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Interaction of cimetidine and ranitidine with the FAD monooxygenase in pig liver microsomes

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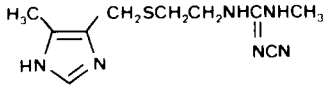
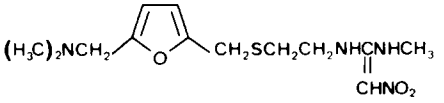
The purified flavin monooxygenase (E.C. 1.14 13.8) from pig liver catalyzes the oxygenation of nucleophilic nitrogen and sulphur compounds and in particular secondary and tertiary amines and divalent sulphur-containing compounds to form the corresponding hydroxylamines and nitrones, amine oxides and sulphoxides. Both cimetidine and ranitidine contain divalent sulphur, each also contains two secondary amine groups and in addition, ranitidine also contains a tertiary amine group (see Fig. 1). About 10% of an administered dose of cimetidine is recovered in urine as its sulphoxide [1]. Both the N-oxide and S-oxide of ranitidine have been identified in urine after oral or intravenous administration of ranitidine and account for approximately 5 and 2% respectively of the administered dose [2].



cimetidine



ranitidine

Fig. 1. Formulae of cimetidine and ranitidine.

Previous studies [3] have indicated that in comparison with cimetidine, ranitidine is far less potent at interacting with the cytochrome P-450 system although both form ligand complexes with rat liver microsomes [4]. The studies reported here were designed to examine the *in vitro* interaction of cimetidine and ranitidine with the pig hepatic flavin monooxygenase, both to compare their abilities to act as substrates for this system and to determine whether they could competitively inhibit the oxygenation of methimazole, a substrate which, at concentrations below 2 mM, has been reported to be metabolized solely by this enzyme system [5].

Materials and methods

Materials. Methimazole, *n*-octylamine, ADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Sigma (Poole, Dorset, U.K.). Zantac injection (ranitidine hydrochloride, 10 mg/ml) was used as the solution or as a lyophilized powder. Cimetidine hydrochloride was obtained from Smith Kline & French Research Ltd. Tricine buffer (*N*-[Tris(hydroxymethyl)-methyl]-glycine) was obtained from Aldrich Chemical Company (Dorset, U.K.).

Preparation of porcine microsomes. Pig livers were obtained from Playle and Sons, Butchers (Bassingbourn, Royston). Six-month-old female pigs were chosen and livers were removed within 5 min of death. Microsomes were prepared essentially as described by Ziegler and Poulsen [6] and protein measured by the method of Lowry *et al.* [7].

Assay of flavin monooxygenase activity. Flavin monooxygenase activity was assayed in pig liver microsomes by measuring oxygen consumption in the presence or absence of substrate using an oxygen electrode (Rank Bros., Bottisham, Cambridge). The oxygen electron incorporated a 2 ml Perspex incubation chamber surrounded by a water jacket connected to a Churchill pump to maintain a constant temperature of 37°. A bubble cap comprised the top of the incubation chamber and solutions could be introduced into the chamber via a hole in the centre of the cap. Changes in oxygen consumption were recorded on a Perkin Elmer 56 chart recorder. The assay mixture contained (final concentrations in 2 ml) NADP (0.25 mM), glucose-6-phosphate (2.0 mM), glucose-6-phosphate dehydrogenase (0.7–1.0 units), Tricine buffer (0.1 M, pH 8.35–8.40), *n*-octylamine (3 mM) and pig liver microsomes (200 μl, 4.71 mg protein). Varying concentrations of methimazole, cimetidine and ranitidine were used as substrates.

Calibration curve. A calibration curve was constructed by adding known concentrations of phenylhydrazine hydrochloride (using a stock solution of 10 mM in water) to 2 ml potassium ferricyanide (1.0 mM) in potassium phosphate buffer (50 mM) according to the method of Misra and Fridovich [8]. Increasing concentrations of phenylhydrazine resulted in increasing oxygen consumption which was expressed in terms of distance travelled on the chart paper. A calibration curve was plotted of mm chart paper against oxygen consumption (nmoles/min). Values obtained in the assay resulted in a gradient on the chart paper of O_2 consumption/min and activity was finally expressed in terms of protein concentration as nmoles O_2 consumed/min/mg protein.

Treatment of data. Kinetic data were analysed by various computer curve fitting routines. The Michaelis–Menten kinetic parameters, K_m and V_{max}, were determined by a rectangular hyperbolae iterative fit programme from Wilkinson [9]. The ALLFIT programme (NICHD, NIH, Bethesda, U.S.A.) was utilized to estimate IC_{50} values.

