Sites of First-Pass Bioactivation (Hydrolysis) of Orally Administered Zofenopril Calcium in Dogs

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Received January 3, 1990; accepted September 5, 1990

The relative contribution of the gut, liver, and lungs as sites of first-pass bioactivation (hydrolysis) of the orally administered ester prodrug, zofenopril calcium (SQ 26,991), to the active angiotensin converting enzyme (ACE) inhibitor, SQ 26,333, was determined. With a five-way study design, two dogs each received a single 1.6-mg/kg dose of zofenopril [as its soluble potassium salt (SQ 26,900)] via the following routes of administration: intraarterial, intravenous, intraportal, and oral. Each dog also received an equimolar oral dose of zofenopril calcium (1.5 mg/kg). Concentrations of zofenopril in plasma were quantitated with a GC/MSD assay. Extraction ratios (E) for zofenopril by the gut, liver, and lungs were calculated based on the ratios of the area under the curve (AUC) values of zofenopril in arterial plasma after administration by the various routes. As individual eliminating organs, the gut and liver each had a high intrinsic capability to hydrolyze zofenopril; E values ranged from 45 to 89%. The lungs were found to have low, but measurable, hydrolytic activity with estimated E values that ranged from 5 to 26%. Overall, about 95% of the orally administered dose of zofenopril calcium was hydrolyzed during the first pass. Because the prodrug is sequentially exposed to the gut, liver, and lungs, the contribution of the gut to the overall first-pass hydrolysis (ca. 87%) was estimated to be significantly greater than that of the liver (<10%) or lungs (<2%). Zofenopril was rapidly eliminated after parenteral administration; mean residence time values were 2 min and the elimination half-life values (intraarterial route only) were 9 min. The total-body clearance (Cl_{total}) of zofenopril, determined after intraarterial injection, was rapid (ca. 25 ml/min/kg) and was similar to the Cl_{total} value reported previously for fosinopril sodium, another prodrug ACE inhibitor. Although the E_{lungs} value was relatively low, the lungs receive 100% of cardiac output and were estimated to contribute significantly to *systemic* bioactivation of zofenopril. The major sites of *systemic* bioactivation appear to be the lungs (ca. 44 to 65%) and liver (ca. 31%), whereas the hydrolysis of zofenopril by the blood per se does not apparently contribute to Cl_{total}.

KEY WORDS: (ACE) angiotensin converting enzyme inhibitor; prodrug; esterase activity; bioactivation sites; gut; liver.

INTRODUCTION

Zofenopril calcium is an orally active antihypertensive agent that is the *S*-benzoyl prodrug of SQ 26,333, an ACE inhibitor (Fig. 1). After oral administration of zofenopril to animals and humans, there is extensive hydrolysis to SQ 26,333 (1,2,3).

The objective of this study was to determine the relative contribution of the gut, liver, and lungs as sites of first-pass bioactivation (hydrolysis) of zofenopril after oral administration in dogs. In addition, the contribution of the liver and lungs as sites of *systemic* bioactivation of zofenopril was estimated. The potassium salt of zofenopril (SQ 26,900) was used for the parenteral routes, because zofenopril calcium (SQ 26,991) is not sufficiently soluble in an appropriate vehicle for parenteral administration.

The experimental approach that was selected involves administration of the prodrug by multiple routes that are afferent and efferent to the potential sites of first-pass bioactivation. In addition, the concentrations of prodrug in arterial and central venous plasma were determined after a constant-rate infusion; thus, the efficiency of the lungs to hydrolyze zofenopril was also evaluated under steady-state conditions. This approach was used previously to characterize the first-pass bioactivation of fosinopril sodium in dogs (4).

MATERIALS AND METHODS

Animals. Two male beagle dogs were used (Nos. 7057 and 7077; 10 to 14 kg; White Eagle, Doylestown, PA). Each dog was surgically prepared with an indwelling cannula in the portal vein (5). Each dog was also surgically prepared with two indwelling arterial cannulae, which were inserted into the right carotid artery; one was positioned in the descending abdominal aorta (distal cannula) and the other was positioned in the thoracic aorta (proximal cannula). These cannulae were used for drug administration (intraarterial route) and arterial blood collection, respectively. Immediately after surgery, the portal and arterial cannulae were filled with a radioopaque marker (diatrizoate sodium, 50%; Winthrop-Breon Laboratory, New York) and their positions were visualized by contrast radiography; positions of the cannulae were confirmed at necropsy. For intravenous drug administration and for collection of central venous blood (steady-state infusion experiment only), a temporary cannula was inserted on the day of dosing into the right external jugular vein and positioned at the junction of the superior and inferior vena cava.

