Effect of Omeprazole on Diazepam Disposition in the Rat: *in Vitro* and *in Vivo* Studies

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Purpose. The inhibitory effects of omeprazole on diazepam metabolism *in vitro* and *in vivo* are compared in the rat.

Methods. 3-hydroxylation and N-demethylation of diazepam was investigated in the presence of a range of omeprazole concentrations (2-500μM) in hepatic microsomes and hepatocytes. Zero order infusions together with matched bolus doses of omeprazole were used to achieve a range of steady state plasma concentrations (10-50mg/L) and to study the diazepam-omeprazole interaction *in vivo*.

Results. The 3-hydroxlation pathway was more prone to inhibition (K_Is 108 ± 30 and 28 ± 11 μM in microsomes and hepatocytes, respectively) than the demethylation pathway (K_Is of 226 ± 76 and 59 ± 27 μM in microsomes and hepatocytes, respectively). In both *in vitro* systems, the mechanism of inhibition was competitive with Km/K_I ratios larger than 1 for the 3HDZ pathway and smaller than 1 for the NDZ pathway. There was an omeprazole concentration dependent decrease in diazepam clearance *in vivo* which could be modelled using a simple inhibition equation with a K_I of 57μM (19.8mg/L). In contrast there was no statistically significant change in the steady state volume of distribution for diazepam in the presence of omeprazole.

Conclusions. The *in vivo* K_I for the omeprazole: diazepam inhibition interaction shows closer agreement with the K_I values obtained in hepatocytes than with those observed in microsomes.

KEY WORDS: omeprazole; diazepam; inhibition of cytochrome P450; drug–drug interactions; *in vitro–in vivo* correlations.

INTRODUCTION

Several substituted imidazoles and benzimidazoles are known to inhibit cytochromes P450 (CYP) to a clinically important degree (1,2). We have investigated omeprazole, a substituted benzimidazole used widely for the treatment of gastrointestinal ulcers, as a CYP inhibitor under *in vitro* and *in vivo* conditions. In man and rat, omeprazole is rapidly distributed to extravascular sites, is more than 90% bound to plasma proteins and is rapidly and extensively metabolised by CYP to numerous compounds (3,4). The most substantial inhibitory effect of omeprazole observed to date concerns the elimination of diazepam and results in a 50% reduction in clearance (5,6).

The present study examines the use of *in vitro* screening systems for assessing the inhibitory potency of omeprazole in the rat. The effect of omeprazole on the kinetics of diazepam metabolism to form the primary metabolites 3-hydroxydiazepam (3HDZ) and nordiazepam (NDZ) has been investigated in both liver microsomes and freshly isolated hepatocytes. In addition, *in vivo* studies have been performed to determine diazepam clearance in animals receiving infusions of omeprazole, with matching loading doses, to achieve a range of steady state omeprazole concentrations. As diazepam has a high extraction ratio in rat, this drug was administered via the hepatic portal vein (IP) to avoid blood flow limitations and allow direct assessment of its intrinsic clearance (7).

MATERIALS AND METHODS

Chemicals. Omeprazole, 3HDZ (temazepam) and prazepam (internal standard) were gifts from Astra Pharmaceuticals Ltd (Mölndal, Sweden), Wyeth (Maidenhead, Berks, UK) and Warner & Co. (Pontypool, Gwent, UK) respectively. Diazepam and NDZ were purchased from Sigma (Poole, Dorset, UK). All other chemicals were obtained from either BDH (Lutterworth, Leics, UK) or Sigma (Poole, Dorset, UK).

Animals. Male Sprague-Dawley rats (250-300g), obtained from the Biological Sciences Unit, Medical School, University of Manchester, were housed and cannulated as described previously (8).

Preparation of rat liver microsomes and hepatocytes. The animals were sacrificed by cervical dislocation. Washed microsomal pellets were prepared by standard differential centrifugation techniques and resuspended in buffer (Sucrose 250mM, Trizma base 26mM, EDTA 5.4mM, pH 7.4) and stored at −80°C. Hepatocytes were prepared as described in a previous study (9). Cell viability was assessed by the Trypan Blue exclusion test and preparations in excess of 85% viability were used.

