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Is the Metabolism of Alfentanil Subject to Debrisoquine Polymorphism?

A Study Using Human Liver Microsomes

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The present study was designed to investigate whether the metabolism of the opiate analgesic alfentanil in humans is subject to the debrisoquine 4-hydroxylation polymorphism. The role of a specific cytochrome P-450 form, debrisoquine 4-hydroxylase, in the metabolism of alfentanil was investigated by competitive inhibition experiments over the concentration range 4–100 μM. Alfentanil was incubated with human liver microsomes in the presence of an NADPH-generating system. Alfentanil and its major metabolites were quantified in the incubates by reversed phase high-performance liquid chromatography (HPLC). Alfentanil was rapidly metabolized, yielding noralfentanil as the main metabolite. Kinetically, alfentanil metabolism occurred monophasically and the kinetic parameters were 22.8 μM for $K_{m\ app}$ and 3.86 nmol alfentanil metabolized $min^{-1} \cdot mg\ protein^{-1}$ for $V_{m\ app}$. Debrisoquine was a weak, noncompetitive inhibitor of alfentanil metabolism and of the formation of its major metabolites, with K_i values between 2.00 and 3.21 mM. It can be concluded that alfentanil is not metabolized *in vitro* by the human cytochrome P-450 form involved in debrisoquine 4-hydroxylation; therefore, the *in vivo* disposition of the drug is most likely not affected by deficiency of this enzyme. (Key words: Analgesics, opiate: alfentanil. Anesthetics, intravenous: alfentanil. Metabolism: debrisoquine; genetic polymorphism; human liver microsomes.)

ALFENTANIL is a short-acting, kinetically predictable opiate analgesic.[1,2] Its *in vivo* and *in vitro* metabolism were studied extensively in rats and dogs.[3,4] Recent *in vitro* studies suggest that the metabolism of alfentanil in humans might be subject to the debrisoquine 4-hydroxylation polymorphism,[5,¶,**] a genetic defect that affects approximately 10% of the white population.[6] Clinical consequences of this genetic deficiency for alfentanil could be multiple. Reduction of its metabolic elimination in poor metabolizers could lead to elevated plasma levels over a longer period of time, and, consequently, to exaggerated and prolonged pharmacologic and/or adverse effects.[7] Moreover, the broader variability in alfentanil pharmacokinetics would complicate its clinical use, make it less predictable, and necessitate careful individualization of dosage schemes, especially when the drug is used in infusion techniques.

The debrisoquine polymorphism extends to a number of other drugs including sparteine, phenformin, guanoxan, perhexilene, bufuralol, metoprolol, and the high affinity component of the *O*-deethylation of phenacetin.[8,9] There is ample evidence that the enzymatic basis of the debrisoquine polymorphism of drug oxidation in humans depends on a deficiency and/or alteration of a specific form of cytochrome P-450,[10] *viz.*, debrisoquine hydroxylase or cytochrome $P\text{-}450_{DB}$, which has recently been purified from human liver microsomes.[11] Most information regarding the linkage of the polymorphic oxidation of the drugs mentioned above to that of debrisoquine has been gathered from competitive inhibition experiments with human liver microsomes. Drugs that were known to be poorly metabolized by subjects with the genetic deficiency of debrisoquine hydroxylation were potent competitive inhibitors of debrisoquine 4-hydroxylase activity, whereas compounds not affected by the polymorphism were found to be weak noncompetitive inhibitors.[12] It was therefore reasonable to conclude that competitive inhibition experiments *in vitro* might be suitable for the early recognition of an impairment of the oxidation of a certain compound in humans affected by the debrisoquine polymorphism.[13] The present study with human liver microsomes was undertaken to further evaluate the role of cytochrome $P\text{-}450_{DB}$ in alfentanil metabolism, *i.e.*, whether the disposition of the drug is affected by the debrisoquine polymorphism. An *in vivo* study already showed that there were no significant differences in the pharmacokinetic parameters of alfentanil or in the excretion of urinary metabolites in three subjects, one of whom was characterized as a poor hydroxylator of debrisoquine.[14]

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¶ McDonnell TE, Bartkowski RR, Kahn C: Evidence for polymorphic oxidation of alfentanil in man (abstract). ANESTHESIOLOGY 61:A284, 1984.