The dogs were anesthetized with halothane during surgery and allowed to recover for at least 1 week prior to drug administration. Food was withheld for about 16 hr before dosing and until 6 hr after dosing; water was available *ad libitum.* Clinical chemistry tests of blood and serum were performed on 2 different days during the study to ensure that the dogs remained healthy. Each dog was housed unrestrained in a metabolic cage. Patency of the exteriorized cannulae was maintained using heparin (100 U/ml) in sterile saline.

Drug Administration. The anatomical sites of drug administration and blood sample collection are shown in Fig. 2. With a five-way study design, each dog received an aqueous solution of SQ 26,900 (Batch No. 452003) as a single 1.6-mg/kg dose via the following routes of administration: in-

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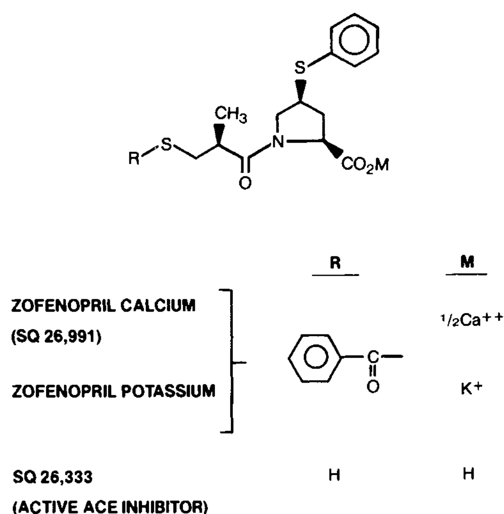

Fig. 1. Chemical structures of zofenopril and SQ 26,333.

traarterial, intravenous, intraportal, and oral. Each dog also received an equimolar 1.5-mg/kg oral dose of zofenopril calcium (Batch No. RR004NC), dissolved in propylene glycol: water (1:1). The dose was selected to be comparable to a therapeutic dose in man, when adjusted for body surface areas. The parenteral doses were administered into the appropriate cannula and the oral doses were administered by gavage. In the infusion experiment, Dog No. 7077 received SQ 26,900 as a constant-rate infusion (0.18 mg/min for 2 hr) via a temporary peripheral venous cannula. At least 3 days elapsed between doses.

Sample Collection and Analysis. For the bolus dose experiments, heparinized blood samples were collected serially from the arterial cannula for up to 6 hr after dosing. For the steady-state infusion experiment (Dog No. 7077 only), arterial blood samples were collected from the proximal arterial cannula and venous blood samples were simultaneously collected from the temporary central venous cannula during the 2-hr infusion and for 30 min thereafter.

Blood samples were stored in crushed ice prior to centrifugation (3500 rpm for 15 min at 5°C) to obtain plasma. Aliquots of plasma (0.1 to 0.5 ml) were extracted and analyzed for zofenopril with a specific capillary GC/MSD assay (6). Plasma samples were kept frozen (−20°C) for up to 2 months prior to analysis. In the present study, the quantitation limit for zofenopril in plasma was 10 ng/ml.

Stability of Zofenopril in Whole Blood and Association with Cellular Elements. Blood obtained from two healthy beagles was pooled. For determination of the stability of zofenopril, SQ 26,900 was added to fresh pooled blood to obtain a concentration of 2 μg/ml. The spiked blood was incubated at 37°C for 3 hr. During the incubation, aliquots of blood were collected and plasma was prepared and analyzed for zofenopril. For determination of the association of zofenopril with cellular elements of blood, ^{14}C-zofenopril calcium (Batch No. NN033IL, specific activity of 4.46 μCi/mg, radiochemical purity of 95%) was added to a separate sample of whole blood to obtain a concentration of 2 μg/ml and was incubated at 37°C for 3 hr. During the incubation, aliquots of blood and plasma were collected and analyzed for total radioactivity by liquid scintillation spectrometry. The association of radioactivity with cellular elements of blood was calculated with the following equation: % association = [blood conc. − plasma conc. × (1-H)]/blood conc. × 100, where H corresponds to the hematocrit. Both *in vitro* incubations were conducted in duplicate.

