results. *In vitro* intestinal uptake/permeability of enalapril was not saturable nor inhibited by β-lactam antibiotics, gly-L-pro, or SQ-29852. Moreover, a 20,000-fold molar excess of enalapril did not inhibit the uptake of [^{3}H]-gly-L-pro by Caco-2 cells. The *in vivo* studies in rats and dogs did not demonstrate saturable absorption.

Conclusions. The present *in vitro* and *in vivo* results indicated that enalapril is primarily absorbed by a non-saturable, passive diffusion process and it is not a suitable model compound for studying the DTS.

KEY WORDS: enalapril; absorption; Caco-2; rats; dogs.

INTRODUCTION

Enalapril maleate (hereafter referred to as enalapril) is an ester prodrug that is converted *in vivo* to enalaprilat, which is the active angiotensin converting enzyme (ACE) inhibitor. A previous report, based on *in situ* intestinal perfusion in rats, concluded that the intestinal absorption of enalapril was a saturable carrier-mediated process *via* the dipeptide transporter system (DTS) (1). Additional studies *in vitro* (2,3) with rabbit brush border membrane vesicles and Caco-2 cells (4) also concluded that enalapril was absorbed *via* the DTS.

Our objective was to use a variety of experimental systems to evaluate the suitability of enalapril as a model compound for studying the DTS. This involved determination of: 1) the *in vitro* uptake (everted rat intestinal rings) and permeability (Caco-2) of enalapril over a wide concentration range and 2) the uptake/permeability of enalapril in the presence of compounds that are known substrates of the DTS. When these *in vitro* studies failed to demonstrate concentration-dependent uptake/permeability or involvement of the DTS, *in vivo* studies were then conducted in rats (1 to 50 mg/kg) and dogs (0.06 to 6 mg/kg) over a wide dose range. The current results with enalapril are compared to those reported previously for SQ-29852, an ACE inhibitor that is a stable and specific probe of the DTS (5); this comparison addresses the suitability of the various *in vitro, in situ,* and *in vivo* model systems that are currently being used to characterize the DTS.

MATERIAL AND METHODS

Materials

[^{14}C]-Enalapril (5.3 mCi/mmol, radiochemical purity of 97%) and SQ-29852 were synthesized at Bristol-Myers Squibb. [^{3}H]-glycyl-L-proline (proline-3, 4-^{3}H; 50 Ci/mmole) and [^{14}C]-Methoxyinulin (5.6 mCi/g) were purchased from NEN Research Products (Boston, MA). Enalapril, β-lactams (Table I), glycyl-L-proline (gly-L-pro), sodium azide, triton X-100 (TX-100), Hank's balanced salt solution (HBSS), 2-N-morpholinoethanesulfonic acid (MES) and N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) were purchased from Sigma Chemical Co. (St. Louis, MO). TX-114 (National Diagnostics, NJ) or Ecolite® (ICN, CA) scintillation cocktail was used for counting radioactive samples. Fetal bovine serum was obtained from Hyclone Lab. Inc. (Logan, Utah). Rat tail collagen-type I was purchased from Collaborative Research Inc. (Bedford, MA). Caco-2 cells (passage #17) were obtained from ATCC (Rockville, MD). Dulbecco's modified Eagle's medium, nonessential amino acids, L-glutamine, penicillin-G, trypsin/EDTA, and streptomycin were purchased from JH Biosciences (Lenexa, KS). Transwell® inserts (surface area: 4.71 cm^2) with a polycarbonate membrane (3 μm pores) were purchased from Costar (Cambridge, MA). All other reagents were at least analytical grade.

Uptake of [^{14}C]-Enalapril by Rat Everted Intestinal Rings

The present research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985). The everted intestinal rings were prepared as follows. Male rats (Sprague-Dawley; 250 to 350 g, Harlan MD) were anesthetized with ether, and 5- to 10-cm segments of jejunum were removed, rinsed with Krebs-Henseleit (KH) buffer and everted with a glass rod. The rings were prepared by cutting several transverse slices (*ca.* 2 mm, 20 to 30 mg). The time-course of uptake of enalapril (90 cycles/min; 37°C) was determined at selected times up to 3 min and a 45-sec incubation time was selected for subsequent studies. Three rings were transferred into a tube (13 × 100 mm) containing [^{14}C]-enalapril (0.01 to 0.08 mM) and one of the β-lactam antibiotics (6 mM, Table I) in pH 6.0 phosphate buffer. Control experiments were conducted with [^{14}C]-enalapril alone at three bath concentrations: 0.01, 0.08, and 6 mM.