Results and discussion

The effect of substrate concentration on oxygen consumption by pig liver microsomes when methimazole, cimetidine and ranitidine are incubated singly as substrates in the flavin monooxygenase assay system are shown in Fig 2. Kinetic estimates were repeated on several occasions throughout the study period and the values obtained are shown in Table 1. The K_m values for cimetidine and ranitidine are 33 and 44 times greater respectively than the value for methimazole, suggesting that neither of the two histamine H_2-antagonists have such a high affinity for the flavin monooxygenase as does methimazole. V_{max} values for cimetidine and ranitidine are approximately one third of the value obtained for methimazole. Although K_m values for cimetidine and ranitidine are high they are comparable with values obtained for antipyrine (900 μM) [10] in pig liver microsomes *in vitro*.

The assay was carried out at pH 8.4 to minimize interference by other microsomal oxygenases and also because this pH has been reported to be the pH optimum of the flavin monooxygenase [5]. In addition all substrate-induced oxygen consumption was measured in the presence of *n*-octylamine, which is an inhibitor of cytochrome P_{450}

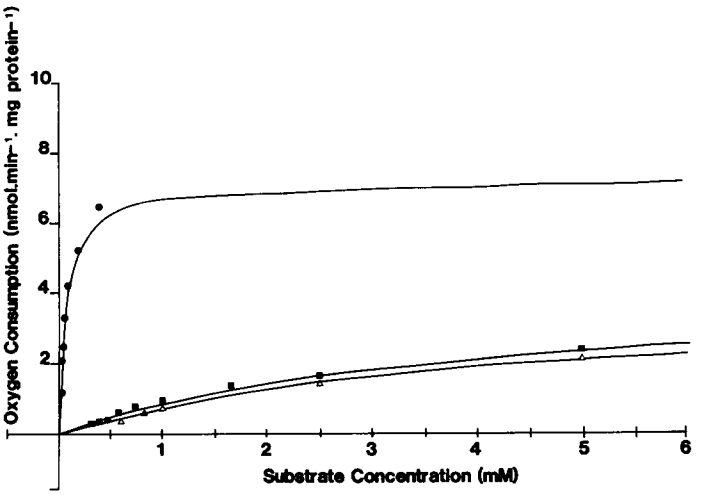


Fig. 2. Flavin monooxygenase activity in pig liver microsomes with methimazole, cimetidine or ranitidine as substrate. ● methimazole, ■ cimetidine, △ ranitidine.

Table 1. K_m and V_{max} estimates for methimazole, cimetidine and ranitidine (means ± S.E.M)

Substrate	N	K_m(μM)	V_{max}(nmoles/min/mg protein)
Methimazole	2	58.9 ± 7.0 (range 51.9–65.9)	8.69 ± 1.69 (range 6.99–10.38)
Cimetidine	4	1928.0 ± 522.0 (range 1020–3430)	3.34 ± 0.20 (range 2.75–3.60)
Ranitidine	4	2600.0 ± 790.0 (range 350–3940)	3.15 ± 0.48 (range 1.91–4.13)

mediated oxidations. However, despite these precautions, the measurement of enzyme activity by stimulation of oxygen consumption is relatively unspecific. Oxygen consumption may be the result of numerous processes including metabolism of endogenous substrates by both flavin and cytochrome P_{450} monooxygenases and by various peroxidative mechanisms. Two further experiments were therefore carried out to test the hypothesis that cimetidine and ranitidine are metabolized by the same enzyme system as methimazole.

Table 2 shows the results of an experiment in which cimetidine or ranitidine was added in combination with methimazole at their K_m concentrations. If the substrates are metabolized by independent enzyme systems, then mixing at K_m concentrations of both substrates will result in a total rate of reaction (V_T) which is exactly the sum of the two rates determined separately [equation (1)]

$$V_T = V^A + V^B.$$

Assuming Michaelis–Menten kinetics at K_m, for separate enzymes:

$$V_T = \frac{V^A_{max}}{2} + \frac{V^B_{max}}{2}. \quad (1)$$

Conversely, if the drugs are metabolized by the same enzyme system and each inhibits the other in a competitive manner, then the total rate on mixing at K_m concentrations of both substrates will be two thirds of the rate observed with separate enzymes [equation (2)]; assuming a competitive interactions, this is derived as follows

for each substrate

$$V^A = \frac{V^A_{max} \cdot [A]}{[A] + K^A_m(1 + \frac{[B]}{K^B_m})}, \quad V^B = \frac{V^B_{max} \cdot [B]}{[B] + K^B_m\left(1 + \frac{[A]}{K^A_m}\right)}$$

at K_m concentrations,

$$V_T = \frac{V^A_{max} \cdot K^A_m}{K^A_m + 2K^A_m} + \frac{V^B_{max} \cdot K^B_m}{K^B_m + 2K^B_m}$$

$$= \frac{V^A_{max}}{3} + \frac{V^B_{max}}{3}$$

$$V_T = \tfrac{2}{3}\left[\frac{V^A_{max}}{2} + \frac{V^B_{max}}{2}\right]. \quad (2)$$

Table 2 shows that the observed value of oxygen consumption in the presence of methimazole was 78.5% of the predicted rate for separate enzymes with cimetidine and 81.9% of the predicted rate for separate enzymes with ranitidine. The spread of the observed values for cimetidine + methimazole and ranitidine + methimazole suggest that they are more likely to be metabolized by the same enzyme system than by independent systems. However, considerable variability was noted in our determinations of oxygen consumption and therefore a more rigorous approach was adopted.