Data Analysis. Concentrations of zofenopril in plasma were based on standard curves prepared with control dog plasma containing known concentrations of zofenopril. The elimination half-life in the terminal phase was calculated only for the intraarterial route ($t_{1/2ia}$) because the concentrations of zofenopril were below the limit of detection in the terminal phase for the other routes of administration. The $t_{1/2ia}$ was calculated by log-linear regression of the last three detectable concentration–time points. The area under the concentration-vs-time curve from time zero to the time corresponding to the last detectable concentration of zofenopril ($AUC_{0\rightarrow t}$) was calculated by the integration method of Lagrange (7). The $AUC_{0\rightarrow t}$ values for all routes of administration were extrapolated to infinity ($AUC_{0\rightarrow\infty}$) using the $t_{1/2ia}$ value for each dog. Other pharmacokinetic parameters were calculated by standard methods (8).

Extraction ratios (E) for zofenopril by the gut (E_{gut}), liver (E_{liver}), and lungs (E_{lungs}) were calculated based on the relationships reported by Cassidy and Houston (9). The AUC values in the equations below correspond to the $AUC_{0\rightarrow\infty}$ for zofenopril in arterial plasma after the various routes of administration (the subscript denotes the route of administration). These calculations assume that there is complete absorption of the oral dose, which was shown to be the case in a previous study in dogs, in which ^{14}C-zofenopril calcium was used (1).

E_{gut}, E_{liver}, and E_{lungs} were calculated from the following equations:

$$E_{gut}(\%) = [1 - (AUC_{oral}/AUC_{intraportal})] \times 100$$

$$E_{liver}(\%) = [1 - (AUC_{intraportal}/AUC_{intravenous})] \times 100$$

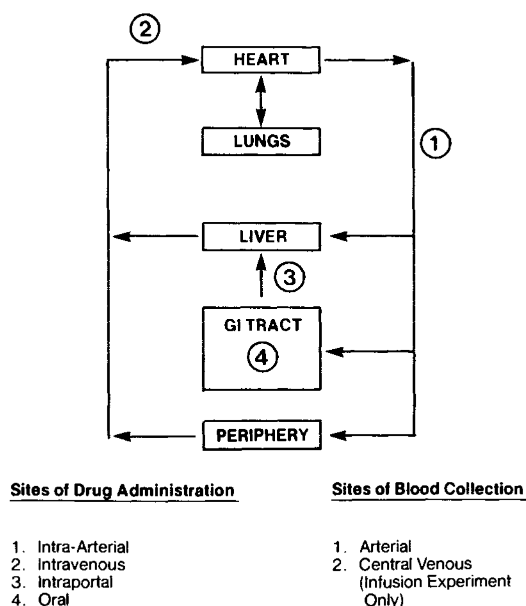

Fig. 2. Anatomical sites of drug administration and blood sample collection.

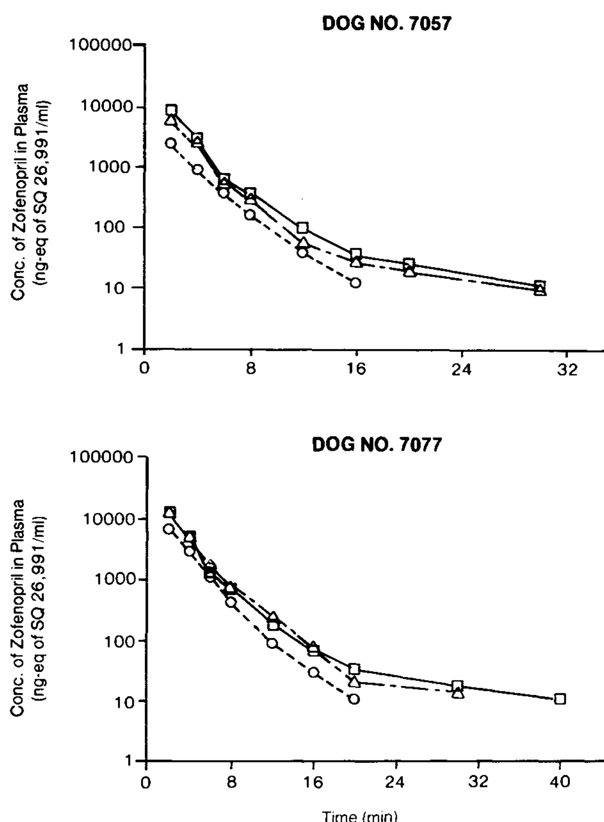

Fig. 3. Concentrations of zofenopril in plasma after intraarterial (□), intraportal (○), and intravenous (△) administration.

$$E_{\text{lungs}}(\%) = [1 - (\text{AUC}_{\text{intravenous}}/\text{AUC}_{\text{intraarterial}})] \times 100$$

For the steady-state infusion experiment, E_{lungs} was calculated by

$$E_{\text{lungs}}(\%) = [(\text{AUC}_{\text{venous}} - \text{AUC}_{\text{arterial}})/\text{AUC}_{\text{venous}}] \times 100$$

where, in this case, the subscript for AUC denotes the sampling site of plasma.

