FENTANYL METABOLISM BY HUMAN HEPATIC AND INTESTINAL CYTOCHROME P450 3A4: IMPLICATIONS FOR INTERINDIVIDUAL VARIABILITY IN DISPOSITION, EFFICACY, AND DRUG INTERACTIONS

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ABSTRACT:
The synthetic opioid fentanyl undergoes extensive metabolism in humans. Systemic elimination occurs primarily by hepatic metabolism. When administered as a lozenge for oral transmucosal absorption, swallowed fentanyl is subject to first pass metabolism in the liver and possibly small intestine. Little is known, however, about the identity and formation of human fentanyl metabolites. This investigation identified routes of human liver microsomal fentanyl metabolism and their relative importance, tested the hypothesis that fentanyl is metabolized by human duodenal microsomes, and identified the predominantly responsible cytochrome P450 isoforms. A GC/MS assay using deuterated internal standards was developed for fentanyl metabolites. Piperidine N-dealkylation to norfentanyl was the predominant pathway of liver microsomal metabolism. Amid hydrolysis to despropionylfentanyl and alkyl hydroxylation to hydroxyfentanyl were comparatively minor pathways. Hydroxynorfentanyl was identified as a minor, secondary metabolite arising from N-dealkylation of hydroxyfentanyl. Liver microsomal norfentanyl formation was significantly inhibited by the mechanism-based P450 3A4 inhibitor troleandomycin and the P450 3A4 substrate and competitive inhibitor midazolam, and was significantly correlated with P450 3A4 protein content and catalytic activity. Of six expressed human P450 isoforms (P450s 1A2, 2B6, 2C9, 2D6, 2E1, and 3A4), only P450 3A4 exhibited significant fentanyl dealkylation to norfentanyl. These results indicate the predominant role of P450 3A4 in the primary route of hepatic fentanyl metabolism. Human duodenal microsomes also catalyzed fentanyl metabolism to norfentanyl; the average rate was approximately half that of hepatic metabolism. Rates of duodenal norfentanyl formation were diminished by troleandomycin and midazolam, and were significantly correlated with P450 3A4 activity, suggesting a prominent role for P450 3A4. These results demonstrate that human intestinal as well as liver microsomes catalyze fentanyl metabolism, and N-dealkylation by P450 3A4 is the predominant route in both organs. The fraction of fentanyl lozenge that is swallowed likely undergoes significant intestinal, as well as hepatic, first-pass metabolism. Intestinal and hepatic first-pass metabolism, as well as systemic metabolism, may be subject to individual variability in P450 3A4 expression and to drug interactions involving P450 3A4.

The synthetic opioid fentanyl, after iv administration, is cleared predominantly by hepatic biotransformation (1,2). Fentanyl metabolism is extensive and rapid. Less than 8% administered iv to volunteers was eliminated unchanged, with approximately 6% appearing in urine and 1% excreted in the stool (1), and only 2% was eliminated intact in the urine of surgical patients (3). Metabolites appeared in plasma within 2 min, and plasma metabolite radioactivity exceeded that of parent drug after 30 min (1). More than 80% of the dose was recovered as metabolites, with 76% appearing in the urine and 8% in feces (1). Although these early investigations established the importance of fentanyl biotransformation, the identity of the metabolite(s) was not established.

A few subsequent investigations identified some fentanyl metabolites in humans (fig. 1). The major route of metabolism is N-dealkylation to norfentanyl [4-N-(N-propionylanilino)piperidine] (4), which has been recovered in plasma (5) and urine (4, 6). Considerably less is known, however, about other routes of human fentanyl metabolism. Van Rooy et al. found norfentanyl and despropionylfentanyl [1-(2-phenylethyl)-4-N-anilinopiperidine] in varying amounts in plasma, but no other metabolites were sought (5). In a more thorough evaluation, norfentanyl was identified as the most abundant metabolite and lesser amounts of hydroxyfentanyl [4-N-(N-hydroxypropionylanilino)piperidine] were found in the urine of all patients (4). Small amounts of hydroxynorfentanyl [1-(2-phenylethyl)-4-N-hydroxypropionylanilino)piperidine] were detected in two of five patients. Hydroxynorfentanyl may be formed either from hydroxyfentanyl or norfentanyl; however, the metabolic origin of hydroxynorfentanyl is unknown. Despropionylfentanyl was not recovered in any patient, in contrast to the report of Van Rooy et al. Unlike fentanyl metabolism in rats (7), no piperidine ring-hydroxylated metabolites were observed in human urine (4). More recently norfentanyl, but not despropionylfentanyl, was found in human urine (6). The fentanyl analogue alfentanil undergoes amide N-dealkylation to N-phenylpropionamide as one of two major routes of metabolism (8); however, it is not known whether fentanyl undergoes similar N-dealkylation. Since fentanyl metabolites possess no significant pharmacological activity (9), the multiple potential routes of fentanyl deactivation and elimination are of substantial therapeutic importance. Nevertheless, there is considerable disagreement and ambiguity regarding human fentanyl metab-
Fentanyl formulation in a solid sucrose matrix designed for oral transmucosal absorption and for the treatment of cancer pain (10, 11). The overall bioavailability of fentanyl in this lozenge form is 50%, representing both rapid transmucosal absorption and more prolonged gastrointestinal absorption of swallowed fentanyl (12). Approximately 25% of the total dose is absorbed from the buccal mucosa, thereby bypassing first-pass metabolism. The remaining 75% of the dose is swallowed, and approximately 1/3 (25% of the total dose) escapes first-pass metabolism. Gastrointestinal absorption of fentanyl is subject to first-pass metabolism in the liver. In addition, human duodenum and jejunum, and to a lesser extent ileum, contain significant amounts of certain cytochrome P450 isoforms (13–17). The role of intestinal P450 in the first-pass metabolism of orally administered drugs has been increasingly recognized (18, 19). Therefore, swallowed fentanyl may also undergo first-pass metabolism in the proximal small intestine. The existence and importance of intestinal fentanyl metabolism, however, is unknown. Therefore, the second objective of this investigation was to establish whether fentanyl is metabolized by human duodenal microsomes. The last objective was to identify the P450 isoform(s) responsible for fentanyl metabolism by human liver and intestinal microsomes. Human hepatic microsomal fentanyl N-dealkylation by P450 3A4 was recently reported (20).

