(Received February 29, 1996; accepted May 28, 1996)

ABSTRACT:

The microsomal metabolism of fentanyl, a synthetic opioid commonly used in anesthesia, was investigated in human liver. Incubation of fentanyl with human hepatic microsomes fortified with NADPH resulted in the formation of a single major metabolite, namely norfentanyl, as determined by GC/MS. No evidence was obtained for the formation of either desproprionylfentanyl or *N*-phenylpropionamide, the latter arising *via* *N*-dealkylation of the fentanyl amide nitrogen. Kinetic analysis of microsomal fentanyl oxidation revealed a single K_m of 117 μM and a V_{max} of 3.86 nmol of norfentanyl formed/min/nmol of cytochrome P450 (P450). Studies using chemical inhibitors of human P450 enzymes revealed that only agents known to inhibit CYP3A4 (*e.g.* ketoconazole and erythromycin) were capable of strongly inhibiting (≥90%) microsomal fentanyl oxidation. Marked inhibition (>90%) of norfentanyl formation by liver microsomes was also observed with polyclonal antibodies to CYP3A4, whereas antibodies to other human P450s were without effect. Furthermore, rates of norfentanyl production by 10 individual human liver samples were highly correlated (r^2 = 0.876, F = 56.46, p < 0.001) with immunochemically determined levels of CYP3A4 present in the samples but not with levels of CYP2C8, CYP2C9, CYP2C19, or CYP2E1. Our results indicate that CYP3A4 is the major catalyst involved in fentanyl oxidation to norfentanyl in human liver. Alterations in CYP3A4 levels or activity, as well as the concomitant administration of other therapeutic agents metabolized by this P450 enzyme, could lead to marked perturbations in fentanyl disposition and, hence, analgesic response.

Fentanyl is a synthetic opioid that is widely used in surgical procedures requiring analgesia. The organ primarily responsible for fentanyl biotransformation is the liver (1–4). Lehmann *et al.* (3) first showed that, upon administration to rats, fentanyl was oxidized to a variety of polar products and that pretreatment of animals with phenobarbital increased fentanyl oxidation by liver homogenates to one such product, namely phenylacetate. In other *in vivo* studies, norfentanyl has been identified as the primary oxidative metabolite of fentanyl (5–7), although desproprionylfentanyl may also be formed (8). Norfentanyl appears to be the major metabolite produced in humans (6) (fig. 1).

Sufentanil and alfentanil are two newer anesthetics that, like fentanyl, belong to the anilidopiperidine class of synthetic opioids. *In vivo* and *in vitro* studies of sufentanil and alfentanil disposition have shown that liver microsomal P450[1] enzymes participate in the metabolism of both of these compounds (9–12). Sufentanil undergoes dealkylation at both the piperidine nitrogen and the piperidine ring, to form norsufentanil and SM6, respectively (10). Alfentanil also undergoes piperidine nitrogen dealkylation, which gives rise to noralfentanil, and the P450 responsible has been identified as CYP3A4[2] (11, 12). Yun *et al.* (11) used chemical inhibitors, CYP3A4 antibodies, and purified P450 enzymes to demonstrate that CYP3A4 was responsible for most noralfentanil formation occurring in human liver. By using Western blot analysis, Kharasch and Thummel (12) found that alfentanil me-

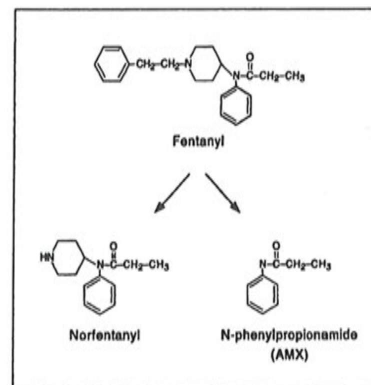

Fig. 1. *Structures of fentanyl and potential oxidative metabolites.*

This work was supported by National Institutes of Health Grant AA07842 (J.M.L.), by a Mount Sinai Department of Anesthesiology Research Development Award (D.E.F.), and by the Liver Tissue Procurement and Distribution System (National Institutes of Health Grant DK62274). Portions of this work were presented at the 22nd Annual Meeting of the American Society for Anesthesiologists, May 1995, in Atlanta, GA.

