FACTORS INVOLVED IN THE N-OXIDATION OF ISOMERIC AROMATIC DIAZINES BY MICROSOMAL PREPARATIONS

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SUMMARY

Factors affecting the metabolism of isomeric aromatic diazines *in vitro* were studied and the conditions which allow maximal metabolism established. The N-oxidation of isomeric diazines was linear with respect to microsomal concentration up to 0.5 g original liver weight per ml using a rabbit microsomal suspension. N-Oxidation was also linear up to between 20-30 minutes depending on substrate studied. The rate of N-oxide production increased with increase in substrate concentration up to about 10 μmol/incubate, after which the rate declined. By using the data obtained the appropriate kinetic factors, K_m and V_{max}, for the N-oxidation of pyrazine, pyrimidine and pyridazine by rabbit hepatic microsomal preparations were calculated. Spectral binding constants, K_s, of substrates to cytochrome P450 were also calculated and appeared to be related to the K_m values.

KEY WORDS

diazines, enzyme concentration, incubation time, substrate concentration, enzyme kinetics, K_m, V_{max}, K_s, *in vitro* metabolism

INTRODUCTION

Metabolic N-oxidation of a wide variety of nitrogenous compounds has received increasing attention during the past twenty years. Some of the reasons for this increased interest are the discovery of the natural occurrence of N-oxidised products in plant and animal tissues /1,2/, the

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use of certain N-oxy compounds (e.g. Minoxidil [2,4-diamino-6-piperidinopyrimidine-3-N-oxide] in therapy /3-5/, and the observation that numerous drugs (e.g. trimethoprim, pyrimethamine, metoprine, aprinocid) and other xenobiotics undergo transformation to the corresponding N-oxidised derivatives after administration to various species /6-10/. Several pyridazine-N-oxides (including cinnoline N-oxides) have been prepared as potential antitumour agents. Various 4-nitropyridazine-1-oxides have been tested for activity against rat ascites hepatoma /11/.

We have recently initiated a study of heteroaromatic N-oxidation using isomeric aromatic diazines (e.g. pyrazine, pyrimidine and pyridazine), in order to establish the occurrence of diazine-N-oxidation and to elucidate the enzymology of the processes involved /12/. Studies *in vitro* using a sensitive HPLC method and mass spectrometry showed that mono oxidation at a diazine nitrogen appears to be a general metabolic route for isomeric diazines /13/.

Many factors affect the rate and pathway of metabolism of drug substrates *in vitro*. These factors include source of enzyme preparation (species differences), enzyme concentration, time of incubation and substrate concentration. The effect of various factors on the C- and N-oxidation of a series of 3-substituted /14,15/ and 2- and 4-substituted pyridines /16/ and 2,4-diamino-6-substituted pyrimidines /17/ has been studied previously. The *in vitro* enzymic oxidation of aliphatic azo compounds (-N=N-) to N-oxides was also demonstrated by Koh and Gorrod /18,19/. The interaction of a substrate with a microsomal preparation is known to give rise to characteristic difference spectra of various types /20,21/. In some cases a correlation between the spectral binding constant K_s and K_m has been observed /20,21/ although this correlation may be fortuitous /21,22/.

A study of factors influencing the *in vitro* metabolism of diazines enabled the establishment of optimal incubation conditions and allowed enzyme kinetic constants involved in their biotransformation to be obtained. We now report the effects of various factors on the N-oxidation of isomeric diazines *in vitro* and the relationship between the physico-chemical properties (logP and pKa) and spectral binding constant (K_s) with the enzyme kinetic constants (K_m and V_{max}) for this metabolic oxidation.

MATERIALS AND METHODS

Materials

The preparation of isomeric diazine N-oxides was as previously described /12/. Pyrazine, pyrimidine and pyridazine were obtained from Aldrich Chemical Company (Dorset, U.K.). Phenobarbitone (sodium salt) was supplied by May and Baker Ltd. (Buckinghamshire, U.K.). Glucose-6-phosphate and nicotinamide adenine dinucleotide phosphate ($NADP^+$) were purchased from Sigma Chemical Company (Dorset, U.K.); glucose-6-phosphate-dehydrogenase suspension grade II from yeast (140 U/mg) was supplied by Boehringer Mannheim Corporation (Sussex, U.K.). Other solvents and chemicals were obtained from British Drug Houses Ltd. (Dorset, U.K.). 6-Chloro-2,4-diamino-pyrimidine was obtained from Aldrich Chemical Company (Dorset, U.K.). Male New Zealand rabbits (2-2.5 kg) were supplied by King's College London Biological Services Facility.