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Table I. Uptake of Radioactivity by Everted Rat Intestinal Rings (Mean ± SD; n = 3) 45 Seconds After Incubation with [^{14}C]-enalapril in the Presence of Various β-lactam Antibiotics (6 mM)

β-Lactam Antibiotic (6 m*M*)	Uptake of Radioactivity[a] (nmol-Eq.[b]/g/min)	
	0.01 m*M*[c]	0.08 m*M*[c]
None (Control)	1.0 ± 0.2	6.3 ± 0.5
Cefaclor	1.2 ± 0.1	6.1 ± 1.3
Cefazolin	0.9 ± 0.2	6.8 ± 1.0
Cefoxitin	1.0 ± 0.2	6.5 ± 2.0
Cephalosporin-C	1.2 ± 0.4	5.4 ± 0.9
Cephalexin	1.0 ± 0.3	4.5 ± 0.1
Cephradine	1.2 ± 0.4	5.1 ± 1.1
Amoxicillin	1.0 ± 0.2	6.2 ± 1.7

[a] ANOVA indicated lack of significant inhibition by the β-lactam antibiotics ($p > 0.05$).
[b] Expressed as equivalents of enalapril.
[c] Bath concentration of [^{14}C]-enalapril.

Prior to analysis of drug uptake, the rings were removed from the bath, rinsed with ice-cold KH buffer, and blotted dry. Each ring was weighed, digested with Soluene-350® (Packard Instruments, Downers Grove, IL), neutralized, and mixed with 15 mL of scintillation cocktail and counted for ^{14}C (Packard LSC Model 1600).

Effect of Enalapril on the Uptake of [^{3}H]-Gly-L-Pro by Caco-2 Cells

Validation of the model used to assess the inhibition of [^{3}H]-gly-L-pro uptake by enalapril is described elsewhere (6). Caco-2 cells (passage number of 25 to 35) were seeded (160,000 cells/well) and grown for 7 to 9 days on 24-well tissue culture plates (2 cm^2). Just prior to conducting the uptake study, the culture medium was aspirated and the monolayers were washed with buffer at 37°C. The incubation medium was HBSS with 25 mM MES, [^{3}H]-gly-L-pro (50 nM), 10 mM proline, 2% DMSO and enalapril (1 or 10 mM). Excess non-radiolabeled proline was added to maximize specific binding because it blocks uptake of the small amount of [^{3}H]-proline that is formed from hydrolysis during the 3-min incubation. Control incubations were done without addition of enalapril. The pH was adjusted to 6 with NaOH (1M). This solution (250 μL) was added to the well and incubated for 3 min at 37°C on an orbital shaker (50 cycles / min). The solution was removed after 3 min and the monolayers were washed with PBS sodium azide 0.05% (w/v) at 4°C. The monolayers were solubilized with 1% TX-100 at 37°C. The cell suspension was mixed with 15 mL of scintillation cocktail and counted for tritium. The data are expressed as the percentage inhibition of [^{3}H]-gly-L-pro uptake by enalapril *vs* control (no enalapril).

Permeability of Enalapril Across Caco-2 Cells

Caco-2 cells (passage number 33) were seeded (80,000 cells/cm^2) onto a collagen coated polycarbonate filter. Growth media was Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, 100 U/ml penicillin-G, and 100 μg/ml streptomycin. The culture medium was replaced every two days for a total of 24 days, and the cells were maintained at 37°C, 90% relative humidity, and 5% CO_2.

The permeability medium was modified Hank's balanced salt solution (MHBS) containing either 10 mM HEPES, pH 7.4 (basolateral side) or 25 mM MES, pH 6.0 (apical side). The permeability studies were initiated by adding 1.5 mL of MHBS (pH 6.0) containing enalapril (the initial concentration ranged from 0.1 to 10 mM) and [^{14}C]-methoxyinulin (0.4 μCi) to the apical side of the monolayer. The permeability of enalapril (0.3 mM) was also examined in the presence of several DTS substrates (e.g., SQ-29852, cephradine, or gly-L-pro, 3 mM). The monolayers were placed on an orbital shaker (50 cycles/min) and incubated up to 3 h at 37°C. At hourly intervals, the Transwell® insert was moved to a new receiver well containing fresh MHBS to maintain sink conditions. Samples were taken from each receiver well and the apical compartment was sampled at the end of the 3-h period.

