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SPECTRAL INTERACTION OF ORPHENADRINE AND ITS METABOLITES WITH OXIDIZED AND REDUCED HEPATIC MICROSOMAL CYTOCHROME P-450 IN THE RAT

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Abstract—Product inhibition has been suggested to be a determinant in orphenadrine pharmacokinetics. Two possibilities for the mechanism of product inhibition in orphenadrine metabolism are explored in this study. Orphenadrine and its metabolites may compete for cytochrome P-450 catalytic binding sites. Therefore the interaction of orphenadrine and some of its metabolites with hepatic microsomal ferri-cytochrome P-450 of the rat was investigated. The spectral dissociation constant for the type I (substrate) interaction of orphenadrine and its metabolites displayed no relationship with the lipophilicity of the compounds. Orphenadrine is only partially displaced from its cytochrome P-450 binding sites by its respective metabolites. For this mechanism to be significant *in vivo*, the metabolites need to reach concentrations near cytochrome P-450 similar to that of orphenadrine. This is not known yet. The significance of this mechanism for the product inhibition phenomenon is therefore uncertain. In this study it is also established that during both *in vitro* as well as *in vivo* metabolism of orphenadrine, a metabolic intermediate is formed, which binds irreversibly to ferrous-cytochrome P-450 (MI complex). *In vitro*, both the rate and extent of the MI complex formation with orphenadrine and metabolites as precursor, decreased in the order *N*-hydroxytofenacine ≫ tofenacine > orphenadrine > bisnororphenadrine. The metabolite orphenadrine-*N*-oxide did not produce an MI complex, *in vitro*. Furthermore, *in vitro*, it was shown that the *N*-demethylation of tofenacine paralleled the concomitant MI complex formation. Together, the data suggest that the first *N*-demethylation step of orphenadrine occurs via α-carbon oxidation, whereas the second *N*-demethylation step mainly comes about via *N*-oxidation. Both metabolic pathways eventually lead to the MI complex forming species. These two parallel pathways also account for the complicated substrate dependency and concentration dependency in MI complex formation. Finally, the formation of the nitroxide radical (the ultimate ligand for MI complexation) has been shown to be susceptible to inhibition by its precursors.

The occurrence of MI complex formation resulting in metabolic inactive cytochrome P-450 is probably the main mechanism for the product inhibition phenomenon in orphenadrine metabolism.

The anticholinergic drug, orphenadrine hydrochloride (Disipal®) has been shown to be effective in the treatment of Parkinson's disease. During studies on the pharmacokinetics of this compound, a discrepancy in the single dose, and multiple dose kinetics was observed [1]. The unexpectedly high plasma concentrations of orphenadrine after repetitive oral administration to humans was explained, assuming product inhibition, which exerts a negative feedback on the metabolism of orphenadrine. This was shown to be a tenable hypothesis, since in addition it was found [1] that the administration of the prevailing metabolite of orphenadrine, tofenacine (nororphenadrine) prolonged the half-life of orphenadrine in the dog.

In the present study two tentative explanations for the mechanism of product inhibition in orphenadrine metabolism are proposed and studied.

Firstly, competition for cytochrome P-450 between orphenadrine and its metabolite(s) may occur. It is known that binding of a substrate to the ferric form of cytochrome P-450 is the first event in oxidative drug metabolism mediated by cytochrome P-450 [2]. Interaction of metabolites with 'the enzyme-active sites' of cytochrome P-450 may inhibit the metabolism of their precursor, by preventing the binding of the precursor to cytochrome P-450. However, literature data about the mutual interaction of substrates and their metabolites with native ferri-cytochrome P-450 are surprisingly scarce [3, 4]. Still, the overall pharmacokinetic profile of a drug and of its metabolites may depend upon the mutual interaction of precursor and metabolites with cytochrome P-450.

Secondly, product inhibition in orphenadrine metabolism may be explained by the formation of an inactive cytochrome P-450–metabolic intermediate (MI) complex. From literature data [5] several amines including diphenhydramine, a structural analogue of orphenadrine, are known to be metabolized by the hepatic microsomal oxidizing system to an intermediate which binds to the reduced form of cytochrome P-450. The complex thus formed is detected spectrally by an absorbance maximum near 455 nm. The MI complexes formed from amines appear to be relatively stable *in vitro* [5–9] and for a limited number of compounds also *in vivo* [9, 10]. Because the cytochrome P-450 that is bound in the complex is prevented from further participation in

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mixed function oxidation reactions, MI complexes have an inhibitory effect on mixed function oxidations. Presumably this includes the metabolism of the MI complex forming compounds themselves [11].

Our interest in product inhibition during drug metabolism [12] in addition to the aforementioned phenomena, prompted us to study the interaction of orphenadrine and some of its known and potential metabolites [13, 14] with cytochrome P-450 in its oxidized (Fe^{3+}) form. Moreover, the occurrence and origin of the MI complex formation both *in vitro* and *in vivo* was studied.

MATERIALS AND METHODS

Chemicals. Orphenadrine and its structural analogues were gifts from GistBrocades N.V. (Haarlem, The Netherlands). SKF 525-A was a gift from Smith Kline and French (Welwyn Garden City, U.K.). NADPH was obtained from Boehringer/Mannheim GmbH (Mannheim, F.R.G.). All other chemicals and solvents used were of analytical grade purity.