To accomplish these objectives, the major putative fentanyl metabolites were synthesized along with their deuterated analogs for use as internal standards for metabolite assays, and analytical methods for the identification and quantitation of fentanyl metabolites were developed. Although analytical techniques for the quantification of some unlabeled fentanyl metabolites have been reported (4–7) and a norfentanyl assay using radiolabeled fentanyl has recently been described (20), our objective was to develop a comprehensive mass spectrometric assay using deuterated internal standards that quantified all known metabolites and avoided radiolabeled fentanyl. Optimum extraction, derivatization, and chromatographic conditions for these metabolites were identified.

Materials and Methods

Materials. PFMA and BSTFA-TMCS were purchased from Pierce (Rockford, IL). Sulfaphenazole was the generous gift of William F. Trager (University of Washington, Seattle, WA). HPLC grade ethyl acetate (J. T. Baker, Phillipsburg, NJ) was used for extractions and Optima grade ethyl acetate (Fisher Scientific, Santa Clara, CA) dried over molecular sieves was used for sample derivatization and reconstitution. Silica gel (200–400 mesh, 60A) from Aldrich (Milwaukee, WI) was used for flash chromatography. Microsomes containing individual cDNA-expressed cytochrome P450 isoforms were purchased from Genest Corp. (Woburn, MA). Unless otherwise specified, all other chemicals were from Aldrich or Sigma (St. Louis, MO).

Synthesis of fentanyl metabolites. Intermediates in multi-step syntheses were identified by thin layer chromatography (TLC) and characterized by 1H NMR (Varian XLR 300) and infrared spectroscopy (Perkin-Elmer Model 1600 FTIR, Norwalk, CT), and gas chromatography-mass spectrometry (GC-MS; Hewlett-Packard 5890 Series II GC-5972 MSD, Wilmington, DE). Unlabeled and d5-labeled compounds were crystallized as oxalate salts. Identification and purity were established by 1H NMR, GC-MS, and fast atom bombardment mass spectrometry, both as native compounds and as PFP or TMS derivatives.

Synthesis of: Norfentanyl [4-N-(N-propionylanilino)piperidine].

N-benzyl-4-anilino piperidine (A): N-benzyl-4-piperidone (3.7 ml, 0.02 mole) was dissolved in anhydrous toluene (20 ml). Freshly distilled aniline (1.8 ml, 0.02 mole) was added along with a catalytic amount of p-toluene sulfonic acid. The mixture was stirred at 35°C for 2 hr. Toluene was removed under vacuum, and the mixture was cooled in ice water and sodium borohydride (0.9 g, 0.025 mole) was added in portions. The reaction mixture was allowed to reach room temperature and stirred for 2 hr. Reaction progress was monitored by TLC (silica, 50% ethyl acetate). Methanol was removed on a rotary evaporator and the residue was dissolved in ethyl acetate (50 ml). The organic layer was washed with water (2 × 10 ml) and brine (10 ml), dried with anhydrous sodium sulfate, filtered, and concentrated in vacuo. The compound was purified by flash chromatography (ethyl acetate/hexane 1:1) and collected in 20-ml fractions. Pure fractions were pooled and concentrated to yield 77% of (A) as a brownish yellow solid. 1H NMR (CDCl3): δ 1.4–1.55 (m, 2H, —CH2 —), 1.98–2.2 (two sets of m, 4H, two —CH2 —), 2.8–2.9 (m, 2H, —CH2 —), 3.23–3.35 (m, 1H, tertiary proton), 3.51–3.52 (s, 2H, —CH2—CH2—), 6.55–6.70 and 7.1–7.4 (two sets of multiplets, 10 aromatic protons). GC-MS: m/z: 91 (—CH2—CH2—), 266 (M+173 (M+—C6H5—)).

N-benzyl-4-propionylanilido piperidine (B): N-benzyl-4-anilino piperidine (1.6 g, 0.006 mole) was dissolved in anhydrous dichloromethane (10 ml). Pyridine (0.48 ml, 0.006 mole) was added, followed by propionyl chloride (1.04 ml, 0.012 mole) at ice-cold temperature. The progress of the reaction was followed by TLC (silica, ethyl acetate/hexane 1:1). The reaction was complete in 2–3 hr. The mixture was washed with water and brine, and the organic layer was dried over molecular sieves and concentrated in vacuo. The compound was purified by flash chromatography (ethyl acetate/hexane 1:1) and collected in 20-ml fractions. Pure fractions were pooled and concentrated to yield 77% of (B) as a brownish yellow solid. 1H NMR (CDCl3): δ 1.3–1.55 (m, 2H, —CH2—), 1.98–2.2 (two sets of m, 4H, two —CH2—), 2.8–2.9 (m, 2H, —CH2 —), 3.2–3.35 (m, 1H, tertiary proton), 3.51–3.52 (s, 2H, —CH2—CH2—), 6.55–6.70 and 7.1–7.4 (two sets of multiplets, 10 aromatic protons). GC-MS: m/z: 91 (—CH2—CH2—), 266 (M+173 (M+—C6H5—)).

