Development of a Gas Chromatographic–Mass Spectrometric Drug Screening Method for the *N*-Dealkylated Metabolites of Fentanyl, Sufentanil, and Alfentanil

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Abstract

A sensitive, specific urinary assay for fentanyl, sufentanil, and alfentanil based on their *N*-dealkylated metabolites is described. Norfentanyl, norsufentanil–noralfentanil, and $^2H_5$-norfentanyl are synthesized and characterized by standard analytical techniques. Derivatization of these secondary amines to yield the pentafluorobenzamides produces stable products with good gas chromatographic properties and unique, high-mass fragments in their mass spectra. These properties are utilized to develop a drug screening procedure based on gas chromatography–mass spectrometry to detect these major metabolites in human urine. The metabolites are isolated from urine samples by a liquid–liquid extraction procedure. The method allows for detection of metabolite concentrations as low as 0.3 ng/mL.

Introduction

Fentanyl, sufentanil, and alfentanil are members of the 4-propananilido-piperidine class of narcotic analgesics. These compounds are more potent than the prototype analgesic morphine, have shorter durations, and are less likely to produce complicating adverse reactions upon acute administration. As a result of these properties, these drugs are among the most widely used agents to produce surgical anesthesia. Fentanyl, sufentanil, and alfentanil differ structurally in the nature of the 1-substituent and the degree of substitution at the 4-position; these differences affect relative potency and duration of action. Sufentanil is the most potent of the series (2,000 times as potent as morphine), followed by fentanyl (200 times as potent as morphine) and alfentanil (30 times as potent as morphine). Sufentanil and alfentanil are shorter acting than fentanyl and are less likely to produce respiratory depression. All of these compounds are routinely used in hospitalized patients and are accessible to many hospital personnel. There is growing concern about the potential for abuse by health care workers as well as the general public. Because of their high potencies, relatively low doses are used to produce significant pharmacological effects, both in therapeutic and drug abuse situations, making routine screening procedures based on chromatography and immunoassay of little value in detecting these drugs.

Fentanyl and sufentanil are extensively metabolized in humans, and very low concentrations of the parent drugs are excreted in human urine (1). Oxidative *N*-dealkylation to produce the nor-metabolites is the major metabolic pathway for fentanyl, sufentanil, and alfentanil (Figure 1). Reports indicate that 0.4–6% of fentanyl and about 2% of sufentanil is excreted unchanged in human urine (2). In the case of fentanyl, 26–55% of the human dose is excreted in urine as the norfentanyl metabolite (2). The major urinary metabolites of sufentanil are norsufentanil and *O*-desmethyl alcohol (1). These metabolic profiles suggest that considerable sensitivity gain can be made if the urinary assay is based on drug metabolites and not the parent drugs. The presence of significant *N*-dealkylated secondary amine metabolites further suggests that a single analytical procedure involving isolation and derivatization could be designed to detect the metabolites of the parent drugs. Additionally, sufentanil and alfentanil produce the same secondary amine metabolite upon oxidative *N*-dealkylation. The secondary amine metabolites of all three drugs can be acylated with various reagents to further enhance sensitivity and specificity of analytical procedures.

Experimental

Synthesis

A solution of 1-benzylpiperidin-4-one and aniline or pentadeuterio aniline in benzene was heated at reflux under a Dean-Stark trap for 24 h. The resulting imine was isolated by evaporation and treated with sodium borohydride in methanol

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for 4 h to yield 1-benzyl-4-anilinopiperidine. This intermediate was treated with propionic anhydride or acetic anhydride to produce the 1-benzyl-4-propananilido- or 1-benzyl-4-acetanilidopiperidine intermediates. Hydrogenolysis of these intermediates afforded the 4-propananilido- (norfentanyl) and 4-acetanilidopiperidine standards. The norsufentanil metabolite was synthesized by a modification of the methods of Taber and Rahimizadeh (3) and Van Daele et al. (4), which is described elsewhere. The structures of all products were established by nuclear magnetic resonance (NMR), infrared (IR) and gas chromatography–mass spectrometry (GC–MS); purity was confirmed chromatographically and by elemental analysis.