Methods

The HPLC isocratic system comprised a LDC Analytical constaMetric 3200 solvent delivery system, a Rheodyne syringe loading sample injector valve (model 7125) fitted with a 20 µl sample loop (Thames Chromatography, Berks, U.K.), a LDC spectromonitor 3100 variable wavelength UV detector and a LDC model 4000 computing integrator (LDC Analytical, Staffordshire, U.K.). The HPLC column used (Spherisorb ODS2 5 µm, 25 cm length 4.6 mm i.d.) was coupled to guard column (packed with Whatman pellicular ODS). Ammonium acetate (0.01 M) and triethylamine (100:0.3 v/v, pH 7.5) was used as mobile phase with a flow rate of 1 ml/min. A wavelength of 252 nm was used for detection.

Male rabbits were used as enzyme source. Pretreatment of rabbits was carried out using phenobarbitone (PB) (80 mg/kg i.p. in 0.5 ml isotonic sahne once daily for 3 days). The animals were sacrificed 24 h after the last injection. Hepatic microsomes were prepared at 0°C using the calcium chloride precipitation method /23/. Incubations were carried out in a shaking water bath at 37°C using a standard cofactor solution at pH 7.4. Cofactors consisting of $NADP^+$ (2 µmol), glucose-6-phosphate (10 µmol), glucose-6-phosphate-dehydrogenase (1 unit), $MgCl_2$ (20 µmol) in phosphate buffer (2 ml, 0.2 M, pH 7.4) were preincubated for 5 minutes before the addition of microsomes and

substrate. In certain experiments the incubation time or the concentration of substrate or amount of enzyme preparation was varied.

The incubation was terminated by transferring the incubation flasks onto crushed ice, the internal standard (6-chloro-2,4-diamino-pyrimidine, 50 nmol in 50 µl of methanol) was added to the incubate prior to the addition of extraction solvent. Incubation mixtures were then extracted by the addition of acetonitrile (5 ml) in the presence of sodium chloride (2 g). The incubates were extracted further with 3x5 ml of acetonitrile and the combined organic extracts were evaporated to dryness using a stream of N_2 at 40°C. The dried extract was reconstituted in methanol (100 µl) for injection onto the HPLC system.

Metabolic calibration curves were constructed using phosphate buffer (2 ml) and heat inactivated microsomal preparation (1 ml) spiked with known quantities of the metabolites (25-200 nmol) and internal standard (6-chloro-2,4-diaminopyrimidine, 50 nmol in 50 µl methanol). The above method was carried out for extraction and analysis of metabolites. The peak area ratios of metabolite/internal standard were plotted against amount of potential metabolites.

The effect of variation of protein concentrations

The incubation mixture consisted of cofactor solution (2 ml in phosphate buffer, pH 7.4, 0.2 M), isomeric diazines (5 µmoles in 50 µl water) and resuspended microsomes (1 ml with varying concentrations of microsomes). Incubations were carried out as described above. N-Oxidation products formed metabolically were quantified using the HPLC analytical method described above using previously prepared calibration curves.

The effect of variation of substrate concentration

Incubates were prepared containing cofactor solution (2 ml), substrate (1-20 µmoles per flask) in 50 µl water and 1 ml of hepatic microsomes from rabbit equivalent to 0.5 g per ml original liver weight. Incubations were carried out for 30 minutes at 37°C and metabolically produced N-oxides were quantified as above.

The effect of variation of incubation time

Incubations were performed for various times (0-60 minutes) using 10 μmoles of substrate at 37°C with the standard cofactor solution and resuspended microsomes (1 ml equivalent to 0.5 g original weight of liver). Metabolically formed N-oxides from isomeric diazines were quantified using the HPLC analytical method as above.