[1] Abbreviations used are: P450, cytochrome P450; AMX, *N*-phenylpropionamide; MSD, mass-selective detector; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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[2] The P450 enzymes referred to in this report are designated according to the nomenclature of Nelson *et al.* (37).

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correlation analyses. The results described herein indicate that the capacity of human liver microsomes to convert fentanyl to its major oxidative metabolite norfentanyl is indeed due to the presence of CYP3A4.

Methods

Human Liver Specimens. Human liver samples were obtained from the Liver Transplant, Procurement, and Distribution System (University of Minnesota, Minneapolis, MN). None of the subjects had a known history of alcohol or drug abuse. The livers were removed within 30 min of death, frozen in liquid nitrogen, and stored at −80°C until microsomes could be isolated.

Preparation of Microsomes. Liver samples were thawed in ice-cold 100 mM Tris-HCl buffer, pH 7.4, containing 100 mM KCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, and were then homogenized in 4 volumes of the same buffer with two 40-sec bursts in a Waring blender. The mixture was further homogenized with a motor-driven Teflon/glass tissue grinder. Microsomes were then prepared as described elsewhere (14). The pyrophosphate-washed microsomes were resuspended at a protein concentration of 20–40 mg/ml in 10 mM potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose, and were frozen at −80°C until use. Protein and P450 contents were determined using the bicinchoninic acid procedure (15) and according to the method of Omura and Sato (16), respectively.

Enzyme Purification. CYP3A4 was purified from human liver microsomes from subject 920908 with a modification of the methods used for isolating CYP2E1, CYP2C8, and CYP2C9 (14, 17). Potassium phosphate buffers used for the purification contained 1 mM dithiothreitol, 1 mM EDTA, and 20% glycerol unless noted otherwise. As reported elsewhere (14, 17), CYP2E1-enriched fractions eluted from tryptamine CH-Sepharose 4B with 10 mM potassium phosphate buffer, pH 7.4, containing 0.5% cholate and 1.0% Lubrol PX (buffer D), were subjected to hydroxylapatite chromatography on Hypatite C (Clarkson Chemical Co., Williamsport, PA). The P450 recovered (75 nmol) during elution of the Hypatite C column with 500 ml of 50 mM potassium phosphate buffer, pH 7.4, containing 0.5% Lubrol PX, was treated with XAD-2 resin (75 mg of resin/ml) to reduce the detergent content, concentrated 15-fold by ultrafiltration through an Amicon PM-30 membrane, and exhaustively dialyzed against 5 mM potassium phosphate buffer, pH 7.7. The sample was then subjected to anion-exchange chromatography on DE-53 cellulose (1.5 × 28 cm) as described for CYP2E1 (17), except that the column was developed with a linear gradient of 0–0.15 M KCl prepared in 5 column volumes of equilibration buffer. Analysis of column fractions by SDS-PAGE and immunoblotting with anti-HL_p (see below) revealed that the first A_{417} peak, which eluted at 30 mM KCl, contained a single major hemoprotein that reacted strongly with anti-HL_p. These CYP3A4-enriched fractions were pooled (11 nmol of P450 total), dialyzed against 20 volumes of 10 mM potassium phosphate buffer, pH 6.5, containing 0.5% Lubrol PX, for 3 hr at room temperature, and then applied to a 1.5- × 6-cm CM-Sepharose column equilibrated with the same buffer used for dialysis. Upon charging, the column was washed consecutively with 5 volumes of equilibration buffer containing 50 mM KCl and 5 volumes of equilibration buffer containing 100 mM KCl. CYP3A4 was then eluted from the cation-exchange resin as a broad peak with a linear gradient of 100–300 mM KCl prepared in 15 column volumes of equilibration buffer. A flow rate of 0.5 ml/min was used during sample loading, column washing, and gradient elution. Column fractions containing purified CYP3A4, as assessed by SDS-PAGE, were pooled and then subjected to hydroxylapatite chromatography to remove nonionic detergent (17). The final enzyme preparation was extensively dialyzed against 100 mM potassium

were raised in male New Zealand white rabbits, and the IgG fraction was mad[e] monospecific as described elsewhere (19). Polyclonal anti-human CYP2C and anti-human CYP2A6 IgGs were prepared in an identical fashion excep[t] that these antibody preparations were nearly monospecific as isolated and th[us] did not require back-adsorption. Preimmune (control) IgG was prepared fro[m] rabbit serum obtained before immunization. All IgG fractions were purifie[d] from serum by caprylic acid/ammonium sulfate fractionation (20).