Instrumentation and GC–MS conditions

The GC separation and electron impact (EI) mass spectra (MS) were obtained using a Hewlett-Packard 5890/5970 GC–MS (Palo Alto, CA) system. The GC consisted of a 15-m × 0.20-mm-i.d. fused-silica capillary column with 0.33-µm-thick 5% crosslinked phenylmethyl silicone gum phase (HP-5). The GC separations were obtained using a program with an initial oven temperature of 85°C that was held for 0.75 min, then increased at a rate of 22.5°C/min to a final temperature of 315°C. The oven was held at the final temperature for an additional 0.75 min. The GC injection port and transfer line temperatures were maintained at 270°C, and the MS ionization voltage was 70 eV.

Derivatization

Stock solutions (1 mg/mL in methanol) of norfentanyl, norfentanyl-d_5, norsufentanil, and 4-acetanilidopiperidine were prepared, and 100 µL was removed and placed in clean 13 × 100-mm glass test tubes. The tubes were placed in a Pierce Reacti-Therm heatblock at 40°C, and the methanol was removed by evaporation under a stream of clean, dry air. The residue was reconstituted with 200 µL of 0.1M pentafluorobenzoyl chloride or 2,3,5,6-tetrafluorobenzoyl chloride in chloroform, and the tubes were tightly capped and heated at 55°C for 20 min. The solvent was then removed under a stream of dry air, and the residue was reconsituted in 300 µL of chloroform; 1 µL was injected in the GC–MS for analysis.

Urine sample preparation and extraction

Certified negative urine was spiked with norfentanyl and norsufentanil at concentrations of 0.1, 0.3, 0.5, 0.7, 0.9, 1.5, 2.0, 2.5, and 3.0 ng/mL. A 10-mL aliquot of spiked urine sample was placed in a test tube, and 200 µL of a 100-ng/mL norfentanyl-d_5 internal standard solution was added. The solution was saturated with KCl, and the pH was adjusted to 12 with ammonium hydroxide. The sample was then extracted with 10 mL of ethyl acetate–*n*-butanol (4:1) for 45 min and then centrifuged at 2,500 rpm for 10 min. The organic layer was removed and evaporated in a stream of dry air to a volume of 2–3 mL. The remaining organic solvent was extracted with 0.3N HCl (2 × 3 mL). The HCl extracts were then combined, washed with hexane (2 × 3 mL), and then saturated with KCl. The pH was adjusted to 12 with ammonium hydroxide. The resulting aqueous basic solution was extracted with *n*-butyl chloride (2 × 3 mL), and the extracts were combined and evaporated to dryness at 40°C under a stream of clean, dry air. The resulting residue was derivatized by adding 100 µL of 0.1M pentafluorobenzoyl chloride and heating at 55°C for 20 min. The solution was then evaporated to dryness, and the residue was dissolved in 20 µL of chloroform. A 3-µL portion was injected onto the GC–MS system.

Results and Discussion

The primary urinary metabolites of fentanyl, sufentanil, and alfentanil are formed as a result of piperidine nitrogen dealkylation. This metabolic pathway yields a common metabolite for sufentanil and alfentanil because these two drugs differ in structure only in the *N*-substituent. Figure 1 shows the structures of the parent drugs and the *N*-dealkylated metabolites that are reported (1) as the primary metabolites in human urine. Assays based on the primary metabolites should have considerable sensitivity advantages over assays for the parent drugs.