Determination of apparent K_m and V_{max} values for the *in vitro* N-oxidation of isomeric aromatic diazines

Having obtained the initial data from the effect of substrate concentration on the N-oxidation of isomeric aromatic diazines, the results were used to determine apparent K_m and V_{max} values by Lineweaver-Burk (LB) reciprocal plot of 1/V versus 1/[S] and the Hanes-Woolf (HW) plot of [S]/V versus [S].

The pKa(calc) and logP(calc) values for each isomeric diazine were obtained using computer program pKalc version 2.0/C and prologP version 4.2/D provided by CompuDrug Chem. Ltd., Hungary (1994). The pKa(exp) and logP(exp) data for isomeric aromatic diazines have been determined previously /24-32/ and are recorded in Table 2.

Spectral binding of isomeric aromatic diazines to hepatic microsomal cytochrome P450

The spectral binding constant for isomeric diazines was determined by the method of Schenkman *et al.* /20/. Washed PB-pretreated rabbit microsomes, 1 ml (equivalent to 0.5 g original liver) were diluted to 10 ml with tris/KCl buffer (pH 7.4) to give a concentration of approximately 1 mg protein/ml. Diluted microsomes (2.5 ml) were placed in each of a pair of matched quartz cells. The baseline was run between 350 nm and 550 nm using a Uvikon-Kontron 860 spectrophotometer (Herts, U.K.). Various amounts of isomeric diazines (10-25 μl) in water were added to the test cell and similar volumes of water were added to the reference cell. The difference spectra was then run between 350 nm and 550 nm. Using this procedure spectra were obtained for pyrazine, pyrimidine and pyridazine at between 0.75 mM and 8.61 mM concentrations and the changes in absorbance (ΔAbs) were determined at the maxima and minima wavelengths. All determinations were carried out at 20±1°C (Table 3).

RESULTS AND DISCUSSION

Under the reverse phase HPLC conditions described before, the retention time (Rt) values for isomeric diazine and their mono-N-oxides were well separated. The values are given in Table 1.

The results of studies investigating the effect of protein concentration, incubation time and substrate concentration on the *in vitro* N-oxidation of isomeric aromatic diazines are shown in Figures 1, 2 and 3 respectively. The N-oxidation of isomeric diazines was linear with respect to microsomal concentration up to 0.5 g original liver weight per incubate using a rabbit microsomal suspension (Fig. 1), and therefore all further incubations were carried out using microsomal preparations of this concentration.

The effect of variation of incubation on the *in vitro* N-oxidation of isomeric diazines is shown in Figure 2. The N-oxidation was linear up to between 20-30 minutes depending on substrate, after which it plateaued. This might indicate that during the metabolic reactions the enzyme had been inactivated in some way or was deprived of co-factors.

The effect of variation of substrate concentration on the *in vitro* N-oxidation of diazines by rabbit liver microsomes is illustrated in Figure 3. The bases all remain essentially (>99.99%) in the unionised form at

TABLE 1

HPLC separation of isomeric aromatic diazines and their N-oxides

Compounds	Retention time(min)
Pyrazine	9.7
Pyrazine N-oxide	4.4
Pyrimidine	8.7
Pyrimidine N-oxide	4.1
Pyridazine	7.3
Pyridazine N-oxide	4.3

Apparatus and stationary phase as described in text.
Mobile phase, ammonium acetate (0.01 M): triethylamine (100:0.3 v/v, pH 7.5), flow rate, 1 ml/min.

TABLE 2

Physico-chemical parameters (logP and pKa) of isomeric diazines

Parameters	Pyrazine	Pyrimidine	Pyridazine
logP(exp)[a]	-0.22, -0.23	-0.40	-0.65, -0.72
logP(calc)[b]	-0.40	-0.40	-0.61
logP(calc)[c]	-0.23	-0.36	-0.57
logP(calc)[d]	-0.37	-0.40	-0.72
pKa(exp)[e]	0.65	1.23, 1.31	2.24, 2.33
pKa(calc)[b]	0.80	1.24	2.25