Preparation of microsomes and pretreatment of animals. Hepatic microsomes were prepared from 250–300 g male Wistar rats as previously described [15]. The rats used were either untreated or received intraperitoneal (i.p.) injections of phenobarbital dissolved in saline. The phenobarbital treatment consisted of three daily injections of 80 mg/kg. On the fourth day the rats were killed by decapitation. In order to form the MI complex *in vivo*, the rat, after the phenobarbital induction, was injected (i.p.) with 30 mg/kg nororphenadrine (tofenacine) dissolved in saline on the fourth day and killed 3 hr thereafter.

Spectral measurements. All spectrophotometric observations were performed with the microsomal fraction (2 mg protein per ml) suspended in 50 mM phosphate buffer (pH 7.4) containing 0.1 mM EDTA. The spectral determinations were performed with an Aminco DW 2 U.V.-Vis spectrophotometer at 37°. All substrates were dissolved in water. Only bisnororphenadrine and *N*-hydroxytofenacine were added in dimethylsulphoxide (DMSO), which was shown to be without influence on the spectral data in the concentrations used. Difference spectra were recorded as previously described [15] with the spectrophotometer in the split beam mode. The MI complex formation was measured with the microsomes suspended at 2 mg of protein per ml in a 50 mM phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 4.2 mM $MgCl_2$ and 400 μM NADPH. Both the rate and the extent of the MI complex formation were mainly determined with dual wavelength spectroscopy unless otherwise stated in the text. Ferricyanide oxidation of the microsomes, as described by Buening *et al.* [10] was used to quantitate the cytochrome P-450–metabolic intermediate complex. A molar extinction coefficient (ε) of 65 $mM^{-1}cm^{-1}$ was used to quantitate the MI complex [10].

The concentration of cytochrome P-450 was estimated by bubbling both cuvettes with CO and by adding sodium dithionite to the sample cuvette thus preventing interference with hemoglobin [16].

Colorimetric formaldehyde assay. Microsomes were incubated at 37° with shaking, air being freely admitted. The incubation mixture contained 400 μM NADPH and 4.2 mM $MgCl_2$. The incubation with tofenacine (333 μM) as substrate was carried out in a total volume of 3 ml and stopped by addition of 0.5 ml $ZnSO_4$ (40% w/v) and 1 ml $Ba(OH)_2$ (saturated at 37°). After mixing and centrifugation, 2 ml of the supernatant was mixed with 1 ml Nash reagent at double strength [17] and incubated at 60° for 30 min. The amount of formaldehyde formed was estimated by determining the absorbance at 415 nm relative to that at 500 nm and subtracting the amount of Nash-positive material formed in the appropriate blank.

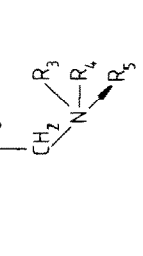
Determination of partition coefficients. The solvents used were *n*-octanol and phosphate buffer (0.1 M, pH 7.4, ionic strength adjusted to 0.3 M by addition of NaCl). The phases were saturated with each other at room temperature. In the experiments 25 ml of buffer and 5 ml of octanol were used. Shaking of the test tubes was performed for 1 hr at room temperature (20 ± 1°). After centrifugation to separate the phases, the concentration of the amine was determined spectrophotometrically in the u.v. region in both phases to ascertain mass balance. The logarithm of the ratio between solute concentration in octanol and phosphate buffer represents the logarithm of the apparent partition coefficient (log P').

RESULTS

Interaction with ferri-cytochrome P-450. Orphenadrine, nororphenadrine (tofenacine) and the aromatic hydroxylated orphenadrine metabolites showed very low spectral dissociation constants (K_s values) as calculated from the type I spectral changes indicating that they have high affinities towards cytochrome P-450. Orphenadrine-*N*-oxide and *N*-hydroxytofenacine showed higher K_s values respectively (Table 1). The spectral dissociation constant for bisnororphenadrine as well as the ΔA_{max} value were not determined as a consequence of the type II (ligand) binding characteristics of this compound. The same kind of type II spectra, with a 410 nm and a 395 nm trough, were observed for example for bisnortriptyline [18] or *n*-octylamine [19]. An outstanding feature of the data in Table 1 is the close similarity of the ΔA_{max} values. One remarkable exception, however, is *N*-hydroxytofenacine which elicits a relatively large ΔA_{max} concomitant with the aforementioned low affinity indicating a rather broad and non-selective binding at cytochrome P-450.

In order to demonstrate a potential quantitative dependency of the K_s upon the log P within this series of structural related compounds, log P values of the free bases were calculated. The computation was performed using hydrophobic fragmental constants obtained from the extended data set published by Rekker *et al.* [20]. The available range of fragmental constants did not allow us to assess the partition coefficients for *N*-hydroxytofenacine and orphenadrine-*N*-oxide. These two compounds, however, displayed an interesting divergence in the obtained K_s values (Table 1). This prompted us to determine the log P' values, estimated at pH 7.4, as described in Materials and Methods, of these compounds and of orphenadrine and tofenacine, the latter being the prevailing metabolite in man [14].

