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BIOTRANSFORMATION OF TRITIATED FENTANYL IN HUMAN LIVER MICROSOMES

MONITORING METABOLISM USING PHENYLACETIC ACID AND 2-PHENYLETHANOL

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Abstract—Norfentanyl has been identified previously as a urinary metabolite of fentanyl. However, at clinically relevant concentrations, norfentanyl concentrations are below the limits of detection. The use of labeled drug in metabolic studies is a standard approach to overcome the limitations imposed by metabolite concentrations that are below detection limits. Unfortunately, the available tritium-labeled fentanyl yields unlabeled norfentanyl following N-dealkylation. Thus, we have developed a technique to monitor the N-dealkylation of fentanyl using the other products of N-dealkylation. The biotransformation of fentanyl was studied in human liver microsomes. After incubation with human liver microsomes for 20 min, almost 50% of a 0.03 μM concentration of [^{3}H]fentanyl was metabolized to the [^{3}H]N-dealkylated metabolite phenylacetaldehyde, which was then converted principally to [^{3}H]2-phenylethanol and to a smaller extent to [^{3}H]phenylacetic acid in microsomal incubates. The apparent $K_{m,app}$ and $V_{max,app}$ for norfentanyl formation were 82 ± 21 μM and 4.7 ± 0.4 nmol product formed/min/nmol cytochrome P450, respectively. Thus, this study defined methodology that can be used to evaluate the metabolism of fentanyl, both *in vivo* and *in vitro*, at clinically relevant concentrations.

Key words: fentanyl; CYP3A4; drug metabolism; opiates; anesthesia; cytochrome P450

Fentanyl, a synthetic opioid, is widely used in anesthesia because of its potent analgesic effect and relatively short duration of action [1]. Although fentanyl was introduced into clinical use in the 1960s, its biotransformation has not been well characterized. This deficiency reflects the difficulties in measuring the low concentrations of fentanyl used in clinical practice. Although norfentanyl has been identified as a major metabolite of fentanyl in urine in both rats [2, 3] and humans [4–6], assay difficulties have prevented *in vitro* and *in vivo* studies of fentanyl metabolism. Fentanyl is a potent opiate that causes marked respiratory depression, so that in the past its metabolism has had to be studied during surgery in patients who were ventilated. In addition, *in vitro* studies have had to use concentrations well above those used therapeutically. The use of tritiated fentanyl has not been particularly helpful because the position of the tritium has led to its loss in metabolism to unlabeled norfentanyl (Fig. 1). The purposes of this study were: (1) to describe the biotransformation [^{3}H]fentanyl in human liver microsomes, (2) to develop a method for monitoring fentanyl N-dealkylation, and (3) to define specifically the N-dealkylated metabolites produced following metabolism of [^{3}H]fentanyl (1-[2,3-^{3}H-*phenethyl*]-4-*N*-[*N*-propionylanilino]piperidine).

MATERIALS AND METHODS

Materials

[^{3}H]Fentanyl (>99% radiochemical purity; 776 GBq/mmol), norfentanyl, and the metabolites M3 and M4 (Fig. 2) were gifts from the Janssen Research Foundation (Beerse, Belgium). The radiolabeled drug was diluted with unlabeled fentanyl to give appropriate specific activities. [^{3}H]Fentanyl was incubated at final drug concentrations ranging from 0.03 μM to 1 mM and a final radioactivity concentration of 135 nCi/sample (0.5 mL). Fentanyl, phenylacetic acid, phenylacetaldehyde, 2-phenylethanol, $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, aldehyde dehydrogenase (from bakers' yeast), alcohol dehydrogenase (from bakers' yeast), NAD^+, and DMSO were purchased from the Sigma Chemical Co. (St Louis, MO). All other chemicals were from various commercial sources and were of analytical grade.

Microsomes and incubation conditions

Human liver samples were obtained from organ donors through the Tennessee Donor Services (Nashville, TN). Microsomes were prepared as described previously from a single human liver [7]. The cytochrome P450 (P450) concentration was determined by Fe^{2+}-CO versus Fe^{2+} difference spectroscopy [8]. Microsomal incubations were carried out in a final volume of 0.5 mL with 100 mM potassium phosphate (pH 7.4). After a 3-min preincubation in the presence of fentanyl, metabolism was initiated by the addition of an NADPH-generating system (10 mM glucose-6-phosphate, 0.5 mM $NADP^+$, and 0.5 IU of glucose-6-phosphate dehydrogenase per sample) using open glass vials in a shaking water bath at 37.5°. The reactions were stopped by the addition of 0.5 mL DMSO. After vortex mixing, centrifugation, and filtration, the mixture was assayed by HPLC as described below.