[a] Ref. /30/
[b] Computer programme pKalc 2.0/C and prologP 4.2/D provided by Compudrug Chem. Ltd., Hungary
[c] Lewis et al. /31/
[d] Ref. /30/
[e] Compiled from references /24-32/

TABLE 3

Spectral interactions of isomeric aromatic diazines with PB-induced male rabbit liver microsomal cytochrome P450

Compounds	Absorption		Ks
	λmax(nm)	λmin(nm)	(M)
Pyrazine	423	388	5.9×10^{-3}
Pyrimidine	422	393	7.0×10^{-3}
Pyridazine	426	392	3.5×10^{-3}

The Ks values were obtained by Hanes-Woolf plot.
Protein concentration of microsomes: 1 mg/ml.

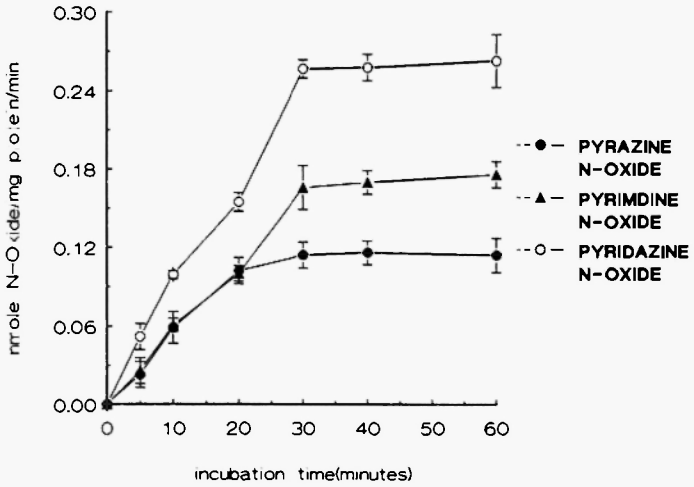

Fig. 1: The effect of microsomal protein concentration on isomeric diazine-N-oxidation using male rabbit hepatic microsomes. The results are an average of duplicate determinations from two rabbits differing by not more than ±10%.

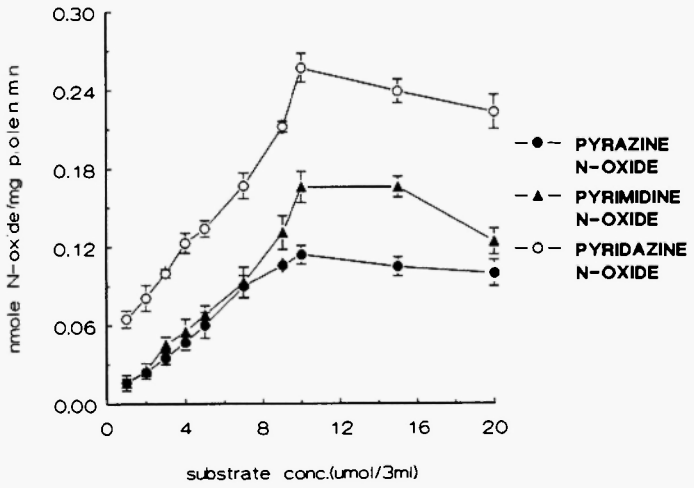

Fig. 2: The effect of incubation time on the formation of isomeric diazine-mono-N-oxides using male rabbit hepatic microsomes. The results are an average of duplicate determinations from two rabbits differing by not more than ±10%.