Table 1. Type I interaction of orphenadrine and some of its metabolites with cytochrome P-450 of rat hepatic microsomes



Compound	Substituents* R_1	R_2	R_3	R_4	R_5	Salt-form	n‡	K_s ± S.E.M. (μM)	ΔA_{max}/nmole P-450/ml ± S.E.M.§	Log P‖	Log P¶
Orphenadrine	H	H	CH_3	CH_3	—	HCl	5	0.224 ± 0.014	0.0082 ± 0.0008	3.86	1.76
Nororphenadrine (tofenacine)	H	H	H	CH_3	—	HCl	5	0.217 ± 0.011	0.0083 ± 0.0006	3.42	1.06
Bisnororphenadrine†	H	H	H	H	—	HCl	—	—	—	3.12	—
N-Hydroxytofenacine	H	H	CH_3	OH	—	Fumarate	4	3.43 ± 0.136	0.0211 ± 0.0009	—	1.74
Orphenadrine-*N*-oxide	H	H	CH_3	CH_3	O	—	6	1.31 ± 0.249	0.0088 ± 0.0007	—	1.99
3′-Hydroxy orphenadrine	H	OH	CH_3	CH_3	—	Maleate	6	0.263 ± 0.028	0.0071 ± 0.0003	3.38	—
5-Hydroxy orphenadrine	OH	H	CH_3	CH_3	—	½ Fumarate	3	0.295 ± 0.024	0.0088 ± 0.0004	3.31	—

* Substituents refer to the general formula:

† Bisnororphenadrine showed a type II (ligand) interaction with cytochrome P-450.

‡ Number of experiments to obtain the spectral characteristics of the compounds.

§ The absorbance was measured between 430 nm trough and 385–390 nm peak.

‖ Log P is the logarithm of partition coefficient between *n*-octanol and water for the free-base form of the amines. The partition coefficient is calculated by means of the hydrophobic fragmental constant, computed by Rekker *et al.* by means of an extended data set [17].

¶ Log P′ is the logarithm of the partition coefficient between *n*-octanol and phosphate buffer, pH 7.4, measured as described in Materials and Methods.

In order to show whether the same or partially the same binding loci of cytochrome P-450 were occupied by orphenadrine and its metabolites the experiment as described in the legend of Fig. 1 was performed. A plot (Fig. 1) of the effect of increasing metabolite concentrations (expressed as multiples of the respective K_s value) on the type I difference spectrum induced by 5 μM orphenadrine shows that only part of the orphenadrine binding sites can be blocked by orphenadrine metabolites.

Interaction with ferrous-cytochrome P-450. In initial experiments liver microsomes from phenobarbital pretreated rats were used in order to facilitate the spectrophotometric detection of the potential MI complex. Incubation of these microsomes with 100 μM tofenacine or with 100 μM *N*-hydroxytofenacine in the presence of 400 μM NADPH resulted in the formation of ferrous (Fe^{2+})-cytochrome P-450–metabolic intermediate complexes (MI complexes). This was shown by an absorbance evolved at 455 nm (Fig. 2). The well known MI complex formation elicited during SKF 525-A metabolism was used as a reference (Fig. 2A). It was shown to be half of the magnitude of the tofenacine MI complex formation.

In order to study the kinetics of the MI complex formation produced by orphenadrine or its metabolites, the spectrophotometer was set in the dual wavelength mode. The results obtained in this manner (Fig. 3) showed that the rate and the extent

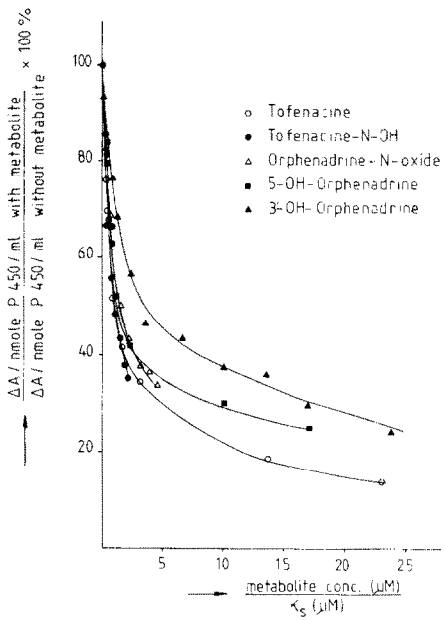


Fig. 1. Influence of metabolites of orphenadrine on orphenadrine type I binding to cytochrome P-450. The sample cuvette contained 5 μM orphenadrine and the contents of both sample and reference cuvettes were titrated with increasing metabolite concentrations. The proportional change in the magnitude of the difference spectrum elicited by 5 μM orphenadrine under influence of the metabolites versus the respective metabolite concentration is plotted. The latter is expressed in multiples of the respective K_s values (which are shown in Table 1).