Assay of fentanyl and metabolites

Fentanyl and its metabolites were measured by HPLC using two pumps (Waters, Marlborough, MA), an AS-

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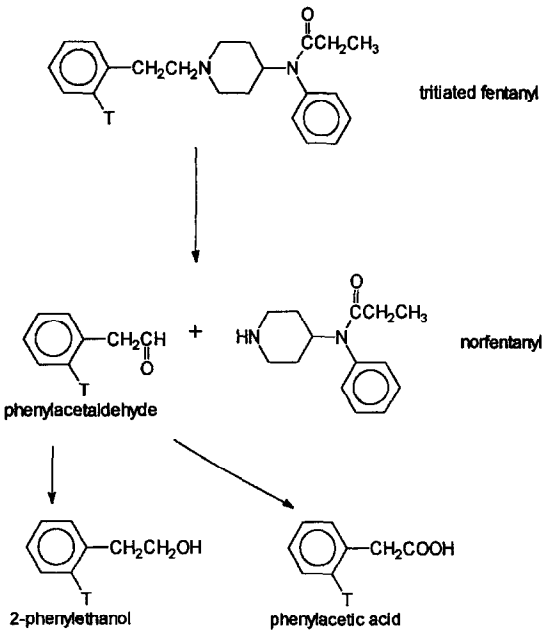

Fig. 1. Postulated metabolic pathways responsible for production of phenylacetaldehyde, 2-phenylethanol, and phenylacetic acid from tritiated fentanyl. T shows the position of tritium.

100 automatic injector (Bio-Rad, Richmond, CA), a 4.6 × 250 mm Spherisorb ODS-2 5-μm column (Alltech, Deerfield, IL), an L-4000 UV detector (Hitachi, Tokyo, Japan), a 746 recorder (Waters), a FOXY fraction collector (ISCO, Lincoln, NE), and a 680 controller (Waters). The absorbance of the eluate was monitored at 230 nm. The mobile phase started with 100% 1.0 M ammonium acetate (solvent system A) and changed over 40 min to 65% 1.0 M ammonium acetate:methanol:acetonitrile:tetrahydrofran (10:20:30:40, by vol.) (solvent system B) and 35% solvent system A at a flow rate of 1.0 mL/min. Following HPLC injection, the effluent was collected in 2-min aliquots and counted in a 1219 Rack-Beta spectral scintillation counter (LKB-Wallac, Turku, Finland).

Assay of phenylacetaldehyde

Phenylacetaldehyde was assayed by a modification of the method of Farrelly [9]. In brief, the incubations were terminated by the addition of 100 μL of a 1:1 mixture of 25% zinc sulfate and 0.8 M semicarbazide. After addition of saturated barium hydroxide solution, vortex mixing, and centrifugation, the supernatant was removed to another tube. One milliliter of water, 100 μL of dinitrophenylhydrazine solution [0.25% (w/v) in 6 N HCl], and 2 mL of hexane were added to the supernatant. After mixing for 30 min, the hexane layer was removed and mixed with 350 μL of acetonitrile. A portion of the acetonitrile layer was injected onto the HPLC system (described above). The mobile phase used consisted of 60% acetonitrile in water at a flow rate of 1.5 mL/min.

Kinetic analysis

$V_{max,app}$ and $K_{m,app}$ were estimated by the nonlinear curve-fitting program K-Cat, version 1.54 (BioMetallics, Inc., Princeton, NJ).

RESULTS

The radiochromatogram obtained following incubation of 5.0 μM tritiated fentanyl with human liver microsomes is shown in Fig. 3. The radiolabeled peaks were identified as phenylacetic acid and 2-phenylethanol by coinjection of unlabeled standards (Fig. 3). The generation of 2-phenylethanol was linear for incubation times of up to 20 min (Fig. 4) and for P450 concentrations of up to 150 pmol/0.5-mL sample. The subsequent incubations were performed with 100 pmol P450 for 20 min. The total radioactivity associated by [^{3}H]phenylacetic acid, [^{3}H]2-phenylethanol and [^{3}H]fentanyl remained constant over the 20 min of the study.