If it is assumed that these drugs interact at the same site on the enzyme, that is at the catalytic site, then it is possible to construct equations which describe the overall rate of reaction (V_T) when a fixed concentration of methimazole is present initially, and the concentration of either cimetidine or ranitidine is increased in a step-wise manner [equation (3)]. When the activity of a good substrate for the enzyme (such as methimazole) in inhibited by a weak substrate such as cimetidine or ranitidine, the following kinetics apply assuming each substrate inhibits the other in a competitive manner

Table 2. A comparison of observed and predicted rates of oxygen consumption by pig liver microsomes with methimazole, cimetidine and ranitidine at K_m concentrations both alone and on mixing

Compound	Oxygen consumption (nmoles/min/mg protein) (Mean ± S.E.M.) N = minimum of 2 Predicted independent*	Dependent†	Observed
Methimazole	4.34‡		4.66 ± 0.00
Cimetidine	1.67‡		1.38 ± 0.02
Ranitidine	1.57‡		1.59 ± 0.21
Methimazole + cimetidine	6.01	4.01	4.72 ± 0.81 (range 3.1–5.8)
Methimazole + ranitidine	5.91	3.94	4.84 ± 0.52 (range 4.3–5.4)

* Independent = separate enzyme systems metabolizing the various substrates.

† Dependent = the same enzyme system metabolizing the various substrates.

‡ Values calculated using $V_{max}/2$ from Table 1.

$$V_T = V^A + V^B$$

$$V_T = \frac{V^A_{max}.[A]}{[A] + K^A_m\left(1 + \frac{[B]}{K^B_m}\right)} + \frac{V^B_{max}.[B]}{[B] + K^B_m\left(1 + \frac{[A]}{K^A_m}\right)}$$

$$= \frac{V^A_{max}.[A]}{K^A_m + [A] + \frac{K^A_m}{K^B_m}.[B]} + \frac{V^B_{max}.[B]}{\frac{K^B_m}{K^A_m}(K^A_m + [A]) + [B]}$$

Multiply top and bottom of left hand term by $\frac{K^B_m}{K^A_m}$

$$= \frac{\frac{V^A_{max}.[A].K^B_m}{K^A_m}}{\frac{K^B_m}{K^A_m}(K^A_m + [A]) + [B]} + \frac{V^B_{max}.[B]}{\frac{K^B_m}{K^A_m}(K^A_m + [A]) + [B]}$$

$$= \frac{\frac{V^A_{max}.[A].K^B_m}{K^A_m} + V^B_{max}.[B]}{\frac{K^B_m}{K^A_m}(k^A_m + [A]) + [B]}.$$

This equation rearranges to

$$V_T + \left[\frac{a/b - V^B_{max}}{1 + [B]/b}\right] + V^B_{max} \qquad (3)$$

where $a = \frac{V^A_{max}.[A].K^B_m}{K^A_m}$, $b = \frac{K^B_m(K^A_m + [A])}{K^A_m}$;

A = methimazole and B = test compound.

In this experiment, one concentration of methimazole (equal to $5 \times K_m$ concentration = 294.5 μM) was chosen, so this will result in a rate of oxygen consumption of 83.3% of V_{max}. After background oxygen consumption was measured in the presence of enzyme alone, the stimulation of oxygen consumption by 294.5 μM methimazole was measured in the absence of cimetidine or ranitidine and then after preincubation with increasing concentrations of cimetidine or ranitidine. It was observed that as increasing concentrations of cimetidine or ranitidine were introduced into the incubation, total oxygen consumption decreased from the oxygen consumption due to methimazole alone towards the V_{max} oxygen consumption for the test compound due to competition between cimetidine or ranitidine with methimazole for enzyme active sites. Preincubation with cimetidine or ranitidine provided another estimate of K_m and V_{max} values for oxygen consumption by cimetidine and ranitidine. By using these values it is possible to predict, using equation (3), the curves for total oxygen consumption assuming a K_m for methimazole of 58.9 μM. Figures 3A and 3B compare experimentally determined values and predicted values for cimetidine and ranitidine respectively. IC_{50} values for the inhibition of methimazole metabolism by cimetidine (Fig. 3A), calculated by the ALLFIT programme, gave a predicted value of 8.9 mM and an experimentally determined value of 7.5 mM.