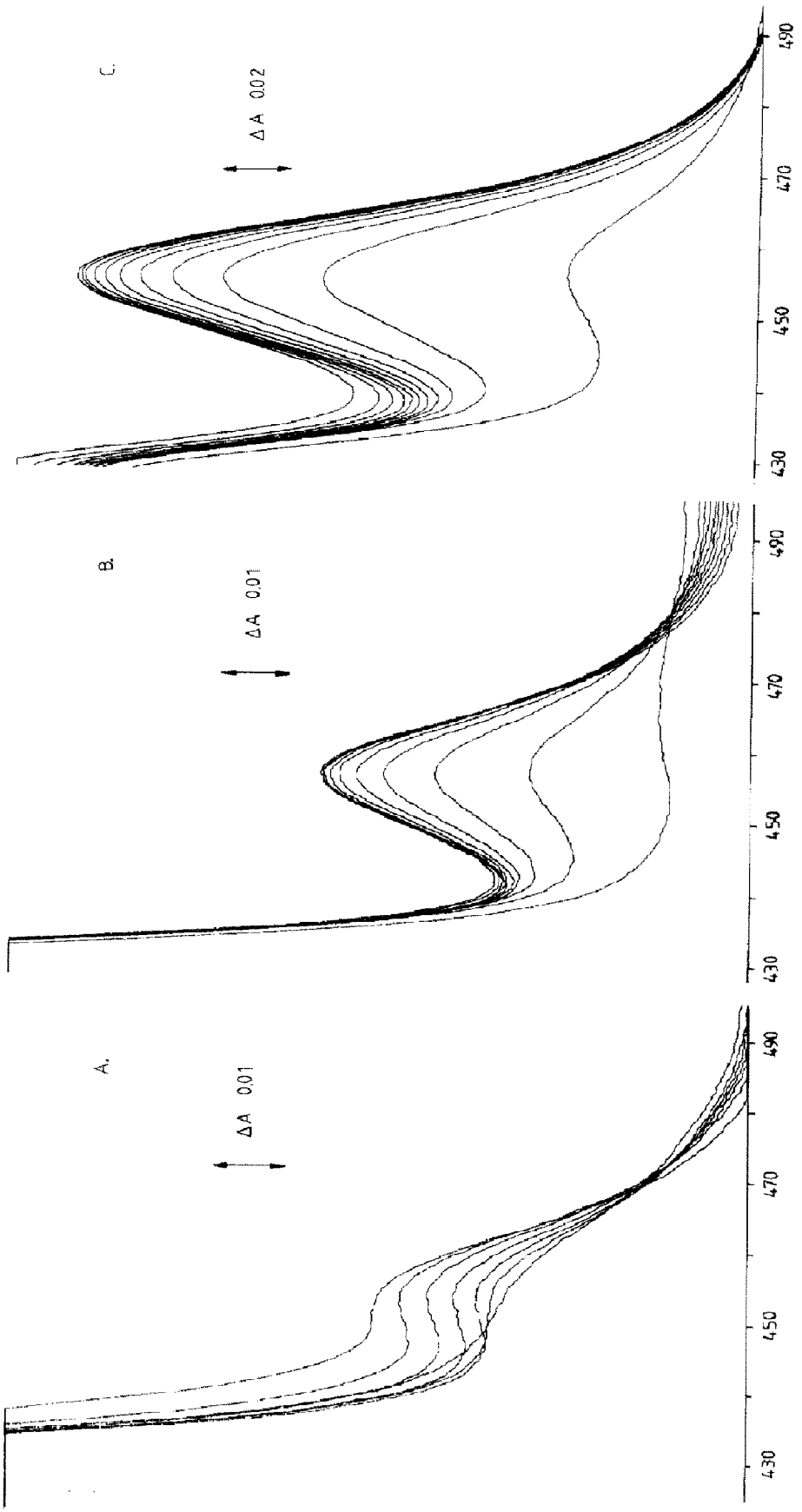


Fig. 2. Repetitive scanning of difference spectra (with a time difference of approximately 70 sec between each scan) produced during NADPH dependent microsomal metabolism of 100 μM SKF 525 A (Fig. 2A), 100 μM tolfenacine (Fig. 2B) or 100 μM N-hydroxytolfenacine (Fig. 2C). The reaction (at 37°) was started with the addition of NADPH, at a final concentration of 400 μM, in the sample cuvette. In this experiment liver microsomes from phenobarbital treated rats were used (7.4 μM cytochrome P-450). Note the difference in attenuation between Figs. 2A and 2B on the one hand and Fig. 2C on the other. The experiments were repeated at least three times.

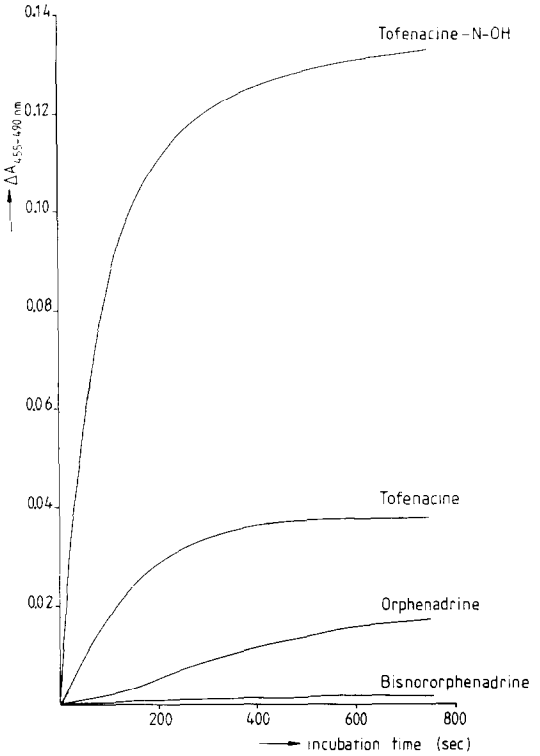


Fig. 3. Formation of cytochrome P-450–metabolic intermediate complexes during microsomal metabolism of 100 μM of bisnororphenadrine, orphenadrine, tofenacine or *N*-hydroxytofenacine. Liver microsomes from phenobarbital treated rats were used (4.3 μM cytochrome P-450). The incubation at 37° was started with the addition of NADPH at a final concentration of 400 μM in the sample cuvette. The change in absorption at 455 nm relative to 490 nm was monitored with the spectrophotometer in the dual wavelength mode. The experiments were repeated at least seven times.