Thus, radiolabeled 2-phenylethanol and phenylacetic acid were identified as metabolites of tritiated fentanyl in human liver microsomes. We postulated the metabolic scheme shown in Fig. 1 in order to explain the pathways responsible for production of these metabolites. To determine if 2-phenylethanol and phenylacetic acid are produced from phenylacetaldehyde, the effect of adding aldehyde dehydrogenase upon the production of the two metabolites was examined. Incubations were performed with 1.7 U of aldehyde dehydrogenase and 0.5 mM NAD^+ using tritiated fentanyl (0.03 μM) with the human liver microsomes as described above. The addition of aldehyde dehydrogenase/NAD^+ decreased production of 2-phenylethanol and increased phenylacetic acid production (Fig. 5).

Phenylacetaldehyde (10 μM) was incubated with hu-

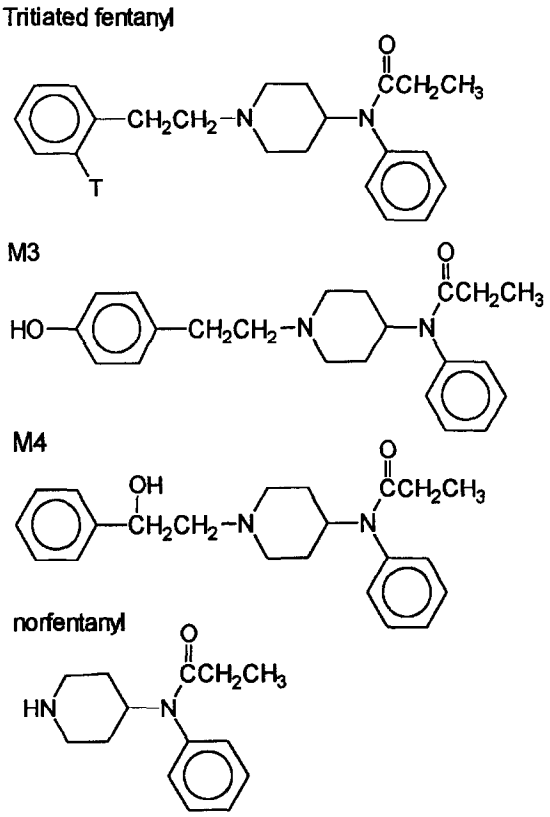

Fig. 2. Chemical structures of tritiated fentanyl, and the metabolites M3, M4, and norfentanyl. T on the fentanyl molecule shows the position of tritium.

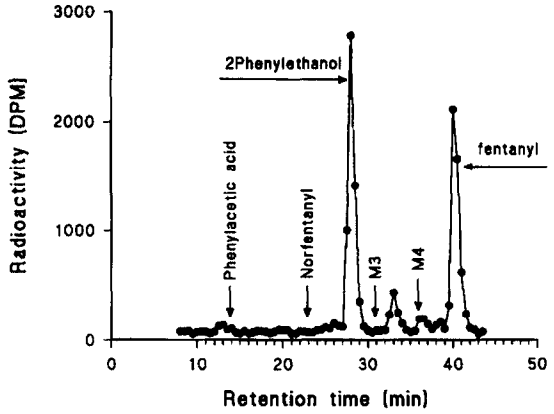

Fig. 3. Radiochromatogram obtained for an incubate of 5.0 μM tritiated fentanyl with human liver microsomes (100 pmol P450); the retention times of cold spiked metabolites are shown by arrows.

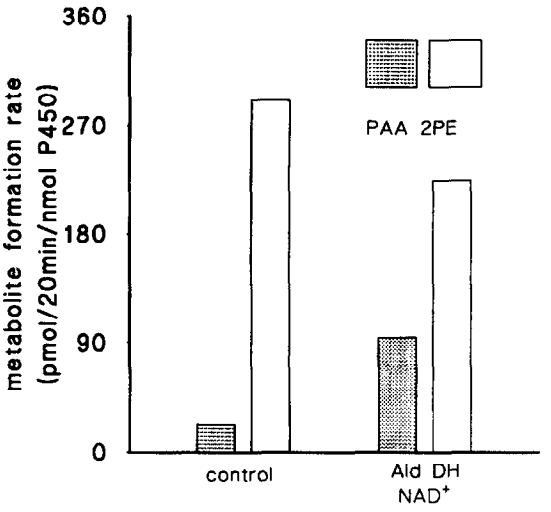

Fig. 5. Effect of 1.7 U of aldehyde dehydrogenase (ALD DH) and 0.5 mM NAD^+ on the formation of phenylacetic acid (PAA) and 2-phenylethanol (2PE). Incubates contained 0.03 μM tritiated fentanyl and liver microsomes prepared from a single human liver (100 pmol P450). (Values are the means of duplicate studies.

man liver microsomes and the NADPH-generating system. The concentration of phenylacetaldehyde derivatized with dinitrophenylhydrazine decreased with the incubation time up to 15 min (Fig. 6). The apparent V_{max} obtained was 4.7 ± 0.4 nmol N-dealkylated metabolite formed/min/nmol P450 and the apparent K_m was 82 ± 21 μM with the one human liver microsomal preparation.