Protein blotting of liver microsomal proteins to nitrocellulose was pe[r]formed as described previously (19, 21). After transfer, the blots were reacte[d] with anti-3A4 IgG (5 μg of IgG/ml) or, in the case of partially purifie[d] CYP3A4 fractions, with anti-HL_p IgG (2 μg/ml) and were then immunochem[i]cally stained using a streptavidin-biotinylated horseradish peroxidase syste[m] (19, 21). Immunoreaction intensity was assessed by first scanning the blo[t] with a Sharp JX-325 flat-bed scanner interfaced to a computer and then usin[g] ImageQuant software (Molecular Dynamics, Sunnyvale, CA) to quantita[te] anti-3A4-immunoreactive areas on the image. Immunochemical staining wa[s] performed under conditions where reaction density on the blots was directl[y] proportional to the amount of microsomal protein originally applied to th[e] polyacrylamide gel.

Microsomal Fentanyl Oxidation. Reaction mixtures contained huma[n] liver microsomes (protein equivalent to 100–200 pmol of P450) and 100 m[M] potassium phosphate buffer, pH 7.4, in a final volume of 2.0 ml. The concen[tration] of fentanyl used was 250 μM except in kinetic analysis experiment[s] where the substrate concentration was varied from 5 to 500 μM. Incubation[s] were initiated with 1 mM NADPH and were terminated after 10 min at 37°[C] with 1.0 ml of 10% NaOH. After addition of 2 μg of the internal standar[d] R031980 (a compound structurally similar to fentanyl), the reaction mixture[s] were extracted either with methylene chloride (22) for assessment of norfen[tanyl] formation or with two 3.0-ml portions of ethyl acetate for assessment o[f] AMX formation. The organic extracts were dried under a stream of argon[,] followed by resolubilization in 30 μl of acetonitrile and placement int[o] microvial inserts. The samples (2.0 μl) were injected automatically onto [a] Hewlett-Packard model 5890 Series II gas chromatograph, with the purg[e] valve closed for the first 1 min (splitless mode), and were resolved by usin[g] either a DB-5 capillary column (30 m × 0.25 mm; 0.25-μm film thickness) o[r] a DB-17 capillary column (15 m × 0.25 mm; 0.25-μm film thickness) (J & W Scientific, Folson, CA). A Hewlett-Packard model 5972 MSD was used in th[e] selective ion mode to quantitate fentanyl metabolites, using the following $m/[z]$ ratios for detection: 83, 159, and 175 for norfentanyl; 44, 118, 146, and 189 fo[r] desproprionylfentanyl; 57, 93, and 149 for AMX; and 140, 187, and 351 for th[e] internal standard. Helium was used as the carrier gas, at a flow rate of 0.[…] ml/min (DB-5) or 2.1 ml/min (DB-17). Initial temperature settings for th[e] oven, injector, and MSD were 100°C, 225°C, and 280°C, respectively. Th[e] oven temperature was increased at a rate of 10°C/min to 300°C over the firs[t] 20 min and was held constant for the remaining 11 min of the analysis; th[e] MSD was usually turned off between 16 min and 21 min to protect the filamen[t] from the very large fentanyl peak. Rates of fentanyl metabolite formation wer[e] determined by comparison with standard curves constructed using know[n] amounts of authentic norfentanyl or AMX (see below).

Reagents. Fentanyl, norfentanyl, and desproprionylfentanyl were purchase[d] from Alltech Inc. (Darfield, IL). The internal standard R031980 was fro[m] Research Diagnostics Inc. (Flanders, NJ). Rabbit antibodies to human HL_p (*i.e.* CYP3A3) were generously provided by Dr. Steven Wrighton, Lilly Researc[h] Laboratories. AMX was synthesized by adding 2 g of aniline to 8 ml o[f] proprionic anhydride and allowing the mixture to react for 5 min at roo[m] temperature. Fifty milliliters of 1 N HCl were then added with vigorous stirrin[g]