decreased in the order *N*-hydroxytofenacine ≫ tofenacine > orphenadrine > bisnororphenadrine. The concentration of the substrates used was 100 μM. From literature data, however, it is known that the rate of formation of the MI complex is strongly dependent upon the substrate concentration [21]. We also found a concentration dependency both for the rate as well as for the extent of MI complex formation (Fig. 4). In these experiments liver microsomes from untreated rats were used. Changing the initial substrate concentrations did not allow us to determine the K_m value for the reaction responsible for the MI complex formation because auto-inhibition occurred at low substrate concentrations. Incubation of *N*-hydroxytofenacine with liver microsomes obtained from non-pretreated rats resulted in a maximum for the rate and the extent of 455 nm absorbance at 5 μM and 66 μM respectively (Fig. 4). For tofenacine the maximum in the formation rate of the 455 nm absorbing species with cytochrome P-450 is obtained at 33 μM (Fig. 4). The extent of the MI complexation with tofenacine as substrate was not determined because a prolonged aerobic incubation would be needed to reach the maximum. Such a prolonged aerobic incubation of microsomes and NADPH may lead to carbon monoxide generation. CO may bind to ferrous-cytochrome P-450, thus obscuring the 455 nm absorbance [22].

The disparity in the rate-curve (Fig. 4) using either *N*-hydroxytofenacine or tofenacine is readily discernible. In order to get more insight into this phenomenon experiments were designed to investigate the prohibitory effect of precursor(s) on MI complex formation. Both the rate and the extent of the absorbance at 455 nm produced during *N*-hydroxytofenacine (20 μM) incubation with liver microsomes of non-pretreated rats could be inhibited

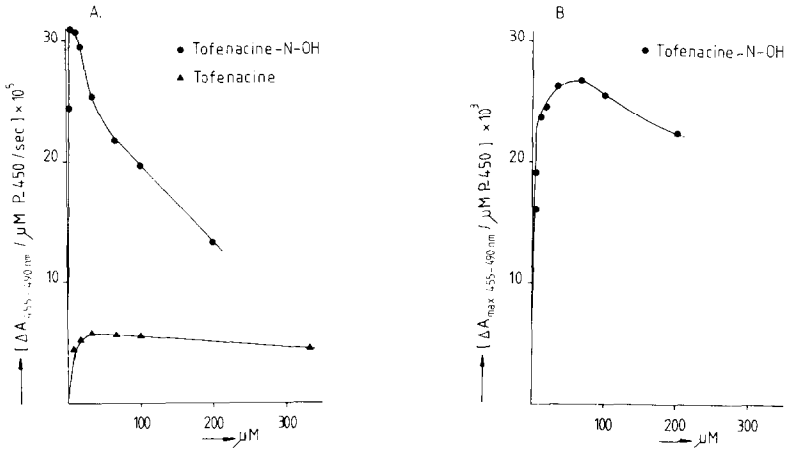


Fig. 4. (A) Concentration dependency in the rate of formation of the cytochrome P-450–metabolic intermediate complex, produced during the metabolism of tofenacine and *N*-hydroxytofenacine. Liver microsomes of untreated rats were used, containing approximately 2.8 μM cytochrome P-450. The reaction performed at 37° was started with the addition of NADPH at a final concentration of 400 μM in the cuvette. The spectrophotometer was set in the dual wavelength mode in order to obtain the change in absorption at 455 nm relative to 490 nm. Each curve is the mean of three experiments. (B) Concentration dependency in the extent of formation of the cytochrome P-450–metabolic intermediate complex, produced during the metabolism of *N*-hydroxytofenacine. Further experimental conditions as in Fig. 4A.

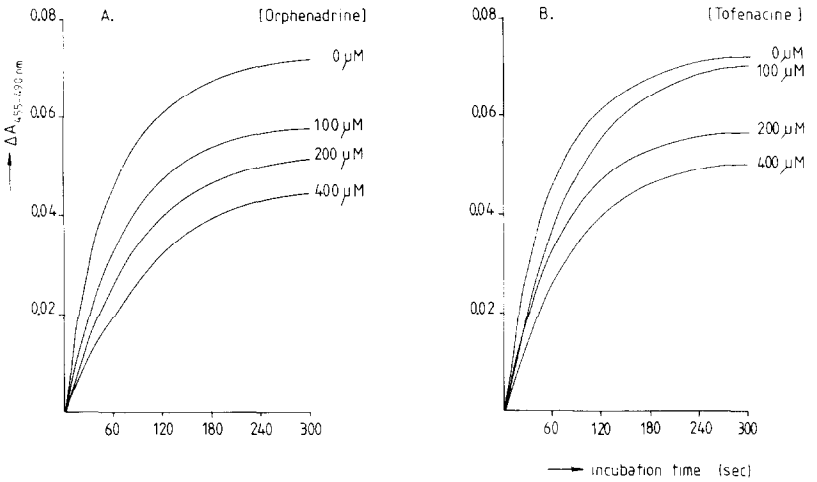


Fig. 5. (A) Inhibition of the MI complex formation from 20 μM *N*-hydroxytofenacine by several concentrations of orphenadrine. Further experimental conditions as in Fig. 4A. (B) As in Fig. 5A except tofenacine was used instead of orphenadrine.

by either orphenadrine or tofenacine (Fig. 5). The inhibition was more pronounced as the concentration of orphenadrine or tofenacine increased. The inhibition patterns using either orphenadrine or tofenacine were very much alike (Fig. 5).