** Kahn C, Rubenfield H, Bartkowski R, McDonnell TE: Competitive inhibition of debrisoquine and phenacetin oxidation by alfentanil in human liver (abstract). North American Symposium on Risk Assessment and the Biological Fate of Xenobiotics, Florida, International Society for the Study of Xenobiotics, 1985, p 31.

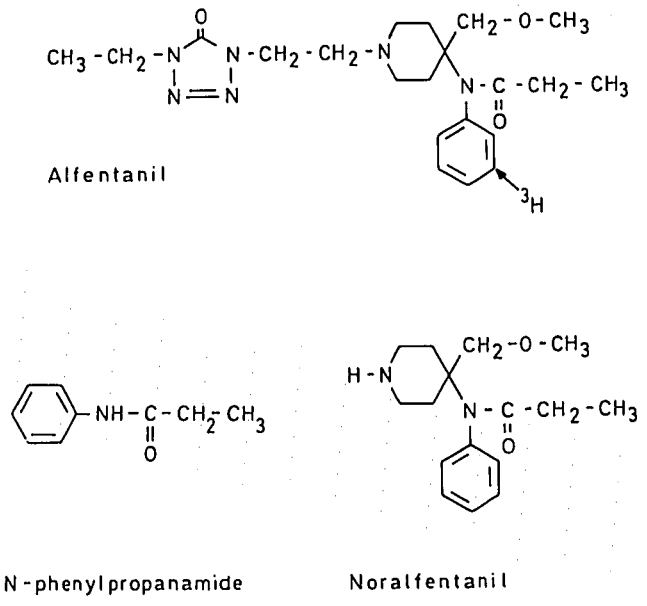

FIG. 1. Chemical structures of alfentanil and its main metabolites with the position of the tritium label.

Methods

A human liver sample, obtained from a kidney donor, was homogenized (1 g of liver in three volumes of 0.01 M Na,K-phosphate buffer, 1.15% KCl *p*H 7.4) with a Potter-Elvejhem homogenizer at 4° C. Liver microsomes were prepared by differential centrifugation at 12,000 and 110,000 ×*g* and stored at −80° C in small fractions. The microsomal protein content was determined by the method of Lowry[15] according to the modification of Miller.[16] The microsomes were characterized by their cytochrome P-450 content,[17] NADPH cytochrome *c*-reductase activity,[18] 7-ethoxycoumarin *O*-deethylase activity,[19] and debrisoquine hydroxylase activity.[20,21] To determine the $K_{m\,app}$ and $V_{m\,app}$ for the 4-hydroxylation of debrisoquine, the substrate concentrations were varied between 0.067 and 1 mM.