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here allowed for sensitive detection of at least three potential fentanyl derivatives, including norfentanyl, desproprionylfentanyl, and AMX (fig. 2*A*). The total-ion chromatogram in fig. 2*C* shows the presence of two prominent peaks, with retention times of 5.0 and 10.8 min, that were formed upon incubation of fentanyl with liver microsomes in the presence of NADPH. Mass spectra of these peaks were essentially identical (97–99% similarity) to the published mass spectra of AMX and norfentanyl, respectively. In contrast to norfentanyl formation, however, AMX formation was not dependent upon microsomal P450 enzymes, because similar amounts of this compound were found in incubations performed in the absence of NADPH (fig. 2*B*) or with microsomes omitted (data not shown). In addition, no evidence for desproprionylfentanyl formation by human liver microsomes was found, at least under the experimental conditions used here.

Norfentanyl production by human liver microsomes was linear with respect to time of incubation (up to 15 min) and microsomal P450 concentration (up to 200 pmol) (fig. 3). Formation of this metabolite, which proceeds *via* dealkylation of the fentanyl piperidine nitrogen, was strictly dependent upon the presence of NADPH as well as substrate in the reaction mixtures. Although some AMX was found in all incubations with hepatic microsomes, amounts of the compound formed bore no relationship to incubation time, microsomal protein concentration, or the presence or absence of NADPH.

Kinetic parameters of microsomal fentanyl oxidation were determined using substrate concentrations ranging from 5 to 500 μM (fig. 4). The conversion of fentanyl to norfentanyl exhibited typical Michaelis-Menton kinetics. The Eadie-Hofstee plot shown in fig. 4, *right*, was used to derive the apparent K_m and V_{max} for the reaction, which were 117 μM fentanyl and 3.86 nmol of norfentanyl formed/min/nmol of microsomal P450, respectively. Regression analysis, which was used to fit the data to a straight line, assuming involvement of a single enzymatic component, gave a correlation coefficient of 0.949.

Inhibition Studies. P450 chemical inhibitors and specific antibodies were used to identify the human P450 enzyme(s) involved in hepatic fentanyl oxidation to norfentanyl. The effects of various chemical inhibitors on fentanyl oxidation by liver microsomes are presented in table 1. Of the different agents tested, only those compounds known to inhibit CYP3A4 activity, namely ketoconazole, 7,8-benzoflavone, and erythromycin, were capable of blocking fentanyl conversion to norfentanyl. Ketoconazole was the most potent, inhibiting essentially all norfentanyl formation at a concentration of 5 μM, whereas the weaker inhibition noted with erythromycin was dose-dependent. In contrast, the CYP1A2 inhibitor furafylline, the CYP2D6 inhibitor quinidine, the CYP2C9 inhibitor tolbutamide, and the CYP2E1 inhibitors 4-methylpyrazole and ethanol either had no effect on fentanyl oxidation or slightly stimulated the reaction.

Because we had hypothesized that fentanyl, like alfentanil, was metabolized primarily by CYP3A4, we purified this P450 from human liver (see *Methods*) and produced antibodies to the enzyme for use as immunochemical probes. Before their use, however, the CYP3A4 antibodies were characterized in terms of their immunological prop-

using an anti-CYP3A4 IgG to microsomal P450 ratio of 7.5 mg/nmo which was optimized in preliminary experiments; ratios greater tha this gave no additional inhibition of the reaction (data not shown). I contrast, antibodies against two other human P450 enzymes, name CYP2A6 and CYP2C9, had essentially no effect on microsom fentanyl metabolism. It should be noted that the failure of ant CYP2C9 to inhibit fentanyl oxidation allows us to also rule o participation of CYP2C8 and CYP2C19 in the reaction, because th CYP2C9 antibodies used here not only cross-react with CYP2C8 ar CYP2C19 but also inhibit their catalytic activity (23).