1 Abbreviations used are: PFMA, pentfluoropropionic anhydride; BSTFA-TMCS, N,O-bis(trimethylsilyl)trifluoroaceticamide with 1% trimethylchlorosilane; PFP, pentfluoropropionyl; TMS, trimethylsilyl.
was dried over anhydrous magnesium sulfate, gravity filtered, and concentrated in vacuo to produce (B) in 50% yield. 1H NMR (CDCl3): δ 0.95–1.5 (t, 3H, –CH3), 1.3–1.45 (m, 2H, –CH2), 1.7–1.8 (m, 2H, –CH2), 1.85–1.95 (q, 2H, –CH2-CH3), 2.05–2.15 (m, 2H, –CH2), 2.8–2.9 (m, 2H, –CH2), 3.4–3.45 (s, 2H, –CH2-CH2CH3), 4.6–4.7 (m, 1H, tertiary proton), 7.0–7.45 (m, 10 aromatic protons). GC-MS: m/z 91 (→CH2CH3) (100), 322 (M+), 265 (M+→COCH2CH3).

Norfentanyl (C): N-benzyl-4-propylinilido piperidine (B) (30 mg) was dissolved in 2 ml of 50% ethanol/water. The compound was hydrogenated with 10% palladium/carbon on a Parr shaker for 20 hr. The catalyst was filtered under suction and the filtrate concentrated to dryness. The residue was dissolved in anhydrous 2-propanol, and one equivalent of oxalic acid in 2-propanol was added. The reaction was monitored by extracting a small sample with a mass spectrum corresponding to norfentanyl. 1H NMR (CDCl3): δ 0.95–1.05 (t, 3H, →CH3), 1.5–1.68 (m, 2H, →CH2), 1.90–2.1 (m, 4H, two methylenes, →CH2 and →CH2-CH3), 3.0–3.18 (m, 2H, →CH2), 3.35–3.45 (m, 2H, →CH2), 4.7–4.85 (m, 1H, tertiary proton), 7.2–7.3 and 7.4–7.6 (m, 5 aromatic protons).

GC-MS: m/z 83 (→CH2CH3 →CH3, CH3NCOCH3) (100), 232 (M+), 175 (M+→COCH2CH3).

Despropionylnorfentanyl [1-(2-phenethyl)-4-N-anilinopiperidine]. N-phenethyl-4-piperidone (4.0 g, 0.02 mole) was dissolved in anhydrous toluene (20 ml). Freshly distilled aniline (1.8 ml, 0.02 mole) was added along with a catalytic amount of p-toluensulfonyl acid. The mixture was stirred at 35°C for 3–4 hr. Toluene was removed under high vacuum and the residual oil was dissolved in anhydrous methanol (15 ml). The solution was cooled in ice water and sodium borohydride (2.2 g, 0.06 mole) was added in small portions. The reaction mixture was warmed and stirred at ambient temperature for 45 min, with monitoring by TLC (silica, 50% ethyl acetate). Methanol was removed in vacuo and the residue was dissolved in ethyl acetate (60 ml). The organic layer was washed with water (2 × 15 ml) and brine (10 ml), dried with anhydrous sodium sulfate, gravity filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (ethyl acetate:hexane 1:1) and collected in 20-ml fractions. Fractions containing the pure product were pooled and concentrated in vacuo to generate despropionylnorfentanyl in 85% yield. The final product showed a single peak on GC with a mass spectrum corresponding to norfentanyl. 1H NMR (CDCl3): δ 1.4–1.6 (m, 2H, →CH2), 2.0–2.28 (m, 4H, two CH2), 2.5–3.0 (three sets of m, 6H, three →CH2), 3.2–3.4 (m, 1H, tertiary proton), 6.5–6.7 and 7.0–7.3 (two sets of m, 10 aromatic protons). GC-MS: m/z 280 (M+), 189 (M+→CH2CH3).

Hydroxynorfentanyl [1-(2-phenethyl)-4-N-(hydroxypropionylamino)piperidine]. N-phenethyl-4-(1-chloropropionylanilido)piperidine (D): Despropionylnorfentanyl (1.68 g, 0.0056 mole) was dissolved in anhydrous dichloromethane (15 ml). Pyridine (0.68 ml, 0.0084 mole) was added, followed by 3-chloropropionyl chloride (800 µl, 0.0084 mole) at ice-cold temperature (0–4°C). The reaction mixture was gradually allowed to reach room temperature and stirred for 3–4 hr. The reaction was monitored by extracting a small sample under basic conditions and monitoring on TLC (silica, ethyl acetate:hexane 2:1). The mixture was extracted with chloroform at neutral pH. The organic layer was washed with water. The product was purified by flash chromatography (ethyl acetate:hexane 1:1) and collected in 20-ml fractions. Fractions containing the pure product were pooled and concentrated to yield (F) as pale oil (0.86 g, 36% yield). 1H NMR (CDCl3): δ 1.3–1.5, 1.7–1.83, 2.0–2.2 and 2.8–2.95 (4m, 8H, four piperidine →CH2), 2.3–2.4 and 3.7–3.8 (two sets of t, 4H, two methylenes →COCH2CH2→), 3.4–3.5 (s, 2H, →CH2N→), 4.6–4.75 (m, 1H, tertiary proton), 7.0–7.5 (m, 10 aromatic protons). GC-MS: m/z 91 (100) (→CH2CH3), 265 (M+→COCH2CH3), 172 (M+→CH2NHCOCH2CH3).