The *in vitro* metabolism of alfentanil was studied by the incubation of 1 μCi alfentanil-^{3}H, specifically labeled with tritium in the 3-position of the phenyl ring (fig. 1), with 1 mg microsomal protein, 0.33 μmol NADP, 3.3 μmol glucose-6-phosphate, 4.6 μmol $MgCl_2$, and 0.5 units glucose-6-phosphate dehydrogenase, in 1 ml 0.255 M phosphate buffer, *p*H 7.4. In some experiments, deuterium-labeled alfentanil (alfentanil-d_5)†† was used to allow the identification of an unknown metabolite X. The five deuterium atoms were positioned in the phenyl moiety of the molecule. A previous study had shown that deuterium substitution did not alter the pharmacokinetic parameters and the metabolism of the drug in humans.†† Final alfentanil concentrations varied between 4 and 100 μM. Debrisoquine was added to the incubation mixtures at final concentrations of 60, 250, and 1,000 μM. The incubations were carried out in a water bath at 37° C under continuous shaking at 100 oscillations/min. At various time points 0.1-ml aliquots were removed from the incubation mixtures and reactions were stopped by freezing the samples in a hexane/dry ice mixture. Samples were stored at −20° C and mixed with an equal volume of dimethyl sulfoxide immediately before analysis by reversed-phase HPLC with on-line radioactivity detection (radio-HPLC). Separation and quantification of alfentanil and its major metabolites were carried out as described elsewhere.[4] The major metabolites were characterized by HPLC co-chromatography with authentic reference compounds as described previously.[4] Metabolite X was identified by reversed-phase HPLC co-chromatography using isocratic elution (1 ml/min) with a mixture of 0.92 M ammonium acetate, *p*H 6.4-methanol-acetonitrile-tetrahydrofuran (32.5/15/22.5/30, v/v/v/v). Final identification of this metabolite was performed by capillary gas chromatographic–mass spectrometric (GC/MS) analysis after HPLC purification from incubates with alfentanil-d_5. The capillary GC/MS was performed by an on-column injection on a 10-m ULTRA-2 column (Hewlett-Packard), which was directly inserted into the ion source of the mass spectrometer. Helium was used as carrier gas and the column-oven temperature was raised from 50° C up to 310° C at 12° C/min. The transfer-line temperature was kept at 300° C. Electron impact (EI) and chemical ionization (CI) mass spectra were obtained on a Finnigan 4500 mass spectrometer coupled to an Incos data system.

The velocities of the alfentanil metabolism and of the formation of noralfentanil (fig. 1) were calculated from the initial rate of the enzymatic reaction. The kinetics of the *in vitro* alfentanil metabolism were analyzed at first by an Eadie-Hofstee plot in order to verify the complexity of the enzyme system. The Michaelis-Menten parameters, the apparent maximum metabolism rate, $V_{m\,app}$, and the apparent Michaelis constant, $K_{m\,app}$, were calculated from Lineweaver-Burk plots. The dissociation constants of the enzyme-inhibitor complex, K_i, were assessed from Dixon plots by linear regression analysis.

Results

The characteristics of the batch of human liver microsomes that was used in the present study are presented in table 1. A comparison is made with some data from

†† Van Beijsterveldt LEC: A study of the pharmacokinetics and metabolism of alfentanil using stable isotopes. Thesis, K. U. Nijmegen, Holland, 1986.

the literature, which were obtained using methods similar to those described above.

Alfentanil was rapidly metabolized in human liver microsomes in the presence of an NADPH-generating system. Figure 2 shows a radio-HPLC chromatogram of an incubate of 4 μM alfentanil with microsomes and cofactors for 6 min. Three radioactive compounds were detected; two of them could be identified by co-chromatography using the previously described method[4]: noralfentanil-*N*-[4-(methoxymethyl)-4-piperidinyl]-*N*-phenylpropanamide (R 30 451) and unchanged alfentanil. Using the modified HPLC method, it could be demonstrated that metabolite X co-chromatographed with authentic *N*-phenylpropanamide (R 50 977; fig. 1). This identification was confirmed by GC/MS analysis of incubates with alfentanil-d_5 as a substrate. The spectra for metabolite X and the authentic reference compound exhibited the same fragment ions, taking into account a shift of 5 daltons for ions containing the phenyl moiety of the d_5-labeled metabolite. The principal fragment ions for the metabolite were at m/z 154 (relative intensity 12), 125 (<1), 98 (100), 82 (4), 57 (16), the latter ion being CH_3CH_2CO. For authentic *N*-phenylpropanamide, they were at m/z 149 (14), 120 (1), 93 (100), 77 (5), and 57 (11). The CI mass spectrum of the deuterated metabolite revealed a protonated molecular ion at m/z 155. No degradation of alfentanil was observed in incubates with boiled microsomes, even after 120 min.