Correlation of Microsomal Fentanyl Oxidation with CYP3A Content. Protein blots similar to that shown in fig. 5 were used t quantitate CYP3A4 levels in 10 individual human liver sample CYP3A4 content was found to vary >7-fold among the specimen (fig. 6). These data were then compared with rates of fentanyl oxida tion in the same samples, which also exhibited considerable variatio (0.28–1.48 nmol of norfentanyl produced/min/mg of protein). Nev ertheless, a strong correlation ($r^2 = 0.876, F = 56.46, p < 0.001$) wa obtained between these two parameters (fig. 6). In fact, in the on subject (UC9402) where the level of CYP3A4 was below detectio rates of norfentanyl formation were also near the limits of detectio (0.01 nmol of norfentanyl produced/min/mg of protein). No signif cant correlation was found between rates of microsomal fentany oxidation and the levels of CYP2E1 ($r^2 = 0.092$), CYP2C8 (r^2 = 0.288), and CYP2C9 ($r^2 = 0.197$) in human liver microsomes; con tents of these latter P450s were quantitated by immunoblotting wit the corresponding antibodies in a manner similar to that used fo CYP3A4 (data not shown).

Discussion

In the present study, we have shown that fentanyl is metabolized *i vitro* to a single major metabolite, namely norfentanyl, by human live microsomes. The kinetic parameters associated with fentanyl dealky lation at the piperidine nitrogen were consistent with participation o a single P450 enzyme in the reaction. This P450 enzyme was subse quently identified as CYP3A4. Chemical inhibitors of CYP3A4 ac tivity, including ketoconazole and 7,8-benzoflavone (24), as well a the CYP3A4 substrate erythromycin (25), proved to potently inhib microsomal fentanyl oxidation to norfentanyl. Antibodies to CYP3A also markedly inhibited norfentanyl formation by human liver micro somes, whereas antibodies to other human P450 enzymes were with out effect. In addition, we found an excellent correlation ($r^2 = 0.876$ between microsomal CYP3A4 content and rates of fentanyl oxidation.

Fentanyl is the synthetic opioid analgesic most often used b

[3] At least three different but highly homologous enzymes belonging to th CYP3A subfamily are expressed in human liver. CYP3A4, CYP3A5, and CYP3A are found in adults, whereas only the latter two P450s are expressed in fetal an neonatal liver (38).

[4] While this manuscript was being prepared, a report appeared implicatin CYP3A4 as the predominant fentanyl *N*-dealkylating enzyme present in huma liver microsomes (40).

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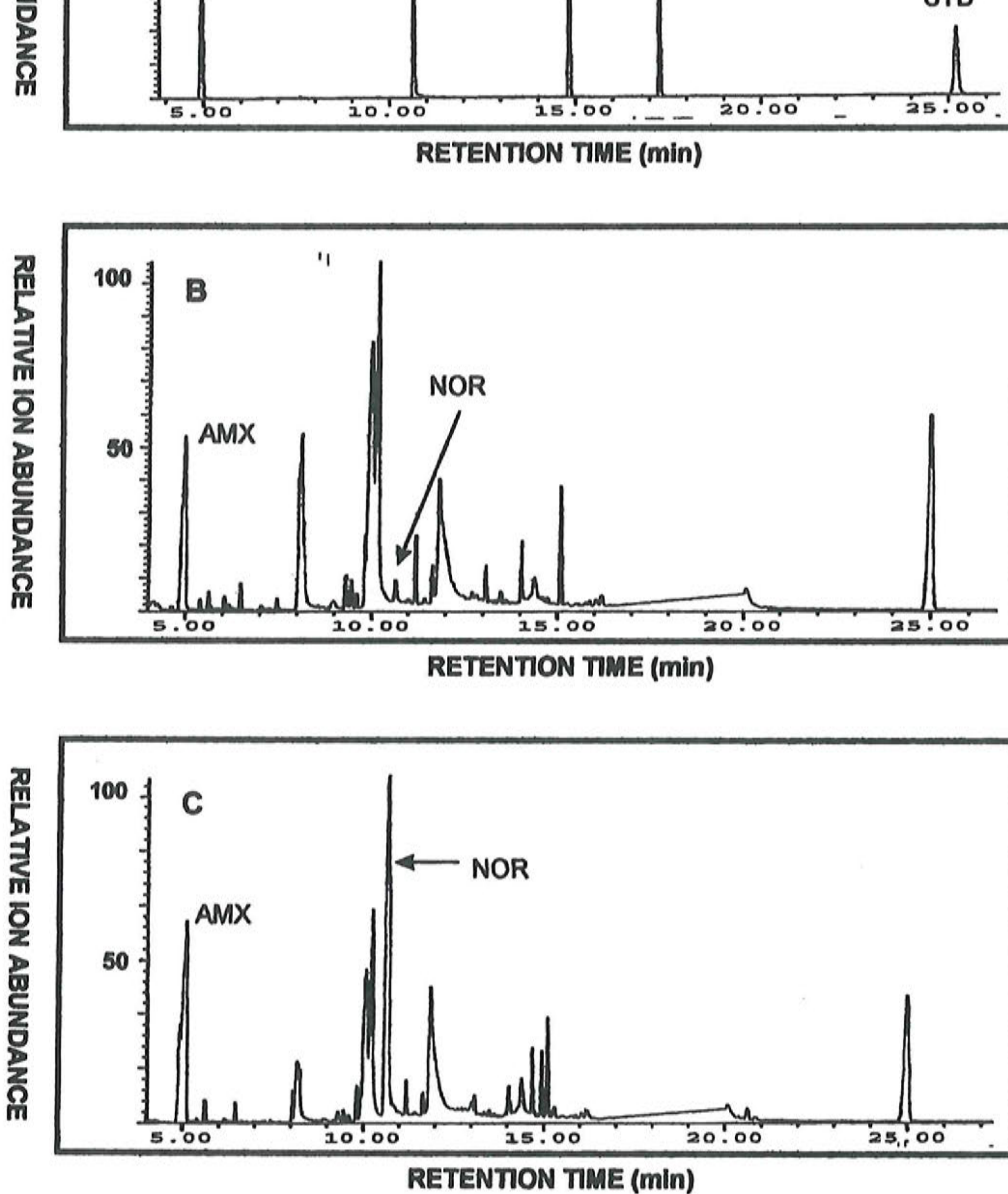