N-benzyl-4(1-benzyloxypropionylanilido)piperidine (G): Sodium metal (460 mg, 0.02 mole), washed with hexane, was added slowly to benzylalcohol (7 ml) and stirred at room temperature until most of the sodium had reacted. N-benzyl-1-chloropropionylanilidopiperidine (F) (0.8 g, 0.002 mole) was added in portions and the reaction was stirred at 68°C for 18 hr. The progress of reaction was monitored by TLC (silica, ethyl acetate:hexane 1:2). The mixture was extracted with ethyl acetate (25 ml), which was then washed with water, dried over anhydrous sodium sulfate, gravity filtered, and concentrated in vacuo. The residual oil was purified by flash chromatography using hexane (250 ml), ethyl acetate:hexane (1:2, 1000 ml) and ethyl acetate:hexane (1:1, 200 ml), collecting 20-ml fractions. Fractions containing the pure product (fractions 15–40) were pooled and concentrated to yield 680 mg (80% yield) of product. The product G was crystallized from anhydrous ether as the oxalate salt. 1H NMR (free base) (CDCl3): δ 1.3–1.45, 1.7–1.8, 2.0–2.15, and 2.8–2.9 (4m, four piperidine →CH2), 2.2–2.29, and 3.65–3.75 (two sets of t, 4H, two methylenes →COCH2CH2→), 4.6–4.7 (m, 1H, tertiary proton), 4.4–4.48 (s, 2H, →OCH2Ph), 3.39–3.45 (s, 2H, →NCH2Ph) 7.0–7.4 (m, 15 aromatic protons).

GC-MS: m/z 91 (100) (→CH2CH3), 265 (M+→COCH2CH3), 172 (M+→CH2NHCOCH2CH3), 320 (M+→CH2OH).

N-phenethyl 4-(1-hydroxypropionylamino)piperidine [hydroxyhydrofentanyl]: The oxalate salt of N-benzyl-4(1-benzyloxypropionylanilidopiperidine (G, 40 mg) was dissolved in 5 ml of ethanol-water (1:1) and hydrogenated on a Parr shaker using 20% Pd(OH)2/C for 20 hr. The reaction was monitored by TLC (silica, CHCl3, MeOH 4:1 v/v). The catalyst was filtered under suction, the filtrate was washed with water, dried over anhydrous sodium sulfate, and concentrated in vacuo to yield (E) as a white solid. The solid was crystallized as the oxalate salt from anhydrous ether to yield 765 mg (76% yield) of the product. 1H NMR (free base) (CDCl3): δ 1.3–1.5, 1.75–1.85, 2.1–2.2, 2.48–2.59, 2.68–2.8 and 2.92–3.05 (6m, 12 H, two phenethyl →CH2 and four piperidine →CH2), 2.2–2.3 and 3.65–3.75 (two sets of t, 4H, two methylenes →COCH2CH2→), 4.6–4.8 (m, 1H, tertiary proton), 4.4–4.5 (s, 2H, →OCH2Ph), 7.1–7.35 (m, 15 aromatic protons).

N-phenethyl 4-(1-hydroxypropionylamino)piperidine [hydroxyhydrofentanyl]: The oxalate salt of N-phenethyl-4(1-benzyloxypropionylanilidopiperidine (E) (100 mg) was dissolved in 5 ml of ethanol-water (1:1) and hydrogenated on a Parr shaker using 20% Pd(OH)2/C for 20 hr. The reaction was monitored by TLC (silica, CHCl3, MeOH 4:1 v/v). The catalyst was filtered under suction, the filtrate was washed with water, dried over anhydrous sodium sulfate, gravity filtered, and concentrated in vacuo (353, M+). GC-MS analysis showed a mass spectrum corresponding to fentanyl alcohol.
concentrated in vacuo, and the product was crystallized from anhydrous diethyl ether to yield norfentanyl alcohol as an off-white solid (20 mg). 1H NMR (oxalate salt) (DMSO): δ 1.35–1.5, 1.75–2.0, 2.8–3.0 and 3.1–3.5 (m, 12 H, four piperidine —CH₂ and two methylene groups —CH₂—CH₂—OH), 4.6–4.75 (m, 1H, tertiary proton), 4.35–4.55 (br s, 1H, —OH) 7.1–7.5 (m, 5 aromatic protons). FAB-MS: molecular weight 249 (M+). GC-MS showed a single peak: m/z 83 (100) (M1−PhNCOCH₂CH₂OH), 248 (M1), 175 (M1−COCH₂CH₂OH).

N-phenylpropionamide was synthesized as described previously (22). Ring-deuterated d₅-norfentanyl, d₅-despropionylfentanyl, d₅-hydroxyfentanyl, and d₅-N-phenylpropionamide were synthesized using similar reaction procedures by replacing aniline with d₅-aniline. Stock solutions of metabolites and standards were prepared with methanol and/or 100 mM potassium phosphate buffer (pH 7.4), as appropriate.

Microsomal Incubations. Microsomes were prepared from livers and mucosal scrapings of duodena obtained from human organ donors as described previously (23, 24). Metabolite formation was determined in 1-ml incubation mixtures containing 0.1M potassium phosphate buffer (pH 7.4), 1 mM NADPH, human liver (routinely 0.05 mg/ml unless indicated) or duodenal (0.1–0.2 mg/ml) microsomes and fentanyl (10 mM unless otherwise indicated) incubated with shaking at 37°C for 7 min. Reactions were initiated by the addition of NADPH after a 3-min preincubation period, and were terminated with 0.2 ml of 0.5 N NaOH. For experiments with CDNA-expressed P450s, the protein concentration was 0.25 mg/ml and the incubation time was 30 min.