The initial phase of this study involved synthesis of the *N*-dealkylated metabolites and the synthesis of deuterated derivatives as reference samples and internal standards, respectively. The *N*-dealkylated metabolite of fentanyl (norfentanyl) was prepared from 1-benzylpiperidin-4-one according to the procedure outlined in Figure 2. The imine was formed with aniline under dehydrating conditions and subsequently reduced using

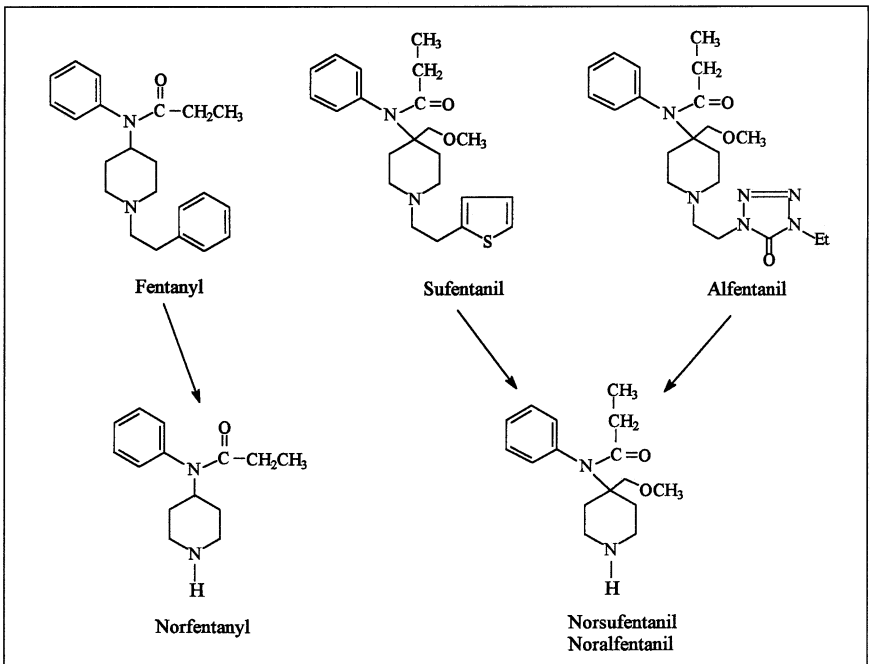

Figure 1. Metabolic *N*-dealkylation of fentanyl, sufentanil, and alfentanil.

sodium borohydride. The resulting secondary amine was allowed to react with propionic anhydride to form the propionamide, which was debenzylated by hydrogenolysis to yield the desired metabolite, norfentanyl. The pentadeuterated derivative was prepared in an analogous procedure using $^2H_5$-aniline for imine formation. The reference standard for norsufentanil and noralfentanil was synthesized in a multistep procedure to be described elsewhere. The final product was fully characterized by NMR, IR, GC–MS, and elemental analysis (CHN).

Each of the metabolites (Figure 1) contain a basic secondary nitrogen that can be derivatized to form stable products with good chromatographic properties and yielding high-mass fragments. These are important properties necessary for the development of sensitive and specific assays based on GC–MS analysis and selective ion monitoring. Many acylating reagents react with primary and secondary amines to produce amide products with excellent GC–MS properties; pentafluoropropionamides (PFPAs) are among the most common (5). The PFPA derivatives were evaluated in this study and compared with the pentafluorobenzamides (PFBAs). The PFBA derivatives showed additional unique high-mass fragments of high relative intensity in their mass spectra, which allowed for additional sensitivity and specificity in the GC–MS assay. Each of the two metabolites and the deuterated internal standard were individually derivatized with pentafluorobenzoyl chloride to form the pentafluorobenzamides. These products were individually characterized by GC–MS; the GC separation of norfentanyl-PFBA and norsufentanil-PFBA is shown in Figure 3. The chromatogram was obtained using a fused-silica column with a 0.33-µm film of 5% phenylmethylsilicone (HP-5) and a temperature program. The PFBA derivatives of norfentanyl and $^2H_5$-norfentanyl coeluted as expected and the norsufentanil-PFBA derivative showed greater retention than norfentanyl-PFBA in this system. The chromatographic separation is sufficient to allow the analysis of samples that might contain a mixture of fentanyl and sufentanil metabolites.