The effects of the initial alfentanil concentrations varying from 4 to 100 μM are shown in figure 3. Linear Eadie-Hofstee plots were obtained for the relationship between v and v/S for the metabolism of alfentanil (r = 0.993), the formation of noralfentanil (r = 0.995) and for the formation of metabolite X (r = 0.884).

Figure 4 shows the Dixon plots for the effect of debrisoquine on the metabolism of alfentanil and on the formation of noralfentanil. Debrisoquine behaved as a weak inhibitor of the metabolism of alfentanil, as indicated by the small slopes of the lines through the experimental points. Figure 5 shows typical Lineweaver-Burk plots for the same data. The lines through the experimental points intersect at the x-axis, which is indicative of noncompetitive inhibition. The kinetic parameters calculated from the Lineweaver-Burk and Dixon plots are presented in table 2. The inhibition constants (K_i) were calculated from the Dixon plots, assuming noncompetitive inhibition. The formation of metabolite X was only inhibited at the highest debrisoquine concentration, 1 mM, yielding parallel curves in the Lineweaver-Burk plot, which is characteristic of uncompetitive inhibition (not shown). The K_i for metabolite X listed in table 2 was calculated from the Lineweaver-Burk plot.

TABLE 1. Characteristics of the Batch of Human Liver Microsomes Used in the Present Study—Comparison with Literature Data

Parameter	This Study	Current Literature Values (with references)
Cytochrome P-450 (nmol · mg protein^{-1})	0.432, 0.456	0.58 ± 0.04[8] 0.31 ± 0.09[22] 0.51 ± 0.16[25]
NADPH-cytochrome *c*-reductase (nmol min^{-1} · mg protein^{-1})	47, 57	117 ± 20[8] 99.8 ± 11.4[22] 121 ± 67[24]
O-deethylation of 7-ethoxycoumarin (nmol · min^{-1} · nmol P-450^{-1})	0.81 ± 0.18	0.75 ± 0.11[24]
4-hydroxylation of debrisoquine		
K_m (μM)	85.5, 90.4	130 ± 24[25]
V_m (pmol · min^{-1} · mg protein^{-1})	50.8, 56.8	69.9 ± 14.3[25]

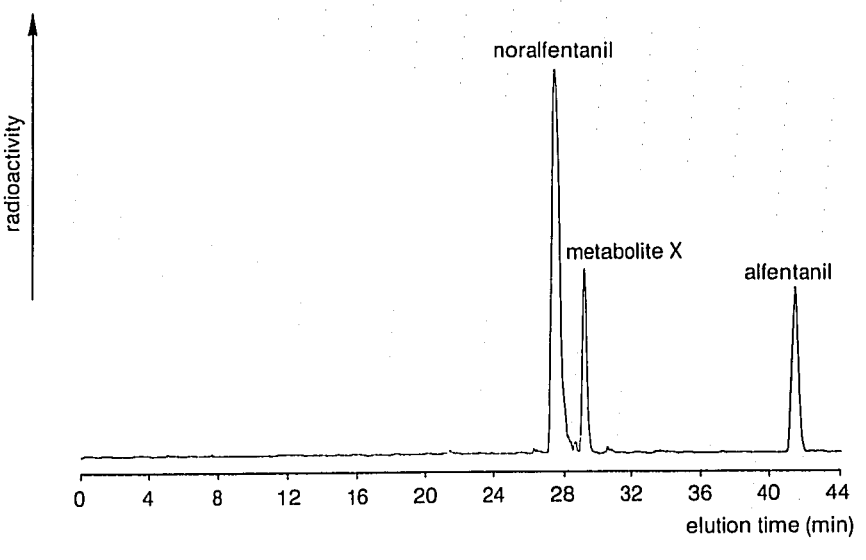

FIG. 2. HPLC chromatogram (radioactivity detection) obtained for an incubate of 4 μM alfentanil with human liver microsomes and an NADPH-generating system for 6 min.