FIG. 2. *GC/MS analysis of fentanyl metabolism by human liver microsomes.*

A, Representative total-ion chromatogram obtained upon GC/MS analysis of a mixture containing authentic fentanyl (*FEN*), AMX, norfentanyl (*NOR* desproprionylfentanyl (*DES*), and the internal standard R031980 (*STD*). *B*, Typical total-ion chromatogram obtained upon incubation of human liver microsome with fentanyl in the absence of NADPH. *C*, Analogous incubation to which NADPH was added. The fentanyl peak cannot be observed in *B* and *C*, because th MSD was shut off during the time period (16.5–20 min) when this compound eluted from the GC column (see *Methods*).

anesthesiologists. Characterization of its metabolic disposition is thus clinically important, because such information can aid in predicting the factors that can affect fentanyl metabolism or that of other drugs given concurrently. Fentanyl is extensively metabolized in patients, and <5% of the drug is excreted unchanged (7). Our finding tha CYP3A4 is the primary fentanyl-oxidizing enzyme in human liver *i vitro* predicts that fentanyl could be subject to extensive drug inte actions *in vivo*, especially when given in the high doses (100 μg/kg

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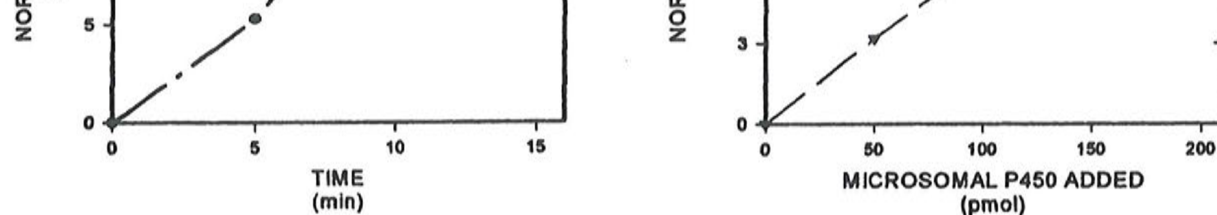

FIG. 3. *Metabolism of fentanyl by human liver microsomes: relationship to time of incubation (A) and P450 concentration (B).*

Fentanyl (250 μM) was incubated with human liver microsomes (protein equivalent of 0.05–0.2 nmol of P450) for 5–15 min at 37°C, in the presence NADPH. Norfentanyl formation was then assessed by GC/MS, as described in *Methods*.