After oral administration, the prodrug is exposed first to the gut, followed by the liver and lungs, prior to entering the systemic circulation. The E values for each eliminating organ (E_{gut}, E_{liver}, and E_{lungs}) were used as indicated in the following equations to account for the fate of orally administered zofenopril. This provides an estimate of the contribution of the gut, liver, and lungs as *sequential* first-pass sites. Since the gut is the first site of hydrolysis, its contribution as a sequential first-pass site ($E_{\text{gut first pass}}$) is simply the E value for the gut: [$E_{\text{gut first pass}}$ (%) = E_{gut} (%)].

The contribution of the liver as the second site of hydrolysis of zofenopril during the first-pass transit of drug ($E_{\text{liver first pass}}$) corresponds to the fraction of the dose that escapes hydrolysis by the gut [100 − $E_{\text{gut first pass}}$] multiplied by the extraction ratio for the liver:

$$E_{\text{liver first pass}}(\%) = [100 - E_{\text{gut first pass}}(\%)] \times [E_{\text{liver}}]$$

It follows that the contribution of the lungs as a sequential first-pass site can be calculated by

$$E_{\text{lungs first pass}}(\%) = [100 - E_{\text{gut first pass}}(\%) - E_{\text{liver first pass}}(\%)] \times [E_{\text{lungs}}]$$

The hepatic clearance of zofenopril (Cl_{liver}) was estimated as the product of E_{liver} and the reported value for hepatic plasma flow rate in dogs [ca. 15 ml/min/kg (10)]. Similarly, the clearance of zofenopril by the lungs (Cl_{lungs}) was estimated as the product of E_{lungs} and the reported value for cardiac output of *plasma* in dogs [ca. 75 ml/min/kg (11)]. These estimates, which are based on *plasma* clearance and *plasma* organ flows, are valid because there is negligible association of zofenopril with cellular elements of blood as discussed below.

RESULTS AND DISCUSSION

The approach of using multiple routes of drug administation to estimate the relative contribution of the gut, liver, and lungs as sites of first-pass metabolism has been used by other investigators (12–17). This approach was used previ-

Table I. Pharmacokinetic Parameters of Zofenopril in Arterial Plasma After Administration by Various Routes

Parameter	Units	Intraarterial	Intravenous	Intraportal	Oral: SQ 26,991 (1/2 Ca⁺⁺)	Oral: SQ 26,900 (K⁺)
Dog No. 7057						
$\text{AUC}_{0\to t}$	ng × hr/ml	832	616	231	33	73
$\text{AUC}_{0\to\infty}$	ng × hr/ml	834	618	233	36	83
MTR[a]	min	2.0	2.1	2.4	17	39
MAT[b]	min	—	—	—	15	37
$C_{\max}$	ng/ml	—	—	—	137	114
Dog No. 7077						
$\text{AUC}_{0\to t}$	ng × hr/ml	1160	1104	612	65	64
$\text{AUC}_{0\to\infty}$	ng × hr/ml	1163	1108	615	70	69
MRT	min	2.3	2.5	2.3	24	30
MAT	min	—	—	—	22	28
$C_{\max}$	ng/ml	—	—	—	182	151

[a] Mean residence time.
[b] Mean absorption time: [$\text{MRT}_{\text{po}} - \text{MRT}_{\text{iv}}$].