Enalapril concentrations were analyzed by a specific HPLC-UV assay. A C_{18} μ-Bondapak column (3.9 mm × 30 cm; Waters, Millipore Corp., Milford, MA) was used. The mobile phase, which consisted of solvent A (water:acetonitrile:trifluoroacetic acid, 95:5:0.1 v/v) and solvent B (water:acetonitrile:trifluoroacetic acid, 20:80:0.1 v/v), was programmed as a linear gradient (flow rate was 1.2 mL/min; 220 nm). The concentrations of [^{14}C]-methoxyinulin were determined by liquid scintillation counting. Permeability coefficient (Pc) values (expressed as cm/sec × 10^{-6}) were calculated as follows: $Pc = dA/(dt \cdot S \cdot C_o)$, where dA/dt is the flux of drug across the monolayer, S is the surface area of the cell monolayer (4.71 cm^2), and C_o is the initial concentration in the apical fluid.

In Vivo Studies on the Oral Absorption of Enalapril over a Wide Range of Doses

Rats

After an overnight fast, each male rat (about 300 g) received an oral aqueous dose of [^{14}C]-enalapril by gavage at the following doses: 1, 5, and 50 mg/kg (n = 4). In addition, an iv dose of [^{14}C]-enalapril (5 mg/kg) was administered to a separate group of rats (n = 4). Urine was quantitatively collected for 72 h and 0.2-mL aliquots were mixed with TX-114 scintillation cocktail prior to counting. The estimate of absolute absorption was calculated by dividing the amount of radioactivity recovered in 0 to 72-h urine for each rat after oral administration by the average recovery of radioactivity after iv administration.

Dogs

After an overnight fast, each male beagle dog (10 to 15 kg; n = 6) received an oral aqueous dose of [^{14}C]-enalapril by gavage at the following doses: 0.06, 0.6 and 6 mg/kg; at least five days elapsed between doses. Urine was quantitatively collected for 72 h and radioactivity was analyzed as described above for rat urine. The minimum estimate of oral absorption was based on the amount of radioactivity recovered in 0 to 72-h urine. Statistical analysis was performed with a paired student's t-test with a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

Uptake of Enalapril by Everted Intestinal Rings

Preliminary experiments indicated that an oscillation rate of 90 cycles/min and an incubation time of 45 sec provided conditions that were within the linear region of the initial uptake of [^{14}C]-enalapril. The initial uptake rate of [^{14}C]-enalapril was not saturable as a function of bath concentration (Figure 1) and was not inhibited by a 600-fold molar excess of several β-lactam antibiotics (Table I). These results were unexpected because of a report, based on *in situ* intestinal perfusion in rats, which indicated that the intestinal permeability of enalapril was saturable and inhibited by cephradine (1).

Effect of Enalapril on the Uptake of [^{3}H]-Gly-L-Pro by Caco-2 Cells

Several investigators have demonstrated the presence of the DTS in Caco-2 cells (7,8). Validation details on the use of Caco-2 cells and [^{3}H]-gly-L-pro as a radioligand for evaluation of binding to the DTS are described elsewhere (6). Briefly, the model system was shown to have high specific binding (>85%) of the radioligand [^{3}H]-gly-L-pro and could reproducibly evaluate the ability of a test compound to displace this radioligand from binding to the DTS carrier protein.