For microsomal inhibition experiments, NADPH in the incubation mixtures was replaced by an NADPH generating system (1 mM NADPH, 10 mM glucose-6-phosphate, 1.0 IU glucose-6-phosphate dehydrogenase, and 5 mM MgCl₂). P450 isofrom-selective substitutes and inhibitors were added to the incubation mixtures to provide the following final concentrations: furafylline (20 μM), coumarin (100 μM), orphenadrine (50 μM), sulfaphenazole (50 μM), quinidine (5 μM), diethylthiocarbamate (100 μM), 4-methylpyrazole (500 μM), trolean-doymycin, midazolam, and sulfaphenazole were added in methanol (final solvent concentration of 0.1%, 0.2%, and 0.2%, respectively) and all other inhibitors/substrates were added in 0.1 M potassium phosphate buffer. The competitive inhibitors (sulfaphenazole, midazolam, coumarin, quinidine, and 4-methyl pyrazole) were co-incubated with fentanyl in the reaction mixture. The reaction was initiated by adding the NADPH generating system after a 3-min preincubation at 37°C and terminated after 7 min. The mechanism-based inhibitors (trolean-doymycin, furafylline, diethylthiocarbamate, and orphenadrine) were incubated with microsomes and the NADPH generating system for 10 min. Fentanyl was then added and the mixtures were incubated for an additional 7 min. Rates of metabolite formation were compared with those of controls in which the inhibitor was replaced with buffer or solvent alone.

Analytical Procedures. For norfentanyl and despropionylfentanyl determination, the internal standards d₅-norfentanyl and d₅-despropionylfentanyl (100–200 ng) were added to the alkaline mixture (pH 12–13), followed by sodium chloride (0.5–0.7 g) to saturation. The mixture was then extracted twice with 4-ml ethyl acetate by vortexing for 2 min. The ethyl acetate layers were pooled and dried over anhydrous sodium sulfate for 30–45 min. The ethyl acetate layer was decanted into a clean conical tube, evaporated to dryness at 45°C under a nitrogen stream, and reconstituted in 100-μl ethyl acetate. The PFP derivatives of norfentanyl and despropionylfentanyl and their respective d₅-analogs were prepared by adding 100 μl of pentafluoropropionic anhydride to the ethyl acetate solution and heating at 67°C for 60 min. The samples were evaporated to dryness under a nitrogen stream and dissolved in ethyl acetate (80–100 μl) for GC-MS analysis. For hydroxyfentanyl and hydroxynorfentanyl determination, the corresponding internal standards d₅-hydroxyfentanyl and d₅-hydroxynorfentanyl (200 ng each) were added to the alkaline incubation samples. The extraction procedure was similar to that for norfentanyl and despropionylfentanyl. After evaporating the ethyl acetate, the residue was reconstituted in 50 μl ethyl acetate and derivatized with 50 μl BSTFA-TMCS at 70°C for 2 hr. The samples were injected without removing the excess derivatizing agent. Recovery of norfentanyl, despropionylfentanyl, hydroxyfentanyl, and hydroxynorfentanyl was 50, 83, 87, and 90%, respectively. The fentanyl metabolites were not derivatized with pentafluoropropionic anhydride because both mono- and di-PFP derivatives of hydroxynorfentanyl were obtained, similar to that observed previously for alfentanil metabolites (22). In contrast, silylation afforded only the di-TMS derivatives. N-phenylpropionamide was determined as described previously (22).

GC-MS analyses were performed on a HP 5890 Series II gas chromatograph interfaced to an HP 5972 mass selective detector using a DB-5 or DB-17 fused-silica capillary column (15 m × 0.32 mm ID × 0.25 um or 0.5 um film thickness) (J & W, Folsom, CA). The injector, containing a quartz liner, was operated in the splitless mode. The helium flow rate was 60 ml/min and the column head pressure was 5 psi. Optimum injector and transfer line temperatures for d₅- and d₅-norfentanyl-PFP and d₅- and d₅-despropionylfentanyl, based on maximum peak areas, were 270°C and 270°C, respectively. The initial oven temperature (100°C) was held for 1 min, then increased to 280°C at 8°C/min, and maintained at 280°C for 10 min (unless otherwise indicated in figure legends). Norfentanyl was quantified by selected ion monitoring of norfentanyl-PFP at m/z 150 (M1−piperidine ring) or m/z 322 (M1−COCH₂CH₂OH) and d₅-norfentanyl-PFP at m/z 155 or 327 (fig. 2). Despropionylfentanyl was quantified by selected-ion monitoring of despropionylfentanyl-PFP (m/z 335, M1−CH₂CH₂H₂) and d₅-despropionylfentanyl-PFP (m/z 340) (fig. 2). Optimum injector and transfer line temperatures for hydroxyfentanyl- and hydroxynorfentanyl-TMS and their corresponding d₅-analogs, based on maximum peak areas, were 250°C and 290°C, respectively. The initial oven temperature (70°C) was held for 1 min, then increased to 200°C at 12°C/min, then to 280°C at 70°C/min (unless otherwise indicated in figure legends). Hydroxyfentanyl was quantified by selected-ion monitoring of hydroxyfentanyl-TMS (m/z 333, M1−CH₂CH₂H₂) and d₅-hydroxyfentanyl-TMS (m/z 338) (fig. 2). Hydroxynorfentanyl was quantified by monitoring hydroxynorfentanyl-diTMS at m/z 247 (M1−COCH₂CH₂OTMS) and d₅-hydroxynorfentanyl-diTMS at m/z 252 (fig. 2). N-phenylpropionamide was monitored as the TMS derivative at m/z 221 (M1) or m/z 206 (M1−CH₂H₂), and the deuterated standard was monitored at m/z 226 or m/z 211 (22).