Table II. Extraction Ratios of Zofenopril by the Gut, Liver, and Lungs and Their Relative Contribution as First-Pass Hydrolysis Sites for Orally Administered Zofenopril Calcium

Dog No.	Extraction ratio $(E)^a$ of zofenopril (% hydrolysis)			Extent of first-pass hydrolysis of the oral dose of zofenopril calcium (% of dose)[b]			
	E_{gut}	E_{liver}	E_{lungs}	$E_{gut\ first\ pass}$	$E_{liver\ first\ pass}$	$E_{lungs\ first\ pass}$	Total first-pass hydrolysis
7057	84.7 (64.2)[c]	62.3	25.9	84.7	9.5	1.5	95.7
7077	88.7 (88.7)	44.5	12.5[d]	88.7	5.0	0.8	94.5

[a] Corresponds to the hydrolytic activity of each eliminating organ.
[b] Contribution of organs when considered as sequential first-pass elimination sites. Estimates correspond to data obtained after oral adminstration of zofenopril calcium.
[c] Values in parentheses were obtained after oral administration of the potassium salt of zofenopril (SQ 26,900).
[d] Average E value from injection (5%) and infusion (20%) experiments.

ously to characterize the first-pass bioactivation of fosinopril sodium in dogs (4). Alternative methods to evaluate first-pass metabolism in animals include surgical procedures such as partial hepatectomy and portal–caval shunt; however, these approaches can alter the systemic clearance of drug (18) and might alter the determination of the liver's contribution to the total first-pass metabolism.

Extraction Ratios. The concentrations of zofenopril in plasma after administration by the various routes are shown in Fig. 3 and the pharmacokinetic results are shown in Table I. Calculation of the extraction ratio (E) of zofenopril by the gut, liver, and lungs provides an estimate of their intrinsic efficiency as individual hydrolytic sites. The gut and liver each had a high intrinsic capability to hydrolyze zofenopril as reflected by E values that ranged from 45 to 89% (Table II). The lungs were found to have low, but measurable, hydrolytic activity with estimated E values that ranged from about 5 to 26%. Hydrolysis of zofenopril by the lungs, as determined after a bolus injection of zofenopril, was confirmed in Dog No. 7077 with a constant-rate infusion; this allowed for evaluation of E_{lungs} under steady-state conditions (Fig. 4). Based on the AUC data in Table III, the value for E_{lungs}, determined at steady state, was 20%.

First-Pass Bioactivation of Zofenopril. The contribution of the gut, liver, and lungs as *sequential* first-pass bioactivation sites of orally administered zofenopril can be estimated from the individual E values. Accordingly, the contribution of the gut to the overall first-pass hydrolysis (ca. 87%) was found to be significantly greater than for the liver (<10%) or lungs (<2%) (Table II). The first-pass bioactivation of zofenopril calcium is similar to that reported previously for fosinopril sodium, for which the gut was the major site (ca. 78 to 91%), and the liver (ca. 9 to 23%) and lungs (~0) were minor sites (4).

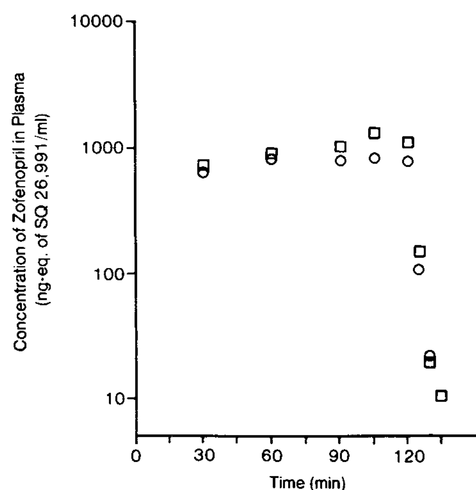

Fig. 4. Concentrations of zofenopril in arterial (○) and central venous (□) plasma after a constant-rate infusion to Dog No. 7077.

Systemic Bioactivation and Pharmacokinetics of Zofenopril. It was important to determine accurately the pharmacokinetics of zofenopril after intraarterial administration because it is the baseline route that is devoid of any first-pass metabolism sites. The Cl_{total} of zofenopril, estimated after intraarterial injection, was rapid [ca. 25 ml/min/kg (Table IV)] and was similar to the Cl_{total} value reported previously for fosinopril sodium (4), another prodrug ACE inhibitor. The Cl_{total} values determined after intraarterial injection were corroborated by the results obtained after a constant-rate infusion in Dog No. 7077 (Table III).

The estimated $t_{1/2ia}$ values, corresponding to the terminal elimination phase, were 8.8 and 10.0 min for Dog Nos. 7057 and 7077, respectively (Table IV). However, the mean residence time (MRT) was only about 2.0 to 2.5 min after parenteral administration. In either case, these values indicated that the prodrug was rapidly hydrolyzed *in vivo* after parenteral administration.