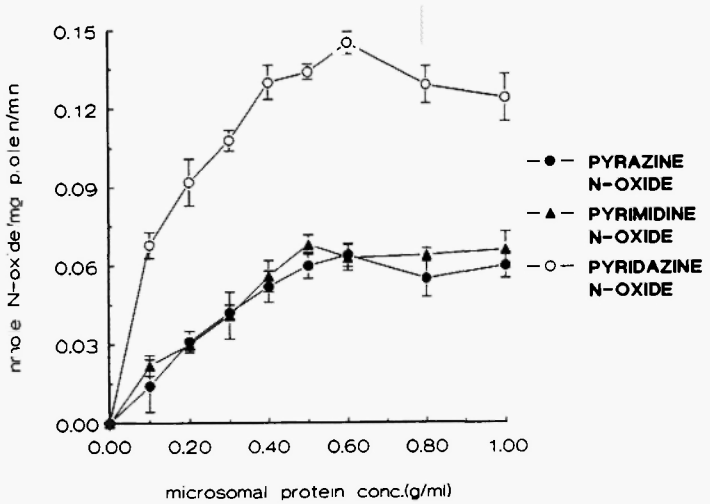

Fig. 3: The effect of substrate concentration on the formation of isomeric diazine-mono-N-oxides using male rabbit hepatic microsomes. The results are an average of duplicate determinations from two rabbits differing by not more than ±10%.

the physiological pH of 7.4 used in our microsomal oxidation experiments. In general, the rate of N-oxide production increased with increase in substrate concentration to about 10 μmoles/incubate, after which the rate declined. The decline in the rate of N-oxide formation could be due to excess substrate inhibition. This may occur as a result of excess substrate molecules binding to a different part of the enzyme causing a change in enzyme conformation. The results showed that for optimal N-oxide formation 10 μmoles of diazine per incubate should be used for further studies.

By using the data obtained by variation of substrate concentration on the rate of N-oxidation, it was possible to obtain kinetic parameters (K_m and V_{max}). In this study, Lineweaver-Burk and Hanes-Woolf plots were used for the determination of apparent K_m and V_{max} values (Table 4). The difference in K_m values observed between different substrates reflected the difference in the affinity of these substrates for the enzymes and might, in turn, be related to the lipophilic characteristics or the pKa of that substrate. The pKa(exp) and pKa(calc) are recorded in Table 2; whilst there are slight differences in the values of pKa for the three bases obtained by the two methods they are

TABLE 4

Enzyme kinetic constants (K_m and V_{max}) for the rabbit hepatic microsomal N-oxidative metabolism of diazines

Plot	Kinetic constant	Pyrazine	Pyrimidine	Pyridazine
LB	Km	3.150	7.100	1.000
	Vmax	0.170	0.360	0.240
HW	Km	2.820	4.450	1.600
	Vmax	0.160	0.260	0.300

K_m is expressed as mM and V_{max} as nmol/mg protein/min
LB = Lineweaver-Burk plot
HW = Hanes-Woolf plot

insignificant for the present comparisons. The data show that pyridazine is the strongest base of those studied, pyrimidine intermediate and pyrazine the weakest. It should be noted, however, that these bases are all much weaker than monoheteroaromatic bases, i.e. pyridine and quinoline. Little difference was observed in the logP values (Table 2) of the three bases whether determined experimentally or by the computer programme. The three bases were almost identical with regard to this parameter, being poorly lipophilic, and therefore this property could not account for the differences in kinetic constants for the N-oxidation of isomeric aromatic diazines.

The K_m values of pyridazine N-oxidation from both plots used were lower compared to those calculated for the other isomeric diazines, indicating that pyridazine had a greater affinity for the enzyme, resulting in higher N-oxide formation. This could be related to the pKa value of the compound which was more basic (pKa 2.33) compared to pyrazine (pKa 0.65) and pyrimidine (pKa 1.31). The V_{max} value of this compound was also higher than the other isomeric aromatic diazines. If the pKa of the base did influence the kinetic parameters of hetero-aromatic N-oxidation it would be predicted that the more basic compound pyridine (pKa 5.22) would have a lower K_m for N-oxidation than the bases presently studied. As the K_m for pyridine N-oxidation from rat hepatic microsomal suspension has been reported to be 0.633 mM /33/ this supports our suggestion that the pKa may influence the kinetic parameters.