DISCUSSION

The purpose of the present study was to define the N-dealkylated metabolites produced from [^{3}H]fentanyl metabolism in human liver microsomes at concentrations of tritiated fentanyl (0.03 μM) similar to those found in plasma (0.06 μM [10]) following clinical use of fentanyl, to allow methodology to be developed to monitor the N-dealkylation of fentanyl. Because the concentrations of norfentanyl are not detectable following low concentrations of fentanyl achieved after clinical dosing, an alternative approach was required. For that reason we chose to examine the metabolism of ^{3}H-labeled fentanyl. The position of the tritium (see Fig. 1) means that norfentanyl, one of the N-dealkylated metabolites, would not be labeled and, therefore, would not be detectable by standard chromatography, UV detection, or measurement of radioactivity. The other product of N-dealkylation, phenylacetaldehyde, was metabolized further to phenylethanol and phenylacetic acid. To demonstrate that phenylethanol and phenylacetic acid were products of phenylacetaldehyde and not vice versa, we first demonstrated that phenylacetaldehyde was indeed metabolized when it was incubated with human liver microsomes and an NADPH-generating system. By adding aldehyde dehydrogenase and NAD^+ to the microsomal incubation of fentanyl, the metabolism of fentanyl was shifted to phenylacetic acid while the metabolism to 2-phenylethanol was decreased. At higher concentrations of fentanyl (1.0

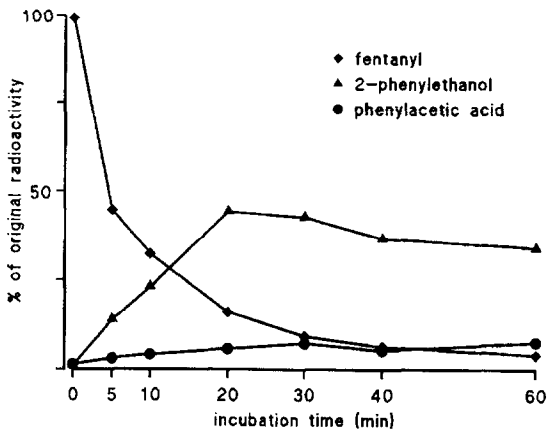

Fig. 4. Formation of phenylacetic acid and 2-phenylethanol after incubation with 0.03 μM fentanyl and human liver microsomes obtained from a single liver (100 pmol P450), as a function of the incubation time. Values are the means of duplicate studies and are shown as a percentage of the original radioactivity.

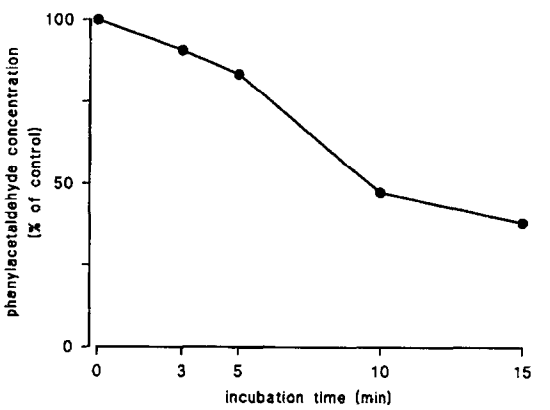

Fig. 6. Elimination of phenylacetaldehyde after incubation with liver microsomes obtained from a single human liver (100 pmol P450) and 10 μM phenylacetaldehyde as a function of incubation time. Values are the means of duplicate studies.

mM), it was possible to identify a radioactive peak with the same retention time as phenylacetaldehyde.

In summary, we have shown N-dealkylation to be the major metabolic pathway for fentanyl metabolism in human liver microsomes, and that the activity of this pathway can be monitored by measuring phenylacetic acid and 2-phenylethanol produced after N-dealkylation of fentanyl. Norfentanyl is the other product of N-dealkylation, and although not identified in this study because it would not be labeled, it has been identified previously in urine. Our results in human microsomes, therefore, are consistent with previous *in vivo* studies and will allow the use of this approach to evaluate fentanyl's metabolism *in vivo* and *in vitro* at therapeutically relevant concentrations.

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