Zofenopril was relatively stable ($t_{1/2}$ of approximately 7

Table III. Pharmacokinetic Parameters of Zofenopril in Arterial and Central Venous Plasma after a Constant-Rate Infusion (0.18 mg/min for 120 min) to Dog No. 7077

Parameter	(Units)	Arterial	Central venous
$AUC_{0\to t}$	μg × hr/ml	1.48	1.86
$AUC_{0\to\infty}$	μg × hr/ml	1.48	1.86
$t_{1/2}$	min	3.6	3.3
$C_{ss}{}^a$	ng/ml	842	1200
Clearance[b]	ml/min/kg	24.3	19.4

[a] Average concentration between 90 and 120 min.
[b] Calculated as dose divided by $AUC_{0\to\infty}$.

Table IV. Pharmacokinetic Parameters of Zofenopril After Intraarterial Injection of 1.6 mg/kg of SQ 26,900: Characterization of Systemic Bioactivation

Parameter	Units	Dog No. 7057	Dog No. 7077
Cl_{total}	ml/min/kg	30.0	21.5
Cl_{liver}	ml/min/kg	9.4	6.7
		(31%)[a]	(31%)
Cl_{lungs}	ml/min/kg	19.4	9.4
		(65%)	(44%)
Cl_{other}	ml/min/kg	1.2	5.4
		(4%)	(25%)
A	μg/ml	27	33
B	ng/ml	128	179
$t_{1/2(\alpha)}$	min	1.3	1.4
$t_{1/2(\beta)}$	min	8.8	10.0
$F_{R\beta}$[b]	%	3.2	3.7
V_c	ml/kg	55	46
$V_{d_{ss}}$	ml/kg	60	51

[a] Values in parentheses represent percentage of Cl_{total}.
[b] Fraction of dose that is kinetically described by the β phase (see text for details).

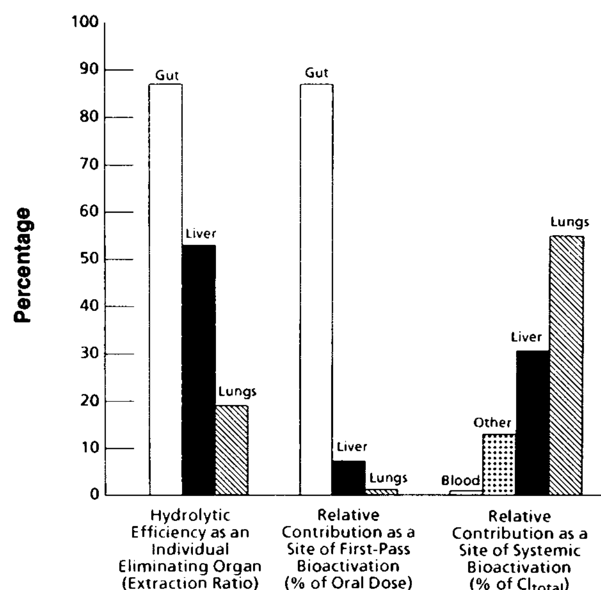

Fig. 5. Efficiency of the gut, liver, and lungs for hydrolysis of zofenopril: Estimation of the sites of first-pass and systemic bioactivation. Data are the average values of two dogs.

hr) *in vitro* when incubated at 37°C in whole blood (data not shown). In addition, the *in vitro* experiment with ^{14}C-zofenopril calcium indicated that there was negligible association of zofenopril with cellular elements of dog blood under these experimental conditions (data not shown). The product of the first-order rate constant for the *in vitro* hydrolysis of zofenopril in whole blood (ca. 0.002 min^{-1}) and the published value for the blood volume of dogs [90 ml/kg (19)] provides a predicted *in vivo* clearance rate of 0.2 ml/min/kg. This allows estimation of the contribution of whole blood as a site of hydrolysis of zofenopril *in vivo*. The value for the predicted *in vivo* clearance of zofenopril by blood, normalized to a value for plasma, is less than 1% of the Cl_{total} for zofenopril. Therefore, blood per se does not significantly contribute to the rapid hydrolysis of zofenopril *in vivo*. In addition, these data indicate that zofenopril was stable in blood during the time required to obtain plasma for the *in vivo* experiments.