Moreover it was shown that the MI complexation induced by tofenacine could be inhibited by orphenadrine. The lag time [8] of MI complexation by tofenacine increased with orphenadrine concentration (data not shown). The rate of MI complexation elicited by high tofenacine concentrations was relatively less affected by its precursor orphenadrine than those produced by low tofenacine concentrations (data not shown). These results may elucidate the cause for concentration dependency in MI complexation as delineated in the discussion.

Our data (Fig. 6) further showed a remarkable correspondence between the MI complexation of cytochrome P-450 and the *N*-demethylation of tofenacine (as measured by formaldehyde formation). These experiments were performed with hepatic microsomes of phenobarbital pretreated rats and with 333 μM tofenacine as substrate. After a 5 min incubation approximately 18% of the total cytochrome P-450 content was complexed and 18.5 μM formaldehyde was formed.

In vivo formation of a stable MI complex could be distinguished also. In order to achieve this MI

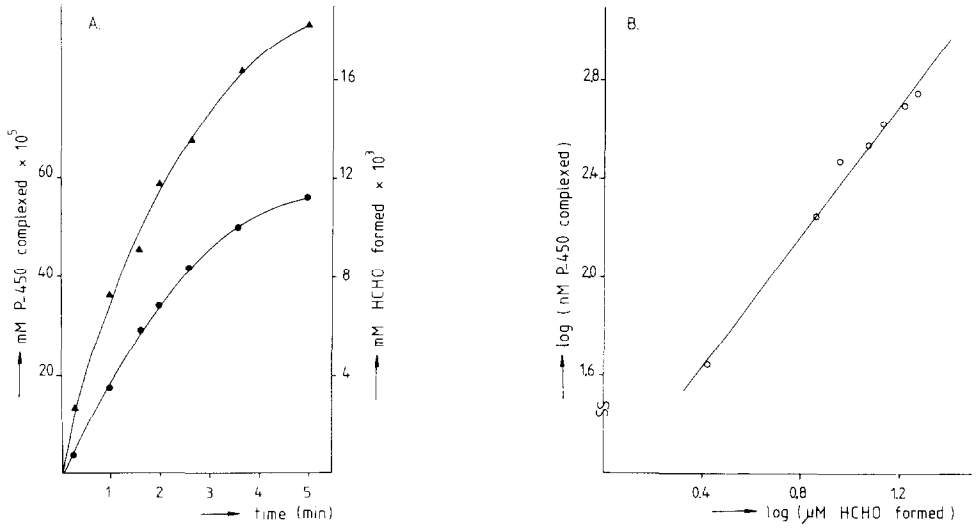


Fig. 6. (A) Comparison of the MI complexation of cytochrome P-450 (●) and formaldehyde production (▲) during NADPH dependent metabolism of 333 μM tofenacine. Microsomes of phenobarbital pretreated rats were used (3 μM cytochrome P-450). In order to calculate the amount of cytochrome P-450 complexed a molar extinction coefficient of 65 mM/cm was used. Data represent one experiment out of two. (B) Log vs log relationship of the data described in Fig. 6A. Further experimental conditions as described in Fig. 6A.

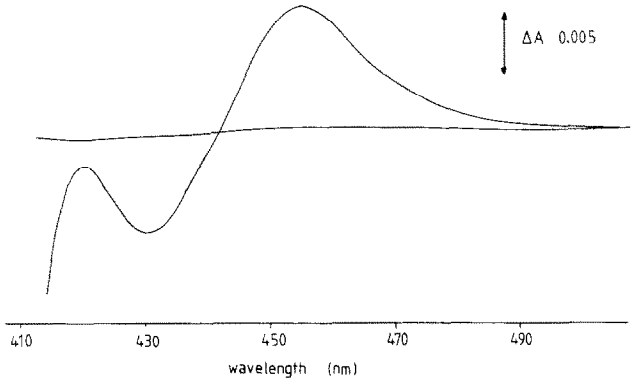


Fig. 7. Illustration of the MI complex formation *in vivo*. A rat was treated with phenobarbital (for 3 days, 80 mg/kg i.p.). The fourth day tofenacine (30 mg/kg i.p.) was administered and after 3 hr the rat was killed and hepatic microsomes were prepared. After establishing a base line, the difference spectrum with a maximum absorbance at 455 nm was obtained 3 min after the addition of potassium ferricyanide (final concentration of 50 μM) to the reference cuvette. In this example (out of ten experiments) 5% of the total cytochrome P-450 content was complexed.

complex *in vivo*, rats were pretreated with phenobarbital for 3 days. After that the rats received 30 mg/kg i.p. tofenacine. It was shown that slightly less than 8% of the total cytochrome P-450 was in the complexed form (Fig. 7).

DISCUSSION

Studies performed by Labout *et al.* [1] showing a discrepancy between the pharmacokinetic behaviour of orphenadrine in single dose and multiple dose experiments, suggested the occurrence of product inhibition under multiple dosage conditions. In order to provide fundamental understanding about product inhibition the interaction of orphenadrine and some of its metabolites with cytochrome P-450 was investigated. In this respect two hypotheses are put forward. Orphenadrine and its metabolites may compete for ferri-cytochrome P-450 or metabolites of orphenadrine may irreversibly interact with ferrous–cytochrome P-450.