For determination of norfentanyl and despropionylfentanyl, standard curves of peak area ratios d₅/d₅ norfentanyl versus norfentanyl concentration (5–10000 ng/ml) and d₅/d₅ despropionylfentanyl versus despropionylfentanyl concentration (2.5–50 ng/ml) were prepared using blank microsomes. Standard curves (r² = 0.99 for both) were used to quantify norfentanyl and despropionylfentanyl in unknown samples. For determination of hydroxyfentanyl, a standard curve of peak area ratio d₅/d₅ hydroxyfentanyl versus hydroxyfentanyl concentration (2.5–50 ng/ml) was similarly prepared (r² = 0.99) and was used to quantify the metabolite formation in unknown samples. The limits of quantification were the lowest point on the standard curve.

Results

Hepatic Microsomal Metabolism

Incubation of fentanyl (100 μM) with human liver microsomes (1 mg/ml) at 37°C for 30 min yielded norfentanyl as the major metabolite (1440 pmol/min/mg), and despropionylfentanyl (1.1 pmol/min/mg) and hydroxyfentanyl (0.58 pmol/min/mg) were identified as minor metabolites using selected-ion monitoring. Neither hydroxynorfentanyl nor N-phenylpropionamide were observed in this experiment. Norfentanyl formation was confirmed by comparing the mass spectrum of the microsomal peak with that of synthetic norfentanyl at the same retention time (fig. 2C). Since despropionyl-fentanyl and hydroxyfentanyl were formed in very minor amounts, full scan mass spectra could not be obtained (scan mode limits of detection were 300 and 1200 ng/ml for hydroxyfentanyl and hydroxynorfentanyl, respectively) and compared with those of synthetic standards. These metabolites were, however, identified in selected-ion mode by comparing diagnostic ions and retention times with those of the synthetic standards.

Separate experiments confirmed the absence of N-phenylpropionamide formation from fentanyl. Fentanyl (100 μM and 500 μM) was incubated with human liver microsomes (1 mg/ml) for 10, 20, and 60 min at 37°C. The deuterated internal standard was added and N-phenylpropionamide determined by selected ion monitoring. Although the internal standard was easily quantified, there was no
N-phenylpropionamide formation detected up to 60 min, within the limits of quantification.

The inability to detect hydroxynorfentanyl in the above experiment was at variance with the report of hydroxynorfentanyl excretion in the urine of patients (4). Therefore, further experiments determined whether hydroxynorfentanyl is formed at all by human liver microsomes, and to identify the metabolic origin of hydroxynorfentanyl.

The potential role of fentanyl, norfentanyl, and hydroxyfentanyl as substrate(s) was examined. Fentanyl, norfentanyl, and hydroxyfentanyl (100 μM each) were separately incubated with liver microsomes (1 mg/ml) at 37°C for 30 min (90 min for fentanyl). Hydroxynorfentanyl (1.1 nmol/min/mg) was formed from hydroxyfentanyl. In contrast, no hydroxynorfentanyl was detected from either fentanyl or norfentanyl.

Subsequent experiments focused on formation of the principal metabolite norfentanyl. The dependence of norfentanyl formation on protein concentration (0.1–1.2 mg/ml) and time (2.5–20 min) was determined (not shown). At 10 μM fentanyl, norfentanyl formation was linear up to approximately 0.5 mg/ml. Because of excellent assay sensitivity, subsequent incubations were conducted at 0.05 mg/ml. At 10 μM fentanyl and 0.05 mg/ml protein, norfentanyl formation was linear for 10 min. Routine incubations therefore contained 10 μM fentanyl and 0.05 mg/ml protein and were terminated after 7 min. There was considerable interindividual variability in rates of fentanyl N-dealkylation. In microsomes from six human livers, the rate of norfentanyl formation was 0.01–2.4 nmol/min/mg.

Cytochrome P450 isoform selective competitive and mechanism-based inhibitors were used to probe the participation of individual isoforms in the metabolism of fentanyl to norfentanyl (fig. 3). The inhibitors used with the P450 isoform(s) they inhibit (in parentheses) were: furafylline (1A2), coumarin (2A6), orphenadrine (2B6), sulfaphenazole (2C9), quinidine (2D6), diethylidithiocarbamate (2E1/2A6), 4-methylpyrazole (2E1), troleandomycin (3A4), and midazolam (3A4). Troleandomycin incubation with microsomes and NADPH, prior to addition of fentanyl, resulted in 63% inhibition of norfentanyl formation. The P450 3A4 substrate and competitive inhibitor midazolam decreased norfentanyl formation by 99%. Significant inhibition of norfentanyl formation by diethylidithiocarbamate was also observed; however, the more selective inhibitors coumarin (2A6) and 4-methylpyrazole (2E1) did not inhibit norfentanyl formation. None of the other selective inhibitors of P450s showed substantial effect on norfentanyl formation, indicating their minimal contribution to fentanyl metabolism.

The role of P450 3A4 in norfentanyl formation was confirmed by comparing norfentanyl formation with P450 3A4 catalytic activity and isoform content in microsomes from a small population of human livers (fig. 4). An excellent correlation between the rates of norfentanyl formation and P450 3A4 activity, measured as R-warfarin 10-hydroxylation (25), was observed in six livers studied (r = 0.98, p < 0.001). No significant correlation with the catalytic activity of other P450 isoforms (1A2, 2A6, 2C9, 2D6, and 2E1) was observed. Norfentanyl formation rates were also correlated with P450 3A4 protein content (r = 0.83, p < 0.02), as determined by Western blot analysis using a rabbit antihuman P450 3A4 antibody as described previously (8).