With respect to the systemic bioactivation of zofenopril, clearances of zofenopril by the liver (Cl_{liver}) and lungs (Cl_{lungs}) were estimated based on appropriate E values and organ plasma flow rates (Table IV). This is a valid approach because zofenopril is not associated with cellular elements of dog blood. Because the values of Cl_{liver} and Cl_{lungs} represent clearance of drug from the *systemic* circulation, they are dependent on plasma flow rates to these eliminating organs. Based on a comparison of Cl_{liver} and Cl_{lungs} to Cl_{total}, it was estimated that the liver accounted for about 31% and the lungs for about 44 to 65% of the Cl_{total} of zofenopril. Although the lungs are a relatively minor site of first-pass hydrolysis, it receives 100% of cardiac output and therefore it contributes significantly to the total *systemic* bioactivation of zofenopril.

CONCLUSIONS

The contribution of the gut, liver, and lungs as organs for bioactivation of zofenopril is summarized in Fig. 5. As individual eliminating organs, the relative efficiency for hydrolysis of zofenopril was in the following order: gut > liver ≫ lungs. With respect to first-pass bioactivation of orally administered zofenopril calcium, the gut was the major site of hydrolysis (ca. 87%), whereas the liver (<10%) and lungs (<2%) were minor sites. At least 75% of the *systemic* bioactivation of zofenopril (Cl_{total}) appears to occur in the lungs (44 to 65%) and liver (ca. 31%), whereas, the hydrolysis of zofenopril by the blood per se does not contribute to Cl_{total}.

ACKNOWLEDGMENTS

We thank E. Ivashkiv, C. J. Mondi, R. J. Ratoff, R. Scalese, J. M. Shaw, A. L. Sibold and D. Wang-Iverson for excellent technical assistance.

REFERENCES

1. G. R. Keim, S. M. Singhvi, K. J. Kripalani, and B. H. Migdalof. 1st European Meeting on Hypertension, Milan, Italy, 1983.
2. A. V. Dean, K. J. Kripalani, and B. H. Migdalof. *Fed. Proc.* 43:349 (1984).
3. R. A. Morrison, J. E. Foley, D. A. Willard, and S. M. Singhvi. *Abstr. 37th Natl. Meet. A.Ph.A. Acad. Sci.* 14(2):256 (1984).
4. R. A. Morrison, S. M. Singhvi, A. E. Peterson, D. A. Pocetti, and B. H. Migdalof. *Drug Metab. Dispos.* (in press).
5. R. Santiesteban. D. Hutson, and R. S. Dombro. *Lab. Anim. Sci.* 33:373–374 (1983).
6. M. Jemal, E. Ivashkiv, T. Teitz, and A. I. Cohen. *J. Chromatogr. Biomed. Appl.* 428:81–92 (1988).
7. K. C. Yeh and K. C. Kwan. *J. Pharmacokin. Biopharm.* 6:79–98 (1978).
8. M. Gibaldi and D. Perrier. *Pharmacokinetics,* Marcel Dekker, New York, 1975.
9. M. K. Cassidy and J. B. Houston. *J. Pharm. Pharmacol.* 32:57–59 (1980).
10. K. B. Bischoff, R. L. Dedrick, D. S. Zaharko, and J. A. Longstreth. *J. Pharm. Sci.* 60:1128–1133 (1971).

11. S. J. Ettinger and P. F. Suter. *Canine Cardiology,* W.B. Saunders, Philadelphia, 1970.
12. R. N. Boyes, H. J. Adams, and B. R. Duce. *J. Pharmacol. Exp. Ther.* 174:1–8 (1970).
13. D. G. Shand, D. M. Kornhauser, and G. R. Wilkinson. *J. Pharmacol. Exp. Ther.* 195:424–432 (1975).
14. D. A. Wiersma and R. A. Roth. *J. Pharmacol. Exp. Ther.* 226:661–667 (1983).
15. M. Mistry and J. B. Houston. *Drug Metab. Dispos.* 13:740–745 (1985).
16. D. Brewster, M. L. Humphrey, and M. A. McLeavy. *J. Pharm. Pharmacol.* 33:500–506 (1981).
17. P. A. Harris and S. Riegelman. *J. Pharm. Sci.* 58:71–75 (1969).
18. R. Gugler, P. Lain, and D. L. Azarnoff. *J. Pharmacol. Exp. Ther.* 195:416–423 (1975).
19. O. W. Schalm, N. C. Jain, and E. J. Carrol. *Veterinary Hematology,* 3rd ed., Lea and Febiger, Philadelphia, PA 1975.