The results indicated that the uptake of [^{3}H]-gly-L-pro by Caco-2 cells was not significantly inhibited ($p > 0.05$) by a 20,000-fold molar excess of enalapril (1 mM); this concentration of enalapril should have had an effect if the K_m value determined previously *in situ* in rats (0.07 mM; (1)), translated to the present *in vitro* situation in Caco-2 cells. In contrast to this result, the inhibition of [^{3}H]-gly-L-pro uptake by 1 mM of SQ-29852 averaged 59.2 ± 5.1% (Mean ± SD; n = 21) and by 1 mM of gly-L-pro averaged 85.2 ± 1.3% (Mean ± SD; n = 3; (5)) under identical experimental conditions. The result with SQ-29852 was consistent with the K_m value reported previously (0.08 mM; (9)). When a relatively high concentration of enalapril (10 mM; corresponding to about 140-fold higher than the previously reported K_m value) was evaluated, inhibition of [^{3}H]-gly-L-pro was observed (33.3 ± 2.2% inhibition). These data suggest that there might be a non-specific effect at this high concentration of enalapril or that the K_m value for enalapril in Caco-2 cells significantly exceeds 10 mM. Enalapril significantly inhibited the transcellular permeability of loracarbef (10) and gly-sar (11); however, this inhibitory effect was only reported when enalapril was studied at relatively high concentrations (10 and 20 mM, respectively). These concentrations of enalapril are *40- to 80-fold* higher than would be predicted to occur in the lumen of humans with a typical therapeutic dose (10 mg) when a 100-mL lumenal fluid volume is assumed. Thus, it is possible that non-specific effects could occur with these high concentrations that are not relevant to the *in vivo* situation.

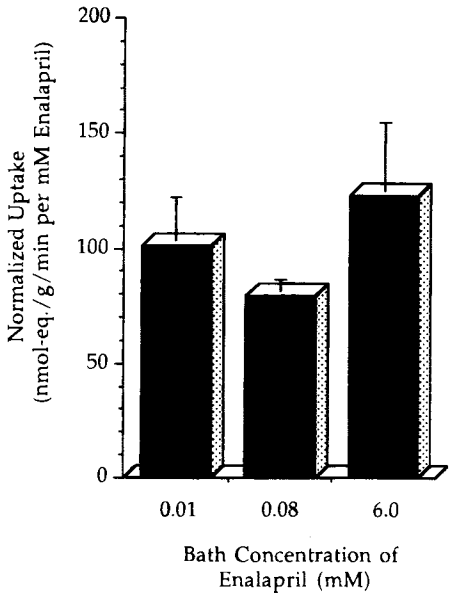

Fig. 1. Uptake (Mean ± SD; n = 3) of radioactivity by everted rat intestinal rings 45 seconds after incubation with enalapril at various concentrations. Uptake was not significantly different (ANOVA; $p > 0.05$).

Permeability of Enalapril Across Caco-2 Cells

The presence of the DTS was demonstrated by evaluation of the transport of gly-L-pro across Caco-2 cells; gly-L-pro transport was concentration- and temperature-dependent and inhibited by gly-sar, cephradine, and SQ-29852 (Table II). The Caco-2 cell permeability of enalapril varied from about 2.5 to 3.5 cm/s × 10^{-6} over the concentration range examined (0.1 to 10 mM) and was not reduced as a function of concentration (Figure 2). Interestingly, at higher enalapril concentrations (*e.g.*, 5 and 10 mM), the permeability appears to be *increased.* This increase might have resulted from a small perturbation of the tight junctions by enalapril since the permeability of methoxyinulin was also increased at higher enalapril concentration (ANOVA; $p < 0.05$); however, the ratio of enalapril to methoxyinulin permeability was independent of enalapril concentration (ANOVA; $p > 0.05$). The Caco-2 permeability of enalapril was not inhibited by a 10-fold molar excess of several DTS substrates (SQ-29852, cephradine, and gly-L-pro) (Figure 3). These data indicate that the Caco-2 cell permeability of enalapril was a passive, non-saturable process, and was not inhibited

Table II. Concentration and Temperature Dependence of Gly-L-Pro Permeability Through Caco-2 Cells (Mean ± SD; n = 3) and Inhibition by Various DTS Substrates

Condition	Concentration of Gly-L-Pro (μM)	Permeability Coefficient of Gly-L-Pro (cm/s × 10^{-6}) 37°C	4°C
I. gly-L-pro alone[a]	1000	6.6 ± 0.2	0.90 ± 0.40
	10	13.5 ± 0.6	1.5 ± 0.50
	0.50	35.6 ± 0.9	ND[b]
II. gly-L-pro + 500 μM of:			
gly-sar	0.50	18.4 ± 0.6[c]	ND
cephradine	0.50	26.5 ± 1.0[c]	ND
SQ-29852	0.50	2.5 ± 0.2[c]	ND

[a] ANOVA indicated gly-L-pro permeability was different as a function of concentration and temperature ($P < 0.05$).
[b] Not determined.
[c] Significantly different than gly-L-pro alone by ANOVA ($p < 0.05$).