Microsomes from cells expressing individual P450s were used as the third method to identify the P450 isoform(s) responsible for fentanyl N-dealkylation. Microsomes containing P450 3A4 (with expressed P450 reductase) showed the highest rate of norfentanyl formation (61 pmol/min/mg protein). No other isoform (P450s 1A2, 2B6, 2C9, 2D6, 2E1) catalyzed significant amounts of norfentanyl formation (all < 10 pmol/min/mg).
Duodenal Microsomal Metabolism

Experiments with human liver microsomes demonstrated that the predominant pathway of fentanyl metabolism is N-dealkylation to norfentanyl. The highest concentration of P450 in the intestinal tract is contained in the duodenum (14). Therefore, subsequent experiments determined whether fentanyl undergoes similar piperidine N-dealkylation in human duodenal microsomes. Fentanyl piperidine N-dealkylation was observed in all six microsomal samples. There was considerable variability in the rate of norfentanyl formation, which ranged from 0.01–0.41 nmol/min/mg.

Experiments were conducted to identify the predominant P450 responsible for duodenal fentanyl metabolism to norfentanyl. Since P450 3A4 was the major isoform responsible for fentanyl metabolism to norfentanyl in human liver microsomes and P450 3A4 seems to be the major P450 enzyme present in human duodenal microsomes (14), we tested the hypothesis that this enzyme also catalyzes norfentanyl formation in human duodenal microsomes. Incubation of troleandomycin (100 μM) with duodenal microsomes and NADPH, prior to addition of fentanyl (10 μM), reduced norfentanyl formation to 22 ± 6 and 45 ± 6% of control in microsomes from two different donors. Midazolam (100 μM) co-incubation also decreased norfentanyl formation to 44 ± 1 and 64 ± 9% of control in the two sets of microsomes. Lesser midazolam inhibition of norfentanyl formation in intestinal compared with hepatic microsomes is noted but cannot be explained by the available data. The role of P450 3A4 in duodenal fentanyl formation was confirmed by comparing norfentanyl formation rates with P450 3A4 catalytic activity in microsomes from a small population of human duodena (fig. 5). An excellent correlation...
Norfentanyl formation was determined by incubating fentanyl (10 μM) with human duodenal microsomes (0.1–0.2 mg) and NADPH (1 mM) at 37°C for 7 min. Each data point represents the mean of triplicate determinations from a single human duodenum. 1'-hydroxy-midazolam formation was determined in incubations containing 8 μM midazolam.

between the rates of norfentanyl formation and P450 3A4 activity, measured as midazolam-1'-hydroxylation, was observed in 6 duodenal samples studied (r = 0.82, p < 0.05).

Discussion

The first objective of this investigation was to characterize the various pathways of fentanyl metabolism in human liver microsomes. Potential pathways included piperidine N-dealkylation to norfentanyl, amide hydrolysis to despropionylfentanyl, terminal methyl hydroxylation to hydroxyfentanyl or hydroxynorfentanyl, and amide N-dealkylation to N-phenylpropionamide. Evidence was obtained for the formation of norfentanyl, despropionylfentanyl, hydroxyfentanyl, and hydroxynorfentanyl. Norfentanyl was the major metabolite in human liver microsomes, accounting for >99% of metabolism at a substrate concentration of 100 μM. Despropionylfentanyl and hydroxyfentanyl were comparatively minor metabolites. Hydroxynorfentanyl formation from fentanyl was not detected under routine (7-min) incubation conditions, but was observed after longer (90-min) incubations. Hydroxynorfentanyl is a secondary metabolite which may theoretically arise by N-dealkylation of hydroxyfentanyl and/or hydroxylation of norfentanyl. Incubation of each of these primary metabolites with liver microsomes showed hydroxynorfentanyl formation from hydroxyfentanyl but not norfentanyl. Therefore, secondary hydroxynorfentanyl production is limited by the initial formation of hydroxyfentanyl. The absence of hydroxynorfentanyl observed under shorter incubation conditions is explained by the relatively slow rate of hydroxyfentanyl formation. Amide N-dealkylation of fentanyl to N-phenylpropionamide was not detected under any conditions. Thus fentanyl differs markedly from alfentanil, for which amide N-dealkylation to N-phenylpropionamide is one of two major routes of metabolism (8, 22). Amide N-dealkylation of alfentanil but not fentanyl may be explained by the presence of a methoxymethyl functionality at the quaternary carbon of the piperidine ring of alfentanil, while fentanyl contains only a hydrogen atom in the same position. Finer elucidation of the structural factors regulating N-dealkylation of substituted piperidine opioids requires further investigation.

The present in vitro human liver microsomal results are consistent with those from in vivo human investigations. Norfentanyl was the most abundant urine and plasma metabolite in vivo in all patients (4–6) and the predominant pathway of microsomal metabolism in vitro. Although despropionylfentanyl was not detected in human urine (4, 6), it was found in varying amounts in human plasma (5). We identified despropionylfentanyl as a minor metabolite in vitro. Hydroxynorfentanyl was observed in urine in vivo (4), and also in the present microsomal experiments. Thus human liver microsomal fentanyl metabolism in vitro is an excellent model for human fentanyl metabolism in vivo: Fentanyl piperidine N-dealkylation to norfentanyl is the predominant pathway of human metabolism, fentanyl amide hydrolysis to despropionylfentanyl, and alkyl hydroxylation to hydroxyfentanyl are comparatively minor pathways, and hydroxynorfentanyl is subsequently N-dealkylated to hydroxynorfentanyl, another minor metabolite.