Microsomal diazepam incubation conditions. 0.1ml of KCl (1.15%), 0.4ml of microsomal solution (2mg of protein/ml in Trizma buffer 50mM; pH 7.4), 5μl of diazepam (in DMF) and 5μl of omeprazole (in DMF) or vehicle alone were preincubated at 37°C in a shaking water bath for 5 minutes. The reaction was started by adding 0.5ml of regenerating system (0.74mg $NADP^+$, 1.94mg isocitric acid, 0.5units isocitric dehydrogenase and 10μmoles magnesium sulphate in Trizma buffer 50mM) and stopped after 10 minutes by the addition of 20μl of NaOH (10M).

Hepatocyte incubation conditions. 5μl of diazepam (in DMF) and 5μl of omeprazole (in DMF) or vehicle alone were added to 2.5ml of William's Media E (pH 7.4) and incubated at 37°C in a shaking water bath for 5 minutes before starting the reaction with 0.5ml of hepatocytes (3×10^6 cells/ml). The reaction was terminated after 10 minutes by freezing the mixture in liquid nitrogen. Samples were then thawed and 0.5ml of each sample was incubated with 0.5ml of β-glucuronidase (200 units/ml in sodium acetate 60mM; pH 4.5) in a shaking water bath at 37°C for one hour.

Analysis of diazepam metabolites. HPLC was used to assay diazepam and its metabolites simultaneously, according to the method of Reilly *et al* (10). Aliquots (0.5ml) of incubation mixture were extracted with ethylacetate (5ml) after adding the internal standard (prazepam, 100μl, 70μM in methanol) and carbonate buffer (1ml, 100mM; pH 10) by rotary mixing for 25 minutes and centrifuging at 2000rpm for 10 minutes. The organic layer was evaporated to dryness

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under nitrogen at 50°C. The residue was reconstituted in mobile phase and 100μl injected via a Spectra Physics SP8780XR autosampler onto the HPLC system. The system comprised of a Hichrom Spherisorb S5 ODS2 250 × 5mm column, mobile phase of 65% methanol/35% water containing 0.02% triethylamine adjusted to pH 7.0 with phosphoric acid (delivered by a Waters 6000A pump at a flow rate of 1ml/min) and UV wavelength of 236nm (measured by an Applied Biosystems spectroflow 783). Diazepam and metabolite concentrations were determined by peak height ratio with respect to the internal standard (prazepam).

In vivo experiments. Initially, the basic pharmacokinetic parameters of omeprazole were assessed in a small number of preliminary experiments (n = 6). Omeprazole was administered in a mixture of propane-1,2-diol and polyethyleneglycol 400, 1:9 V/V, at doses of 15, 30 and 45mg/kg by a short intravenous (IV) infusion over 5 min via the jugular vein. Blood samples were taken over 90 min using the carotid artery. Convolution of a simple exponential disposition function with a zero order input function describes the plasma concentration-time data for both compounds [1] and this function was used in the nonlinear regression analysis (Siphar, Simed, Créteil, France).

$$C = R_0 \sum_{i=1}^{n} \left(\left[\frac{1 - e^{+\theta\lambda_i}}{-\lambda_i} \right] C_i' \, e^{-\lambda_i t} \right) \qquad (1)$$

where C is the concentration, λ is the rate constant, C′ = C/DOSE, t is the time, θ = t for t ≤ τ or θ = τ for t > τ (R_0 and τ are infusion rate and duration respectively) and i refers to the particular exponential function.