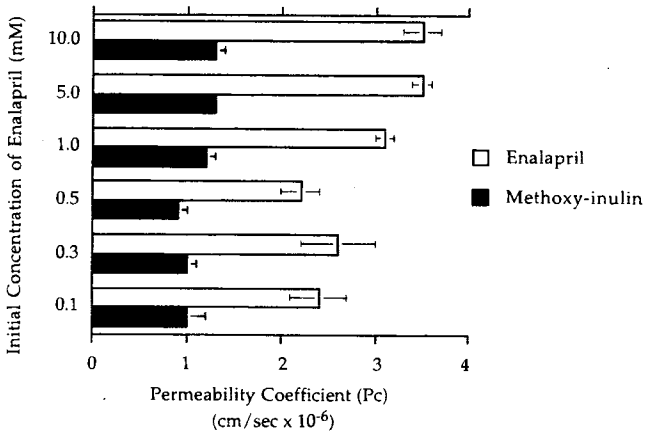

Fig. 2. Permeability of enalapril across Caco-2 cells (Mean ± SD; n = 3) as a function of concentration from 0.1 to 10 mM. ANOVA indicated that the permeability of enalapril and methoxy-inulin were increased as a function of concentration ($p < 0.05$).

by compounds previously demonstrated to be absorbed *via* the DTS.

In Vivo Studies on the Oral Absorption of Enalapril over a Wide Range of Doses

There are no published data that indicate the oral absorption of enalapril is a saturable process *in vivo*. Thus, [^{14}C]-enalapril was given orally to rats and dogs over a wide dose range to evaluate whether there was dose-dependent absorption of enalapril in these species *in vivo*.

The recovery of radioactivity in urine of rats after a 5-mg/kg iv dose of [^{14}C]-enalapril averaged 65.7 ± 2.6%. In rats, the absolute oral absorption of [^{14}C]-enalapril averaged about 52 to 60% and was not reduced at higher doses (Table III). In dogs, the oral absorption of [^{14}C]-enalapril was not reduced when given at higher doses (Table III). Interestingly, the absorption after administration of the 0.6- and 6-mg/kg dose was greater ($p < 0.05$) than after the 0.06-mg/kg dose. These results were consistent with the Caco-2 results described above; however, the reason(s) for the apparent higher absorption with increasing dose was not further investigated.

Thus, the oral absorption of enalapril in rats or dogs was not saturated even at relatively high doses of 50 mg/kg or 6.0 mg/kg, respectively. This corresponds to about *50-fold* (rats) or *20-fold* (dogs) higher than the typical therapeutic dose in humans (10 mg), when normalized for the inter-species difference in body surface area.

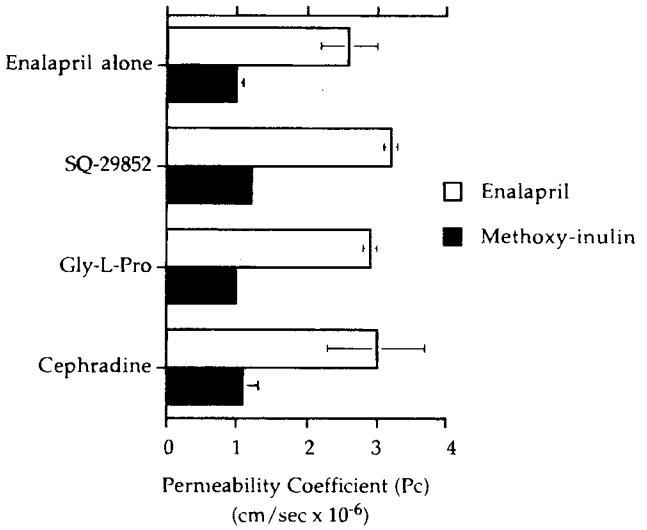

Fig. 3. Permeability of enalapril across Caco-2 cells (Mean ± SD; n = 3) at an initial concentration of 0.3 mM in the presence of compounds known to absorbed *via* the DTS: SQ-29852, gly-L-pro, and cephradine. ANOVA indicated that these DTS substrates did not inhibit enalapril permeability ($P > 0.05$). Permeability of methoxy-inulin was measured simultaneously in each well.