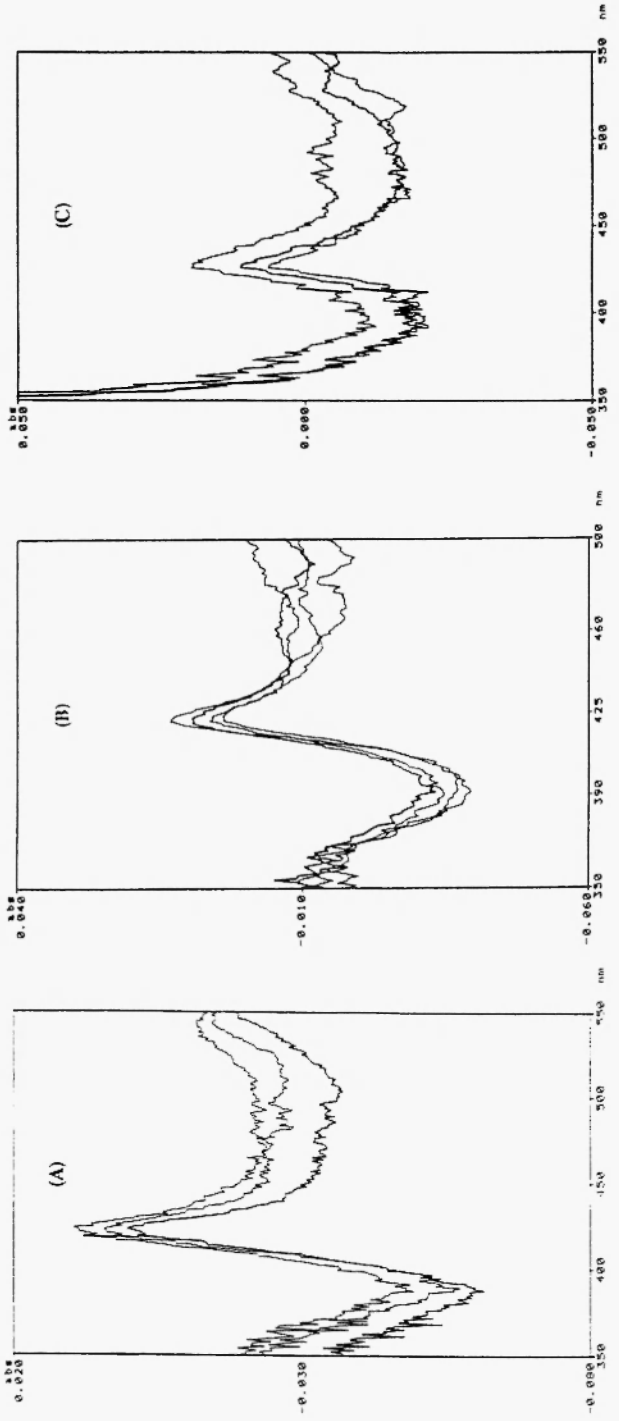

Fig. 4: Spectral binding of isomeric aromatic diazines to microsomal cytochrome P450. PB-induced male rabbit microsomes were used and protein concentration of microsome was 1 mg/ml. (A) Pyrazine; (B) Pyrimidine; (C) Pyridazine.

However, the results from the other isomeric diazines do not support this idea as pyrimidine, which is slightly more basic than pyrazine, has a K_m value greater than the latter compound. Therefore we compared direct evidence for substrate/cytochrome P450 interaction by examining the difference spectra produced by the isomeric diazines. In each case the substrate gave a *type II* spectra (Fig. 4). By varying the concentration of substrate and measuring the ΔAbs between the peak (423-426 nm) and the trough (382-388 nm) it was possible to determine the K_s value by use of a Hanes-Woolf plot. Examination of the K_s values (Table 3) showed that they are related to the K_m values in that the compound having the greatest K_m value (pyrimidine) gave the greatest K_s value, whereas the compound giving the smallest K_m value (pyridazine) gave the smallest K_s value. Pyrazine gave an intermediate K_m value and also intermediate K_s value. The finding of a good correlation between a type II spectra K_s and the K_m value was surprising as previous work /20-22/ has failed to find a correlation except with a few compounds. The present results strongly indicate that the binding of a diazine to microsomes gives a good indication as to its ability to undergo N-oxidation by rabbit hepatic microsomes and may indeed be the rate limiting factor.

Further work is now required to examine the effect of these substrates on the spin state of the cytochrome P450 and to determine the rate constants of the individual reactions /34/. Such data may not only provide evidence of the controlling steps in diazine N-oxidation, but may also help in elucidating the mechanisms involved in the biotransformation of these substrates.

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