The second objective of this investigation was to identify the P450 isoform(s) involved in each major pathway of fentanyl metabolism. Because N-dealkylation to norfentanyl was the predominant route of metabolism, the P450s involved in only this pathway were identified. Experiments using P450 isoform selective inhibitors, correlations of norfentanyl formation with microsomal P450 protein content and catalytic activity, and cDNA expressed enzymes strongly suggest the catalytic predominance of a single isoform, P450 3A4, in the N-dealkylation of fentanyl to norfentanyl in vitro. These results corroborates the recent report identifying P450 3A4 as the major P450 isoform responsible for fentanyl N-dealkylation in human liver microsomes (20). Thus, P450 3A4 is the predominant isoform responsible for the piperidine N-dealkylation of fentanyl, alfentanil, and sufentanil (8, 20, 26–28).

The third objective was to determine whether metabolic biotransformation of fentanyl occurs in human duodenal microsomes. The predominant pathway in liver, N-dealkylation to norfentanyl, was considered the most likely route of duodenal metabolism, particularly since P450 3A4 is the principal duodenal P450 isoform. Results obtained clearly demonstrated that human duodenal microsomes did catalyze fentanyl metabolism to norfentanyl at a rate approximately half that of hepatic microsomes. Evidence for the role of P450 3A4 in human duodenal fentanyl metabolism was also obtained.

There are several potential clinical implications of human hepatic and duodenal fentanyl metabolism by P450 3A enzyme(s), including intestinal first-pass clearance, interindividual variability in oral bioavailability, and drug interactions. Several hepatic P450 3A substrates are also metabolized by human intestinal cells and microsomes in vitro (13, 17, 24, 29–31), and significant human in vivo first-pass intestinal metabolism of 3A substrates has recently been demonstrated (24, 32, 33). Significant (6- to 11-fold) interindividual variability in intestinal P450 3A4, as well as gender differences (1.5 to 2-fold greater in women) are apparent (24,30,31). Modulation of intestinal P450 3A4 activity by erythromycin, rifampin and ketoconazole is thought to contribute to interactions with these drugs (15, 32, 34–37).

Approximately 75% of fentanyl administered by oral transmucosal lozenge is swallowed, 2/3 of which undergoes first-pass metabolism (2). Duodenal microsomal P450 3A4 fentanyl metabolism in vitro suggests that fentanyl undergoes significant intestinal first-pass as well as hepatic first-pass metabolism in vivo. Greater bioavailability of fentanyl in lozenge form (50%), in which buccal absorption partially avoids intestinal and hepatic first-pass extraction, compared

2 Fentanyl Oralet package label.
with oral fentanyl (30%), supports this contention. Interestingly, transmucosal fentanyl lozenge (5000 µg) produced significant sedation, analgesia, and respiratory depression, while oral administration of the same dose resulted in minimal effects (10). While this was attributed to high hepatic systemic fentanyl clearance, it is more likely that gastrointestinal and hepatic first-pass metabolism account for the observed (lack of) effect. Lesser bioavailability of oral compared with lozenge fentanyl has been attributed to hepatic first-pass extraction (12). Again, it is likely that gastrointestinal metabolism also contributes to presystemic clearance of swallowed fentanyl. There is considerable interpatient variability in fentanyl plasma concentrations after oral transmucosal fentanyl citrate (10, 12). This variability has been attributed to variations in mouth pH (38) and the amount of fentanyl-laden saliva swallowed and escaping oral mucosal absorption (12). Analogous to the examples cited above, it is likely that variability in gastrointestinal metabolism of fentanyl, owing to interpatient differences in intestinal P450 3A4 and/or 3A5 activity, also contributes to interindividual variability in bioavailability of fentanyl in lozenges. More importantly, co-administration of drugs known to affect intestinal P450 3A4 activity will probably also affect fentanyl lozenge bioavailability, as described above for several other P450 3A substrates. Human clinical investigations are required to confirm these conjectures.

It is more difficult, however, to predict the impact of variable hepatic P450 3A4 expression on intersubject variability in the systemic clearance of fentanyl, as well as the incidence and severity of P450 3A4-mediated drug interactions after iv fentanyl administration. Fentanyl is a high-extraction drug (2) and therefore theoretically relatively insensitive to changes in hepatic intrinsic clearance caused by altered P450 3A4 activity (39). The effects of altered hepatic P450 3A4 activity on systemic fentanyl elimination, however, are unknown. By analogy, systemic clearance of iv sufentanil, also a high-extraction drug, was unaffected by P450 3A4 inhibition by erythromycin (40). In contrast, systemic clearance of alfentanil, a low-extraction drug, is exquisitely sensitive to changes in P450 3A4 activity (28). Alfentanil clearance was significantly diminished by erythromycin and trolenzomycin (28, 41). Intestinal metabolism of iv midazolam is low (24, 33), and by analogy that of iv fentanyl probably also unimportant. The last consequence of fentanyl metabolism by P450 3A4 may pertain to extrahepatic metabolism. Fentanyl is known to undergo extrahepatic metabolism in animals, with greatest norfentanyl formation in kidneys and intestine (42). This may be explained by intestinal and renal P450 3A expression in animals and may suggest renal fentanyl metabolism in humans (29). Further investigations are required to characterize more fully the clinical implications of fentanyl metabolism by P450 3A4 in liver, intestine, and other organs.

References
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