Comparison of SQ-29852 and Enalapril as a Probe of the DTS

SQ-29852, a lysyl-proline ACE inhibitor, was also reported to be primarily absorbed *via* the DTS based on *in situ* intestinal perfusion in rats (9). It has been subsequently shown that SQ-29852 is an ideal model compound for studying the DTS because it is: 1) a stable and *specific* probe of the DTS (*i.e,* negligible absorption *via* passive diffusion), and 2) provides consistent results on the DTS in a variety of *in vitro* (6,11), *in situ* (9), and *in vivo* models (5). Contrary to what was observed for enalapril, the oral absorption of SQ-29852 was saturable and clearly dose dependent in rats (3 to 3000 mg/kg; (5)), dogs (12), and humans (13). In animals, SQ-29852 is well absorbed (70%) at low doses and its absorption is less than 10% at high doses, suggesting that it has a minimal absorption *via* passive diffusion. In this regard, SQ-29852 appears to be a better probe than the β-lactam aminocephalosporins since the latter have been reported to have a significant passive component to their overall absorption (14).

Despite the fact that enalapril and SQ-29852 were previously reported to have similar K_m values for the DTS based on *in situ* perfusion in rats (*ca.* 0.07 to 0.08 mM; (1,9)), SQ-29852 begins to demonstrate saturable absorption *in vivo* at a dose of 10 mg/kg (5), whereas the present results show that enalapril absorption was not reduced even at a dose of 50 mg/kg. Assuming that the fluid volume within the GI tract is 5 mL in rats, then the projected lumenal concentration at the highest dose used in rats (50 mg/kg) is 8 mM. This estimate of drug concentration in the lumen is one *hundred-fold* higher than the previously reported K_m value. Thus, both enalapril and SQ-29852 should have displayed comparable dose-dependent absorption if the previous *in situ* results translated to *in vivo*.

Comparison of Available Experimental Model Systems for Studying the DTS

Everted rat intestinal rings were previously shown to have significant non-specific uptake of [^{3}H]-SQ-29852 (15), despite the fact that this compound is primarily absorbed *via* the DTS. Non-specific binding to smooth muscle of the intestine appears to be the likely mechanism since there was minimal non-specific uptake of SQ-29852 by Caco-2 cells (11). Although Caco-2 cells appear to be a good model for studying the DTS when conducting uptake experiments (*i.e.,* brush border transporter on apical side; (5,10)),

Table III. Oral Absorption[a] of Enalapril in Rats and Dogs as a Function of Dose

Rats		Dogs	
Dose (mg/kg)	Absolute Absorption[a] (%; Mean ± SD; n = 4)	Dose (mg/kg)	Minimum Estimate of Absorption (%; Mean ± SD; n = 6)
1	51.9 ± 6.5	0.06	20.6 ± 4.4
5	55.5 ± 9.3	0.6	29.6 ± 5.0[b]
50	60.5 ± 5.3	6.0	37.2 ± 10.6[b]

[a] Based on the recovery of radioactivity in 0 to 72-h. These estimates assume that the systemic clearance of enalapril was independent of dose in rats and dogs.

[b] Significantly different ($p < 0.05$) than the absorption at the 0.06-mg/kg dose.

the model can be potentially misleading when evaluating transcellular permeability for compounds that are absorbed primary *via* the DTS (16,17). Although *in situ* intestinal perfusion in rats provided reliable results with SQ-29852 (10), it is not known why the reported *in situ* results with enalapril (1) disagree with the current *in vitro* and *in vivo* findings.

CONCLUSIONS

Previous *in vitro* and *in situ* results with enalapril indicated transport *via* the DTS; however, the current *in vitro* and *in vivo* results indicate that enalapril is absorbed by a non-saturable, passive diffusion process, without involvement of the DTS. A comparison of the current results on enalapril with those reported previously for another ACE inhibitor, SQ-29852, indicate that SQ-29852 is a better model compound for studying the DTS. SQ-29852 provides consistent results in a variety of *in situ, in vitro,* and *in vivo* models, whereas the conclusion regarding enalapril's involvement with the DTS is highly dependent on the model system that is used.

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