Mean values of clearance (CL) and volume of distribution at steady state (Vss), calculated (8) from the preliminary experiments, were used to design loading doses (in the form of a short 5 minute infusion) and infusion rates (over 90 min) for omeprazole in order to achieve various steady state concentrations (Css, Eqs [2] and [3]).

$$Loading\ Dose = Vss.Css \qquad (2)$$

$$Infusion\ Rate = CL.Css \qquad (3)$$

A Sage infusion pump model 355 (A.H. Horwell Ltd., London, England) was used to deliver the infusion into the jugular vein at a flow rate of 0.15-1.1 ml/hour. Diazepam was administered in a mixture of propane-1,2-diol, ethanol and 0.9% sodium chloride solution, 4:1:5 V/V/V, by a short infusion over 5 min at a dose of 5mg/kg into the hepatic portal vein via the lineal vein (IP) immediately after the omeprazole bolus and the start of the infusion. A range of omeprazole concentrations were achieved (10-50mg/L) and the clearance of diazepam assessed at each omeprazole concentration. Each experimental group also included a control infusion of the vehicle without omeprazole. Blood samples were collected over 90 minutes into Eppendorf tubes containing one or two drops of heparinised saline (5000 units/ml) from the carotid artery and plasma (200μl) separated for analysis as described above. Eq [1] was fitted to the data using the Siphar nonlinear regression programme.

Data analysis. Dixon plots were initially used to obtain initial estimates of the inhibitory constant (K_I). In all cases studied, the intersection of the lines lay above the x-axis (competitive inhibition). Furthermore, using nonlinear regression analysis, a model for competitive inhibition [4] was found to fit the data better than noncompetitive or uncompetitive models.

$$V = \frac{S.Vmax}{S + Km\left(1 + \frac{I}{K_I}\right)} \qquad (4)$$

where V is the observed rate of metabolite production (nmoles/min per mg of microsomal protein or per million cells), Vmax is the maximum velocity, Km is the substrate concentration at which the reaction is half of its maximal value, S is the substrate concentration and I is the inhibitor concentration.

Microsomal studies were carried out in two stages. First, detailed studies involving a range of substrate and of inhibitor concentrations were performed in one set of microsomes. Then, in subsequent experiments (with an additional 3 microsomal preparations) a single substrate concentration was used and Eq [5] employed to convert the ICx values (inhibitor concentration causing x% inhibition) to the corresponding K_I values.

$$K_I = \frac{ICx\left(\frac{100 - x}{x}\right)}{1 + \frac{S}{Km}} \qquad (5)$$

For hepatocyte studies a range of substate and inhibitor concentrations were used in 3 different rat preparations. *In vivo* data were analysed to estimate K_I by nonlinear regression (Siphar) using a simple inhibition model [6], described previously (8):

$$CL = \frac{CL_0}{1 + \frac{I_{ss}}{K_I}} \qquad (6)$$

where CL_0 is the clearance of the drug in the absence of the inhibitor and I_{ss} is the steady state plasma concentration of the inhibitor.

RESULTS

In vitro studies. Omeprazole inhibition of 3HDZ formation in microsomes was concentration dependent (Figure 1). Using an inhibitor concentration range of 6-500μM at three different concentrations of diazepam (8, 16 and 31μM), IC50s were found to increase as diazepam concentrations were raised. Qualitatively similar behaviour was observed with the NDZ pathway. K_I values of 163 and 303μM were calculated by nonlinear regression from the 3HDZ and NDZ inhibition data respectively. Also, the Km/K_I ratio for the 3HDZ pathway inhibition was larger than 1 whilst this ratio was smaller than 1 for the NDZ pathway inhibition (Table I).

In the hepatocyte studies, the effect of omeprazole on the formation of 3HDZ and NDZ was also concentration dependent, as illustrated in Figure 2 for the 3HDZ pathway in a typical preparation. Using an omeprazole concentration range of 2-100μM at three different diazepam concentrations