This close agreement suggests that cimetidine does indeed interact with the catalytic site of the flavin monooxygenase. The predicted IC_{50} value for the inhibition of methimazole by ranitidine was 16.7 mM compared with the experimentally determined value of 11.1 mM. It is apparent from Fig. 3B that the experimentally determined points for total oxygen consumption due to ranitidine and methimazole do not closely fit the predicted values as ranitidine appears to decrease total oxygen consumption more than predicted; this may result from the scatter of the experimental points.

These results suggest that both cimetidine and ranitidine are substrates for the flavin monooxygenase. Consequently this enzyme system may be important in the metabolism of both drugs and as a site of potentially important drug interactions.

In summary, in this study we have compared the *in vitro* interaction of cimetidine and ranitidine with pig hepatic microsomal flavin monooxygenase. Flavin monooxygenase activity in pig liver microsomes was measured by the stimulation of oxygen consumption caused by methimazole, a specific substrate for the flavin monooxygenase. Methimazole exhibited a K_m of 58.9 μM whereas cimetidine exhibited a K_m of 1.93 mM and ranitidine 2.60 mM. Mixing experiments suggested that cimetidine and ranitidine interact with the same enzyme system as methimazole.

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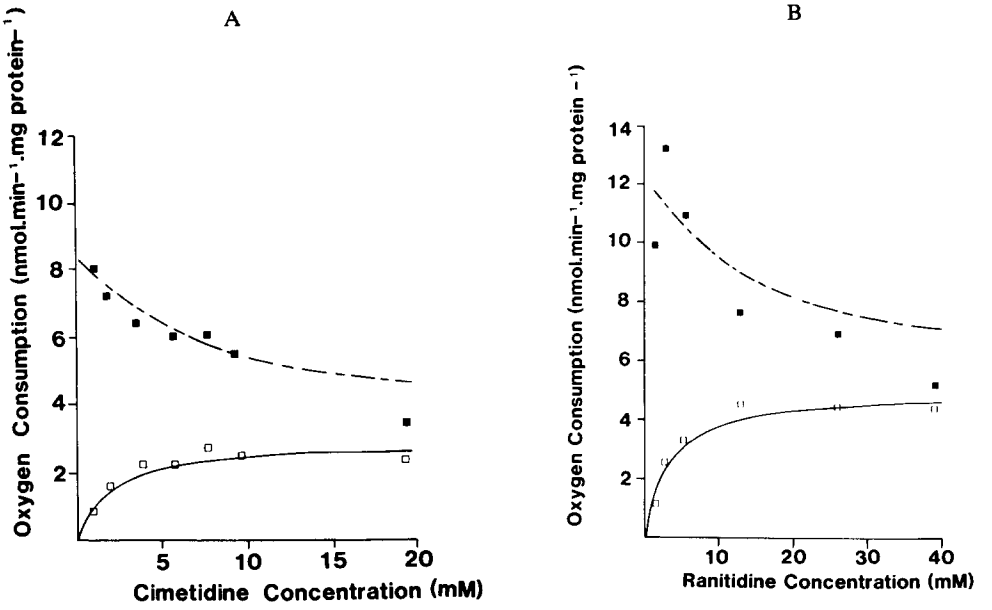

Fig. 3. Total oxygen consumption as a consequence of varying cimetidine or ranitidine concentation in the presence and absence of a fixed methimazole concentration (294.5 μM): (A) Cimetidine plus methimazole: ——— predicted, ■ ■ observed; Cimetidine alone: □ □; (B) Ranitidine plus methimazole: ——— predicted, ■ ■ observed; Ranitidine alone: □ □.

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Natural occurrence of *trans*-gamma hydroxycrotonic acid in rat brain

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γ-Hydroxybutyric acid (GHB) is a naturally occurring substance endowed with potent neuropharmacological and neurophysiological properties [1]. Recently, much evidence has accumulated delineating its role as a possible neurotransmitter (for a review see [2]). While the synthetic pathway of GHB is well established [3, 4], the mechanism of its degradation remains controversial. Its rapid breakdown via the Krebs cycle has been considered [5]. The generation of *trans*-γ-hydroxycrotonic acid (*trans*-HCA) following β-oxidation has also been suggested [6]. Moreover, the existence of GHB and *trans*-HCA in renal tissue has been recently reported [7]. We demonstrated the presence of *trans*-HCA in brain employing capillary gas chromatography, coupled with chemical ionization mass spectrometry, using ammonia as reagent gas.

Materials and methods

Wistar rats, weighing 120–150 g, were stunned by a blow to the head and decapitated. In less than 2 min, the brain was homogenized with the internal standard (β-methyl