The type I binding of the substrates to cytochrome P-450 is determined by measuring the extent of spectral perturbations associated with the low-spin to high-spin transition of the ferric heme protein [19]. The present study showed a lack of correlation between log K_s on the one hand and the true (log P) or the apparent partition coefficient (log P′) on the other (Table 1). This is perhaps most strikingly demonstrated with the K_s and log P′ values for the compounds *N*-hydroxytofenacine and orphenadrine-*N*-oxide. Several reports in the literature, however, described lipophilic structure–activity relationships for various homologous series of compounds in their interactions with cytochrome P-450 [23].

From our results the conclusion that other properties, for example steric factors besides lipid solubility, govern the affinity by which substrates interact with cytochrome P-450 can easily be drawn and substantiates several reports from literature [24–26]. Alternatively, the conclusion that a log P′ vs K_s correlation is absent may well be obscured by the observation that orphenadrine and its metabolites interact with

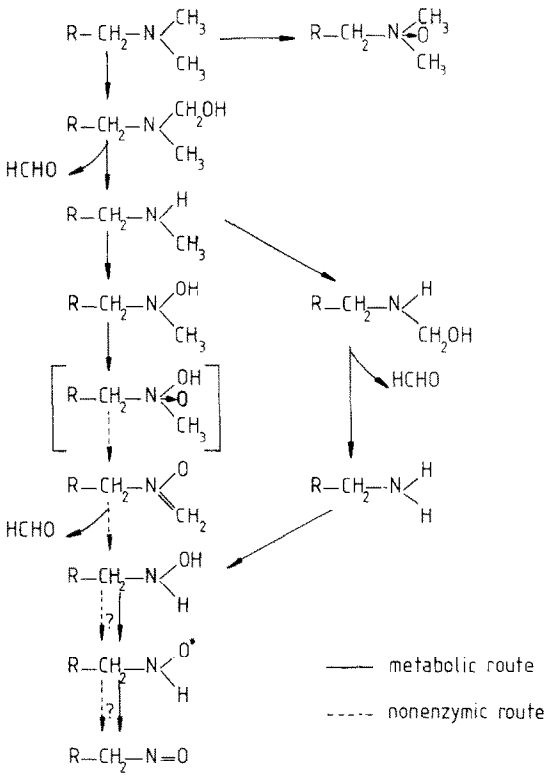


Fig. 8. The metabolic and nonenzymic degradation of orphenadrine leading to the metabolic intermediate which sequesters cytochrome P-450 irreversibly. It is shown that *N*-oxidation of orphenadrine does not lead to the mono-*N*-demethylated metabolite, tofenacine, whereas α-carbon oxidation does. During the microsomal *N*-demethylation formaldehyde is formed. Subsequently tofenacine is *N*-demethylated probably both via *N*-oxidation as well as via α-carbon oxidation (the latter is indicated by the side pathway in this figure). During these steps formaldehyde is formed in microsomes. Both routes lead to *N*-hydroxybisnororphenadrine eventually. This compound may undergo further oxidation leading to the nitroxide radical or/and the nitroso compound.

different (sub)species of cytochrome P-450. This was shown in the present work by the fact that metabolites failed to displace orphenadrine from all of its binding sites (Fig. 1), whereas on the other hand, ΔA_{max} of orphenadrine and its metabolites were identical (Table 1). This means that even this series of orphenadrine metabolites cannot be seen as a homologous series with regard to the heterogenous cytochrome P-450 system. It is emphasized that not all the binding loci at cytochrome P-450 for orphenadrine can be occupied by its metabolites. Figure 1 further shows that the aromatic hydroxylated metabolites and the compounds with metabolic changes around the nitrogen atom respectively inhibit orphenadrine binding differently. This distinctive conclusion is permitted because the concentration of the metabolites is plotted at the abscissa as multiples of their respective K_s values.

If metabolites bind at the same sites where they are formed the outcome of the experiments leading to Fig. 1 further suggests that aromatic hydroxylation involves other sites at cytochrome P-450 or even other cytochrome P-450 species then the *N*-demethylation (or *N*-oxidation). Thus it supports the hypothesis of von Bahr *et al.* [27] which was substantiated by Skånberg *et al.* [28]. They used lidocain and alprenolol respectively and tentatively proposed that aromatic hydroxylation is catalysed by high affinity sites at cytochrome P-450 with a relatively low capacity, in contrast to the *N*-dealkylation of the compounds.

In conclusion, these data present evidence that the competition between orphenadrine and its metabolites for ferri-cytochrome P-450 is only partial. For this mechanism to be significant as a cause for product inhibition, however, relatively high concentrations of the metabolites should be present near cytochrome P-450 *in vivo*, which is not known.

In this study it is also established that a ferrous-cytochrome P-450–metabolic intermediate (MI) complex is formed during orphenadrine metabolism. This inactivation of cytochrome P-450 may lead to product inhibition.

It is known that *N*-oxidation is a prerequisite for MI complex formation from amines. However, the exact nature of the MI complex forming species derived from amines has not been unequivocally established. Two possibilities have been suggested, the nitroso compound [29–32] or the nitroxide radical [32].