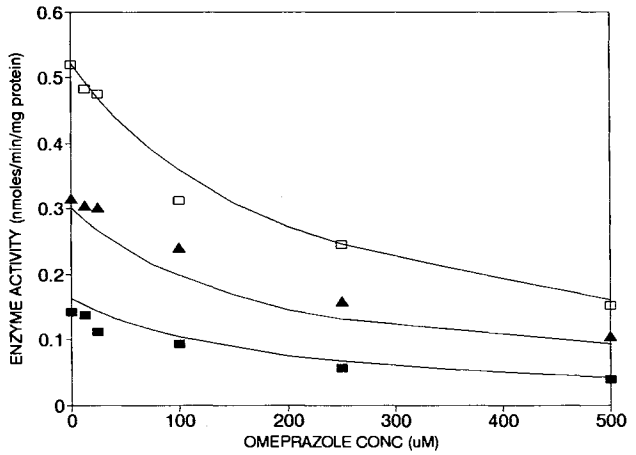

Fig. 1. Omeprazole inhibition of the 3-hydroxylation of diazepam by hepatic microsomes. Diazepam concentrations of 8 (■), 16 (▲) and 31 (□) μM were used and IC50 values of 190, 200 and 250μM, respectively, obtained. The solid lines are the nonlinear regression lines obtained by simultaneously fitting the 3 sets of data to Eq. 4.

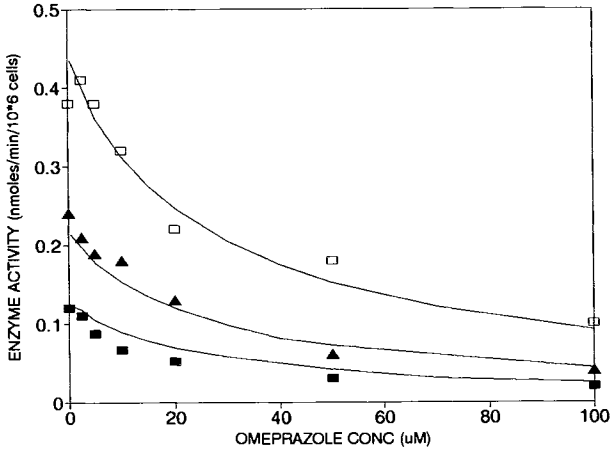

Fig. 2. Omeprazole inhibition of the 3-hydroxylation of diazepam by hepatocytes. Diazepam concentrations of 20 (■), 35 (▲) and 75 (□) μM were used and IC50 values of 29, 31, and 40 μM, respectively, obtained. The solid lines are the nonlinear regression lines obtained by simultaneously fitting the 3 sets of data to Eq. 4.

(20, 35 and 75μM), there was an increase in IC50s as the diazepam concentration was raised. K_I values of 28 and 59μM were obtained by nonlinear regression from the 3HDZ and NDZ inhibition data, respectively. Similar to microsomes, the 3HDZ pathway inhibition was associated with a smaller K_I and a Km/K_I ratio larger than 1 whilst the NDZ pathway inhibition was characterised by a larger K_I and a Km/K_I smaller than 1 (Table I).

In vivo studies. IV bolus dose studies (15, 30 and 45 mg/kg) were performed in order to estimate the primary pharmacokinetic parameters CL and Vss for omeprazole. Dose normalised AUCs indicated that CL was linear over this dose range (50 ± 17 ml/min/kg). In contrast, Vss was dose dependent; the lower two doses showing a statistically significant larger Vss than the higher dose ($p < 0.05$). Mean Vss ranged from 0.34 to 0.70 L/kg but there was no change in the MRT over the dose range (12 ± 3 min).

These data were then used to calculate the loading doses and infusion rates necessary to achieve a range of steady state concentrations of 10-50 mg/L using Eqs [2] and [3]. The relationship between the steady state concentration achieved and infusion rate for these animals (n = 16) was linear and provided a value for clearance of 30 ml/min/kg which is within the range of clearance estimates of the preliminary omeprazole studies (28-69 ml/min/kg).

Figure 3 illustrates diazepam disposition in the presence of two different omeprazole concentrations compared to a control animal. It is evident that the AUC for diazepam increases as the omeprazole steady state concentration increases, and therefore, diazepam clearance decreases. The relationship between diazepam clearance and steady state concentration of omeprazole in 26 animals can be described by a simple inhibition model (Figure 4). Eq [6] was used to obtain estimates of CL_0 and K_I. CL_0 obtained for diazepam (141 ml/min/kg) corresponds well with the clearance values estimated from preliminary studies (166 ± 83 ml/min/kg). Furthermore, the presence of omeprazole inhibits the clearance of diazepam with a K_I of 19.8 ± 1.2 mg/L (57μM). There is no statistically significant change in Vss (4.5 ± 1.5 L/kg) over the omeprazole range used.