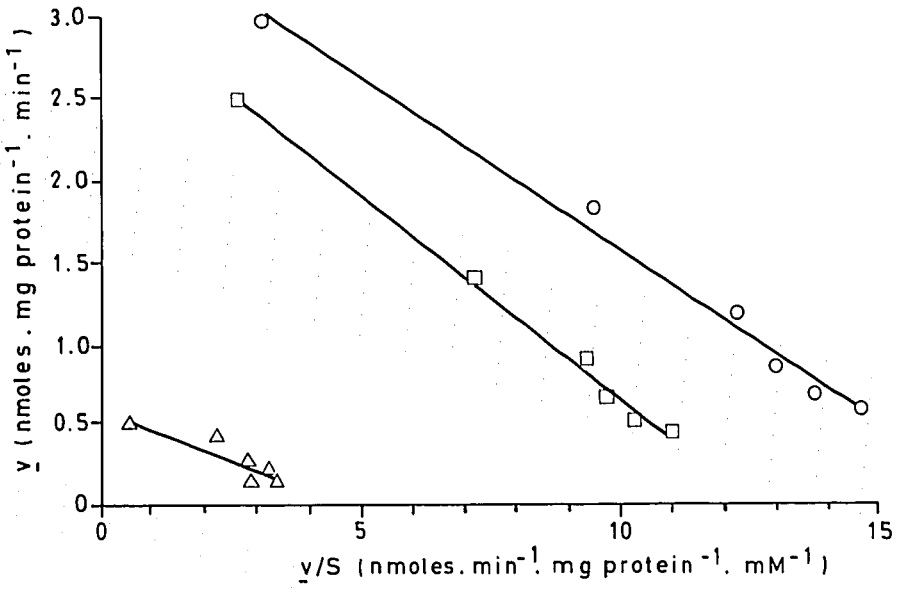

FIG. 3. Eadie-Hofstee plot for the metabolism of alfentanil (– O –) and the formation of its major metabolites, noralfentanil (– □ –) and metabolite X (△), in human liver microsomes. The lines were constructed using linear regression analysis.

Discussion

The biochemical indices determined for the batch of human liver microsomes used in the present study compare well with previously reported literature values obtained in different laboratories (table 1). Only the amount of NADPH-cytochrome *c*-reductase was somewhat low, although this was still in the normal range when compared to NADPH-cytochrome *c*-reductase activities in hepatic microsomes of 33 human subjects.[23]

Alfentanil was readily metabolized *in vitro* by human liver microsomes. The main *in vitro* metabolite could be identified as noralfentanil. The metabolic pathway leading to this metabolite, oxidative *N*-dealkylation at the piperidine nitrogen, was also the main *in vivo* metabolic pathway in humans, noralfentanil accounting for about 30% of the dose in the 0–24 h urine.[14] The second *in vitro* metabolic pathway was oxidative *N*-dealkylation at the amide nitrogen, resulting in *N*-phenylpropanamide. Although this metabolite has not been identified in *in vivo* studies up to now, the metabolic pathway involved had been deduced from the *in vivo* formation of *N*-(4-hydroxyphenyl)-propanamide.[3,14]

The linear relationship between v and v/S obtained in the Eadie-Hofstee plot (fig. 3), essentially indicates that alfentanil metabolism follows monophasic kinetics in human liver microsomes over the concentration range of 4–100 μM alfentanil. A similar observation has previously