Our results point to the conclusion that the metabolic intermediate responsible for the formation of the MI complex generated during the metabolism of orphenadrine does not involve a primary metabolite. Rather metabolites formed as a consequence of further oxidative degradation are responsible for the MI complex. This is clearly demonstrated by using metabolites of orphenadrine as precursors for the MI complex formation (Fig. 3). Both the rate and the extent of MI complex formation decreases in the order *N*-hydroxytofenacine $\gg$ tofenacine $>$ orphenadrine $>$ bisnororphenadrine (Fig. 3). Both the relatively long lag time [8] as well as the low rate and extent of MI complex formation elicited during metabolism with orphenadrine as substrate (Figs. 2 and 3), suggest that this compound is metabolically distant from the MI complex forming species.

With regard to the mechanism of *N*-demethylation of orphenadrine to tofenacine two possibilities exist, *N*-oxidation or α-carbon oxidation. Our data show that the potential *N*-oxidation of orphenadrine does not lead to *N*-demethylation. This because 100 μM orphenadrine-*N*-oxide does not produce an MI complex (in the presence of NADPH), whereas the *N*-demethylated metabolite (tofenacine) already gives a measurable absorption at 455 nm at 10 μM (Fig. 4). Thus the first step leading to the MI complex is α-carbon oxidation of orphenadrine presumably (Fig. 8).

Both the high rate and large extent of MI complexation produced by *N*-hydroxytofenacine in the presence of NADPH (Figs. 2 and 3) indicate that this metabolite is relatively close to the ultimate MI complex forming species. As for the exact pathway of formation of the MI complex forming species, Lindeke *et al.* [33] suggested that during *in vitro* metabolism of the secondary amine, *N*-methylamphetamine, the ultimate ligand is *N*-hydroxyamphetamine. Studies by Werringloer *et al.* [34] with the aim to elucidate the ultimate MI ligand, indicated that the tertiary amine, benzphetamine is initially *N*-demethylated and subsequently metabolized (*N*-dealkylated?) further before the MI complex forming species arises.

Our results showed a very distinct correspondence in the rate of *N*-demethylation and MI complex formation, using the secondary amine tofenacine (Fig. 6). These data in connection with the aforementioned information available in literature suggest that an *N*-oxidized primary amine is responsible for the MI complexation [29–32].

There is plain evidence that the main mechanism involved in the conversion of tofenacine to the MI complex forming species is *N*-oxidation of tofenacine. Firstly, the consideration that the main route leading to MI complexation is via bisnororphenadrine, which is possibly formed via a classical α-carbon oxidation and which is subsequently *N*-oxidized, can be repudiated. This is because both the rate and the extent of MI complexation produced by bisnororphenadrine (and NADPH) is very low (Fig. 3). This means that this route, leading to the MI complex cannot be ruled out completely but is presumably only a minor pathway (Fig. 8). Secondly, as mentioned already, it was shown that *N*-hydroxytofenacine leads to rapid formation of a large amount of MI complex (Figs. 2 and 3); together with the fact that *N*-demethylation and MI complex formation during tofenacine metabolism are closely correlated (Fig. 6), this indicates that *N*-demethylation of tofenacine occurs mainly via *N*-oxidation (Fig. 8) leading to *N*-hydroxybisnororphenadrine eventually. In Fig. 8 it is tentatively suggested that the *N*-hydroxylation of tofenacine is followed by *N*-oxidation [33]. The unstable hydroxylamine-*N*-oxide rearranges presumably and then is dealkylated readily by non-enzymic hydrolysis.

Further, it was shown that orphenadrine as well as tofenacine inhibits the conversion of *N*-hydroxytofenacine to the MI complex forming species (Fig. 5). Also high concentrations of *N*-hydroxytofenacine exert a negative effect on MI complexation, during

the metabolism of *N*-hydroxytofenacine itself (Fig. 4). Both inhibitory processes are probably due to an inhibition of the conversion of *N*-hydroxybisnororphenadrine to the nitroxide radical by competition for ferri-cytochrome P-450 catalytic binding sites. The inhibition of the precursor on the last metabolic steps in the formation of the MI complex forming species can be easily generalized. For example, it is known that ethylmorphine inhibits the MI complexation derived from *N*-hydroxyamphetamine [35]. About nitroxide formation as a metabolic reaction little is known [36] and the general significance of this reaction has yet to be assessed. Our results apparently indicate that it is sensitive to inhibition, particularly by its precursors.

Moreover, our data indicate that *N*-oxidation (*N*-hydroxytofenacine) readily leads to MI complexation whereas α-carbon oxidation (bisnororphenadrine) hardly produces an MI complex (Fig. 3). Thus both the concentration of tofenacine as well as the ratio of *N*-oxidation and α-carbon oxidation determine the ultimate formation of the nitroxide radical.

On account of the intricacy of the concentration- and substrate-dependency in MI complexation (Fig. 4), it cannot be predicted whether *in vivo* MI complexation occurs as well. However, after a single intra-peritoneal administration of tofenacine to phenobarbital pretreated rats, an *in vivo* MI complexation of cytochrome P-450 was also perceived (Fig. 7).

It has been proposed that the cytochrome P-450 forms generating the MI complexing species also catalyse the oxidation of the precursor [34, 37]. Because the metabolic intermediate–ferrous-cytochrome P-450 complex is not available for metabolic reactions, it may well explain the phenomenon of product inhibition. In a subsequent paper it will be demonstrated that the MI complex gives an inhibition of the metabolism of its precursors, thus substantiating the results of this study.

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