Table I. Omeprazole Inhibition of Diazepam Metabolism in Hepatic Microsomes and Hepatocytes

	Pathway	
	3HDZ	NDZ
Microsomal K_I (μM)[a]	108 ± 30	226 ± 76
Hepatocyte K_I (μM)[b]	28 ± 11	59 ± 27
Microsomal Km/K_I[c]	1.1	0.1
Hepatocyte Km/K_I[d]	2.5	0.6

[a] Mean ± SD, n = 4.
[b] Mean ± SD, n = 3.
[c] Km values of 119 and 35 μM for the 3HDZ and NDZ pathways, respectively, taken from Zomorodi *et al* (11).
[d] Km values of 71 and 35 μM for the 3HDZ and NDZ pathways, respectively, taken from Zomorodi *et al* (11).

DISCUSSION

Omeprazole, a substituted benzimidazole, has the potential of forming a ligand with CYP through the lone pair of electrons on its imidazole nitrogen atom. We have demonstrated omeprazole to competitively inhibit two pathways of diazepam metabolism in both hepatic microsomes and hepatocytes. The K_I values for 3HDZ pathway inhibition were smaller than the respective K_I values for NDZ pathway inhibition in both *in vitro* systems. Therefore, omeprazole showed a selectivity towards inhibiting the 3HDZ pathway which was confirmed by the Km/K_I ratios, which were larger than 1 for the 3HDZ pathway and smaller than 1 for the NDZ pathway. These results are in agreement with our previous study on the diazepam-omeprazole interaction in human microsomes (12) which demonstrated the 3HDZ pathway to be susceptible to omeprazole inhibition with a Km/K_I ratio of 3. The NDZ pathway however, was minimally affected by this inhibitor (12). CYP3A is the major isoenzyme family involved in catalysing the 3HDZ pathway (10). Therefore, in

Table II. Disposition of Diazepam in the Presence and Absence of Omeprazole[a]

Group (N)	Infusion rate mg/min/kg	Omeprazole concentration Targeted mg/L	Omeprazole concentration Observed mg/L	Vss L/kg	CL ml/min/kg
A (5)	-	-	-	5.2 ± 1.4	166 ± 83
B (10)[b]	-	-	-	5.3 ± 1.5	139 ± 23
C (4)	0.3	10	9.1 ± 1.5	3.6 ± 0.4	97 ± 26
D (2)	0.6	20	14.4	3.7	70
E (5)	1.05	35	33.1 ± 5.4	5.3 ± 1.9	53 ± 17
F (5)	1.5	50	51.3 ± 7.3	4.3 ± 0.2	36 ± 22

[a] Diazepam dose 5 mg/kg, mean data ± SD.
[b] Received IV infusion of the vehicle.

hibition of CYP3A may be regarded as the major mechanism of the omeprazole-diazepam interaction in microsomes and hepatocytes.

Omeprazole proved to be highly cleared with a short half life of approximately 15 minutes in the rat. Therefore, it was necessary to use the steady state infusion approach for the inhibition studies, otherwise, the inhibitory effects of omeprazole administered in bolus form would have been too transient to enable the study of any possible interactions. The pharmacokinetic parameters reported here for omeprazole are in agreement with those recently reported by Watanabe *et al* (13). Previous diazepam studies (11) showed that 5mg/kg was an appropriate bolus dose for interaction studies since at this dose linear kinetics were operating.