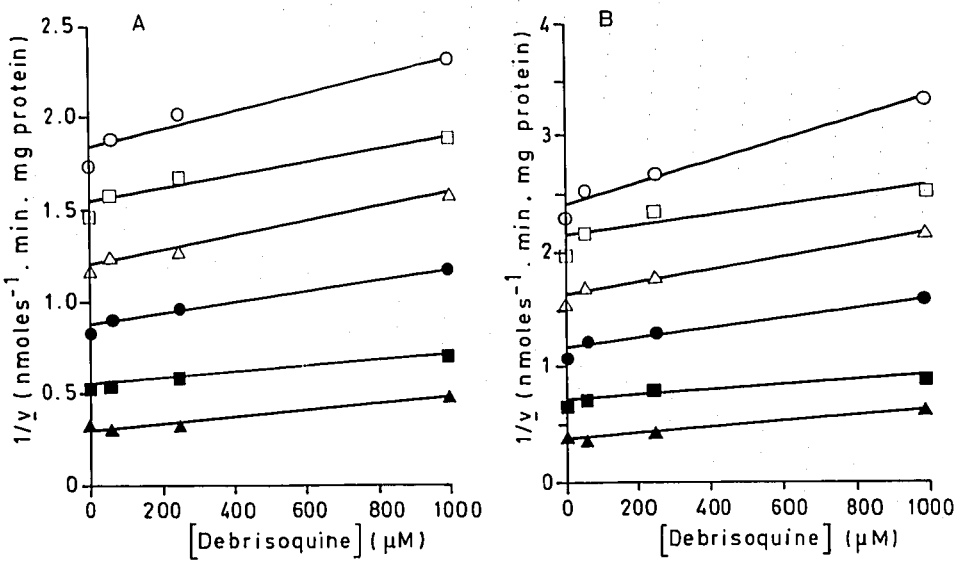

FIG. 4. Dixon plots for the interaction of debrisoquine with the *in vitro* metabolism of alfentanil (*A*) and the formation of the main metabolite noralfentanil (*B*), by human liver microsomes. Initial alfentanil concentrations were 3.94 (○), 4.90 (□), 6.54 (△), 9.73 (●), 19.4 (■), and 96.6 (▲) μM. The lines were constructed using linear regression analysis.

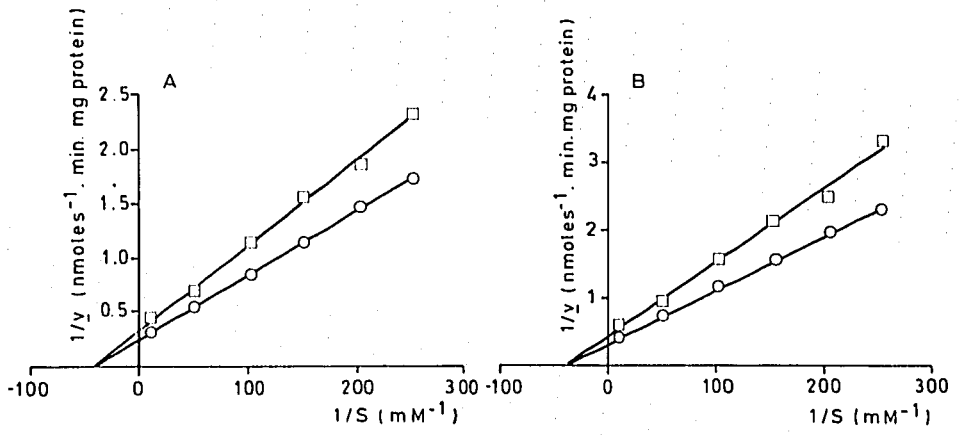

FIG. 5. Lineweaver-Burk plots for the interaction of debrisoquine with the metabolism of alfentanil (*A*) and the formation of its main metabolite noralfentanil (*B*) by human liver microsomes. Incubations were performed in the absence (○) or in the presence of 1 mM debrisoquine (□).

been made for the metabolism of alfentanil in isolated rat hepatocytes in the same concentration range.[4] Although human liver contains multiple cytochrome P-450 forms with considerable overlap in substrate specificity,[8] alfentanil is predominantly metabolized by a single isozyme, although the participation of other isozymes with small differences in substrate affinity ($K_{m\ app}$) cannot be ruled out. Their contribution, however, is thought to be small because differences as large as tenfold would result in biphasic Eadie-Hofstee plots.[26] The predominant involvement of one form of cytochrome P-450 is further illustrated by the linearity of the Lineweaver-Burk plots (fig. 5) and justifies the use of linear regression analysis for the calculation of the kinetic parameters.