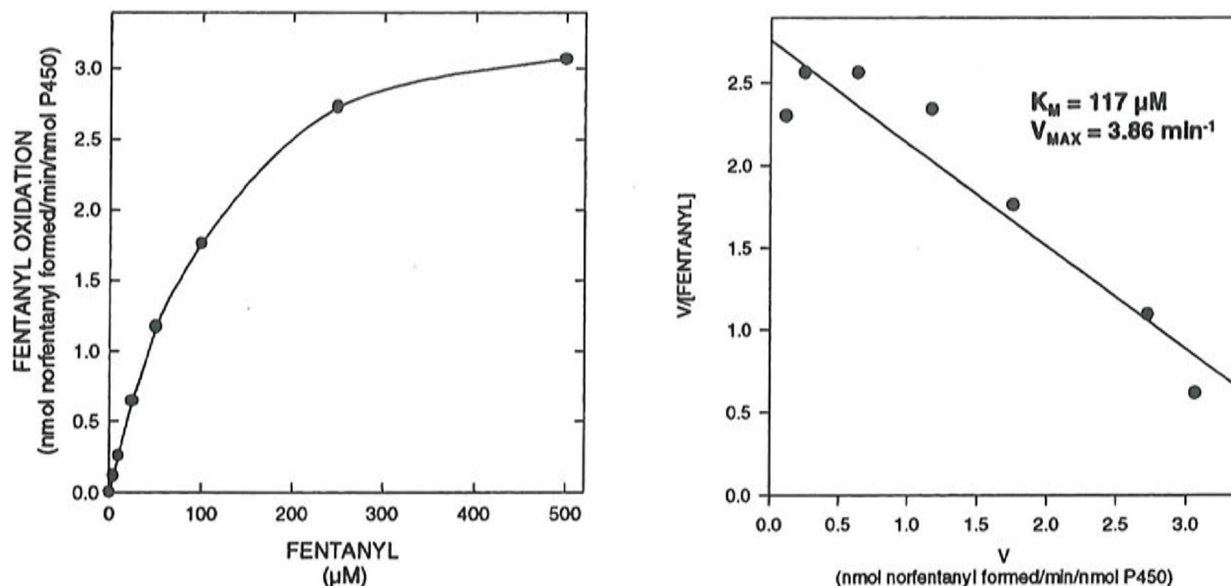

FIG. 4. *Kinetic analysis of fentanyl oxidation by human liver microsomes.*

Fentanyl metabolism was determined in incubation mixtures (2.0 ml) containing 100 mM Tris-HCl buffer, pH 7.4, liver microsomes from subject UC941 (protein equivalent to 100 pmol of P450), 0.5 mM NADPH, and fentanyl in concentrations ranging from 5 to 500 μM. Reactions were initiated with NADP and terminated after 10 min at 37°C. Norfentanyl formation was quantitated by GC/MS, as described in *Methods*. *Left*, plot of reaction velocity *vs.* fentan concentration; *right*, Eadie-Hofstee transformation of the same data. The apparent K_m and V_{max} were derived by fitting the results to a single-compone Michaelis-Menten equation.

required for profound analgesia. In such cases, oxidative metabolism (presumably CYP3A4-mediated) is the primary means for terminating the pharmacological effects of fentanyl. The list of therapeutic agents metabolized by CYP3A4 is exhaustive and includes such commonly used drugs as nifedipine (26), midazolam (27), erythromycin (25), cyclosporine (28), lovastatin (29), and lidocaine (30, 31). Pharmacokinetic interactions (*e.g.* decreased drug clearance) occur frequently among CYP3A4 substrates, such as erythromycin, midazolam, and lovastatin, and also upon concomitant administration of the CYP3A4 inhibitor ketoconazole (32–34). Because the Michaelis constants (*i.e.* K_m) for metabolism of these agents by human liver microsomes (72–119 μM for midazolam 4-hydroxylation and 100 μM for lovastatin 6′-β-hydroxylation) (29, 35)[5] are of the same order of magn tude as that described here for fentanyl (117 μM), the potential fc competitive-type interactions with fentanyl at the site of metabolisı should be considered. Although there have been few published report concerning adverse drug interactions with fentanyl, this is not su prising when one considers that patients receiving high doses of th analgesic require mechanical ventilation during the postoperative pe riod; untoward reactions such as respiratory depression because c decreased fentanyl clearance could be easily overlooked during thi time. In the single case report on drug interactions with the syntheti

[5] R. G. Wang, personal communication.

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