As diazepam and omeprazole are both lipophilic and highly protein bound, co-administration of the two drugs could have caused competition for the protein binding sites with the consequence of dissociation of diazepam from the binding sites and an increase in its Vss. However, no significant increase was observed in Vss of diazepam in the presence of omeprazole. This is in agreement with clinical studies performed by Gugler & Jensen (5) and Andersson *et al* (6), where co-administration of omeprazole had no effect on the protein binding or the volume of distribution of diazepam in the subjects studied. The elimination half life of diazepam showed a progressive increase as the omeprazole concentration increased which is in keeping with the graded decrease in diazepam clearance. Estimates of Vss for diazepam obtained in the present study were also in agreement with those reported previously (11,14).

Eq [6] was found to adequately define the decrease in diazepam clearance as a function of omeprazole steady

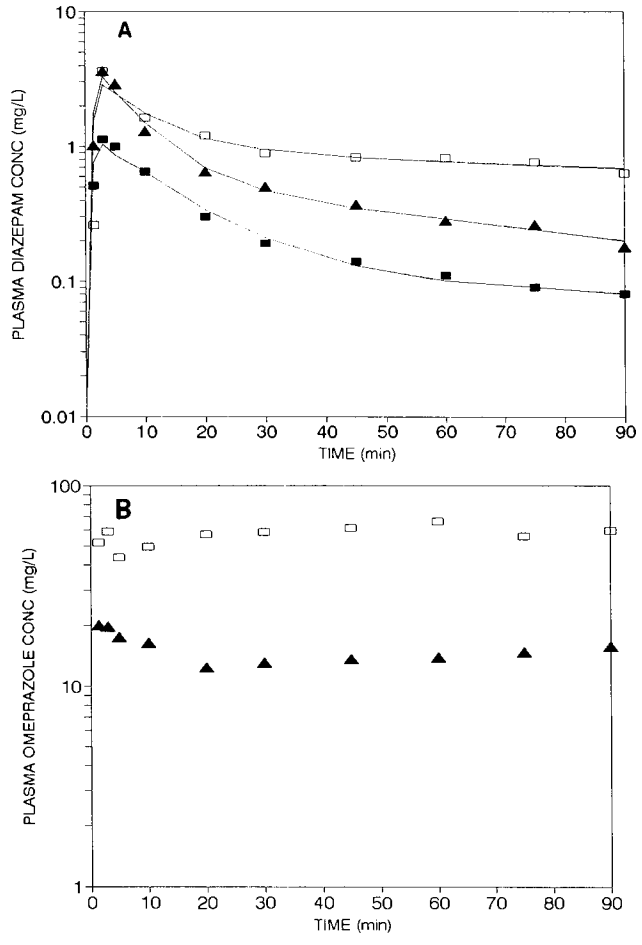

Fig. 3. (A) Diazepam plasma concentration-time profiles in the presence of two different omeprazole steady state concentrations, 13.2 (▲) and 55.3 (□) μM compared to a vehicle infusion (■). (B) Corresponding omeprazole concentration time profile during the infusions. Data shown from three representative animals.

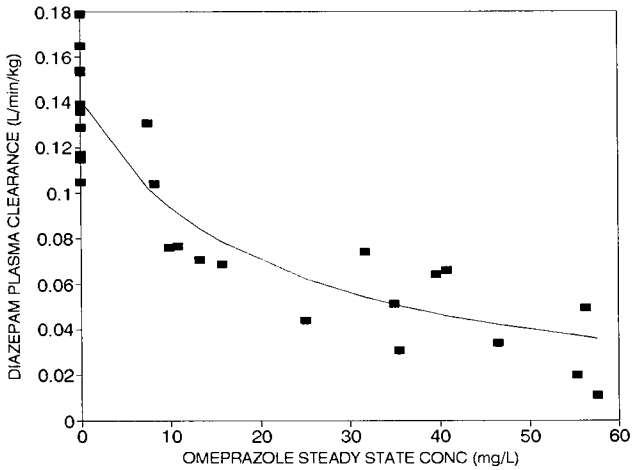

Fig. 4. Relationship between diazepam clearance and steady state concentration of omeprazole. Data from 26 animals which can be described by Eq [6] with a K_I of 19.8mg/L.