The data in table 2 indicate that debrisoquine is only an extremely weak inhibitor of the metabolism of alfentanil and of the formation of its major metabolites. The K_i for inhibition was 23–36 times the K_m for the metabolism of debrisoquine. This suggests that both drugs are not metabolized by the same isozyme. Indeed, numerous *in vitro* studies have shown that drugs that share the debrisoquine polymorphism are potent inhibitors of debrisoquine 4-hydroxylase activity and, conversely, that debrisoquine itself is a potent inhibitor of the *in vitro* metabolism of these drugs. For instance, debrisoquine inhibits the *in vitro* metabolism of sparteine[27] and bufuralol,[28] and the K_i for debrisoquine inhibition is very similar to the K_m for debrisoquine 4-hydroxylation, indicating that the same form of cytochrome P-450 is involved in the catalysis of these three compounds. Boobis *et al.*[12] demonstrated that compounds that show no *in vivo* dependence on the debrisoquine polymorphism have only weak or no inhibitory properties. These compounds also behave as noncompetitive inhibitors,[12] whereas sparteine and bufuralol are competitive inhibitors.[27,28] The noncompetitive inhibitory character of debrisoquine toward alfentanil metabolism further substantiates that both drugs are not metabolized *in vitro* by the same form of cytochrome P-450. This essentially indicates that interindividual variability in the *in vivo* disposition of alfentanil, as a consequence of a genetic deficiency related to the debrisoquine polymorphism, can be excluded. This is in agreement with recent *in vivo* findings of Meuldermans *et al.*,[14] who could not find clear differences in the pharmacokinetic parameters and the excretion pattern of urinary metabolites of alfentanil among three subjects, one of whom was a poor hydroxylator of debrisoquine. Our findings, however, contrast with those of other investigators who concluded on the basis of *in vitro* inhibition experiments that alfentanil was metabolized to a significant extent by the particular form of cytochrome P-450 involved in the 4-hydroxylation of debrisoquine,‡‡ the *O*-deethylation of phenacetin,‡‡ and the 2-hydroxylation of desipramine.[5] The conclusions of these authors were based on the observation of relatively large K_i values that were found for the inhibition by alfentanil of debrisoquine (113 μM), phenacetin (65 μM), and desipramine (171 μM) metabolism. However, these values are 5, 2.8, and 7.5 times the K_m for alfentanil metabolism respectively and, therefore, only a minor metabolic pathway of alfentanil, which was not observed in the present study, could be

TABLE 2. Kinetic Constants for the Metabolism of Alfentanil by Human Liver Microsomes and for Its Inhibition by Debrisoquine (K_i)

Compound	Alfentanil Metabolism		Debrisoquine Inhibition K_i (μM)
	$K_{m\ app}$ (μM)	$V_{m\ app}$ (nmol · min⁻¹ · protein⁻¹)	
Alfentanil	22.8	3.86	3,200 ± 890 (n = 6)
Noralfentanil	25.8	3.21	3,210 ± 1170 (n = 6)
Metabolite X	58.9	1.98	2,000

‡‡ McDonnell TE, Bartkowski RR, Kahn C: Evidence for polymorphic oxidation of alfentanil in man (abstract). ANESTHESIOLOGY 61:A284, 1984.

mediated by debrisoquine 4-hydroxylase. This probably would have no consequences for the *in vivo* metabolism of alfentanil because of its low affinity for this enzyme. Clinical consequences of polymorphic oxidation of a specific drug are only important when the affected metabolic pathway offers a major contribution to the overall elimination of that drug.[29]

In conclusion, the present *in vitro* experiments show that alfentanil is not metabolized by the cytochrome P-450 form involved in debrisoquine 4-hydroxylation. Therefore, interindividual differences in the pharmacokinetics and pharmacodynamics of alfentanil as a consequence of a genetic deficiency related to the debrisoquine polymorphism are not to be expected.

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