state concentration using a simple inhibition model. The K_I from these *in vivo* studies of 57μM is consistent with the K_Is obtained from the hepatocyte studies (28 and 59μM for the 3HDZ and NDZ pathways, respectively). It is however, an order of magnitude smaller than the microsomal values (108 & 226μM for the 3HDZ and NDZ pathways, respectively). Hepatocytes therefore, seem to provide a better *in vitro* model for the *in vivo* situation. This may be related to omeprazole's high tendency to bind to cytosolic proteins providing a cellular reservoir within the hepatocytes (15). The ability of intact hepatocytes to concentrate omeprazole more than microsomes may explain the lower K_I values obtained with the former *in vitro* system.

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REFERENCES

1. O. Pelkonen and J. Puurunen. The effect of cimetidine on *in vitro* and *in vivo* microsomal drug metabolism in the rat. *Biochem. Pharmac.* 29: 3075-3080 (1982).
2. M. Passanen, T. Taskinen, M. Iscan, E. A. Sotaniemi, M. Kairaluoma and O. Pelkonen. Inhibition of human hepatic and placental xenobiotic monooxygenases by imidazole antimycotics. *Biochem. Pharmac.* 37: 3861-3866 (1988).
3. I. Skånberg, K-J. Hoffmann and C. G. Regårdh. Pharmacokinetics and metabolism of omeprazole in animals. *Scan. J. Gastroenterol.* 21: 92-93 (1986).
4. K-J. Hoffmann. Identification of the main urinary metabolites of omeprazole after an oral dose to rats and dogs. *Drug Metab. Disp.* 14: 341-348 (1986).
5. R. Gugler and J. C. Jensen. Omeprazole inhibits oxidative drug metabolism. *Gastroenterol.* 89: 1235-1241 (1985).
6. T. Andersson, K. Andrén, C. Cederberg, G. Edvardsson, A. Heggelund and P. Lundborg. Effect of omeprazole and cimetidine on plasma diazepam levels. *Eur. J. Clin. Pharmac.* 39: 51-54 (1990).
7. G. R. Wilkinson. Clearance approaches in pharmacology. *Pharmac. Rev.* 39: 1-47 (1987).
8. J. D. Davies, L. Aarons and J. B. Houston. Relationship between enoxacin and ciprofloxacin plasma concentrations and theophylline disposition. *Pharm. Res.* 11: 1424-1428 (1994).
9. K. A. Hayes, B. Brennan, R. Chenery and J. B. Houston. *In vivo* disposition of caffeine predicted from hepatic microsomal and hepatocyte data. *Drug Metab. Disp.* 23: 349-353 (1995).
10. P. E. B. Reilly, D. A. Thompson, S. R. Mason and W. D. Hooper. Cytochrome P450IIIA enzymes in rat liver microsomes: involvement in C3-hydroxylation of diazepam and nordazepam but not N-delakylation of diazepam and temazepam. *Mol. Pharmac.* 37: 767-774 (1990).
11. K. Zomorodi, D. J. Carlile and J. B. Houston. Kinetics of diazepam metabolism in rat hepatic microsomes and hepatocytes and their use in predicting *in vivo* hepatic clearance. *Xenobiotica,* in press.
12. K. Zomorodi and J. B. Houston. Diazepam-Omeprazole inhibition interaction: An *in vitro* investigation using human liver microsomes. Submitted for publication.
13. K. Watanabe, K. Furuno, K. Eto, R. Oishi and Y. Gomita. First-pass metabolism of omeprazole in rats. *J. Pharm. Sci.* 83: 1131-1134 (1994).
14. U. Klotz, K. H. Antonin and P. R. Beick. Pharmacokinetics and plasma binding of diazepam in man, dog, rabbit, guinea pig and rat. *J. Pharmac. Exp. Therap.* 199: 67-73 (1976).
15. R. B. Sewell, C. W. Brook, G. W. Mihaly, D. J. Morgan and R. A. Smallwood. Intracellular binding is an important determinant of the avid hepatic uptake of the high clearance drug omeprazole. *Biochem. Pharmac.* 48: 846-849 (1994).