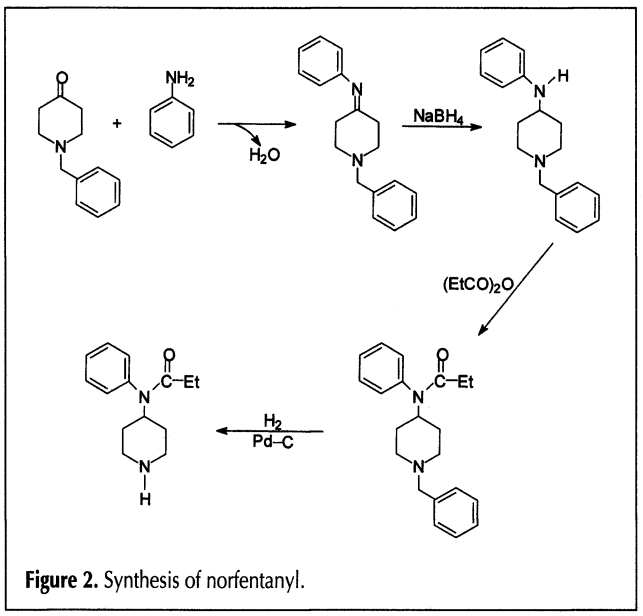

Figure 2. Synthesis of norfentanyl.

The mass spectra for the three PFBA derivatized compounds are shown in Figure 4. Figure 4A shows the spectrum from norfentanyl-PFBA with a molecular ion at 426 and major fragments at 370, 277, 195, 150, 132, and 57 mass units. The fragmentation reactions that yield these ions are illustrated in Figure 5. The *m/z* 370 ion results from the loss of 56 mass units from the molecular ion through a hydrogen-rearranged loss of the acyl (C_2H_4CO) side chain. The major fragment at *m/z* 277 is the pentafluorobenzoylpiperidine radical cation resulting from loss of the propionanilide group at the 4-position of the piperidine ring. The ion at *m/z* 195 is the pentafluorobenzoyl cation, and the fragment at *m/z* 150 is the protonated propionanilide. The ion at 132 is the result of a hydrogen rearrangement of the *m/z* 370 ion involving fragmentation of the piperidine ring.

The mass spectrum of the pentadeuterio-norfentanyl-PFBA derivative in Figure 4B helps to confirm the structural assignments in Figure 5. The ions that do not contain the aniline aromatic ring (*m/z* 277 and 195) are unchanged compared with Figure 4A, whereas the other ions all show the five mass unit isotope effect (*m/z* 431, 375, 155, and 137), indicating the presence of the aniline aromatic ring in these fragments.

The model compounds whose mass spectra are shown in Figures 6A and 6B add additional support to the fragment structures proposed in Figure 5. Norfentanyl derivatized with 2,3,5,6-tetrafluorobenzoyl chloride (Figure 6A) to prepare the tetrafluorobenzamide (TFBA) yields peaks 18 mass units lower for all ions containing the benzamide fragment (*m/z* 352, 259, and 177). The ions not containing the acylated piperidine remain unchanged at *m/z* 150 and 132. The second model compound in Figure 6B is the PFBA-4-acetanilidopiperidine, a replacement of the propionamide moiety with an acetamide group. The resulting mass spectrum shows a fragment at *m/z* 136 for the protonated acetanilide ion and the absence of the protonated propionanilide ion (m/z 150) seen in Figures 4A and 6A. The other major fragments in Figure 6B at *m/z* 277 and 195 remained because these ions do not contain the substituents at the 4-position of the piperidine ring. The *m/z* 132 ion was unchanged also because it does not contain the acyl group at the aniline nitrogen. The spectra for these model compounds support the structural assignments for the major fragments of norfentanyl-PFBA shown in Figure 5.

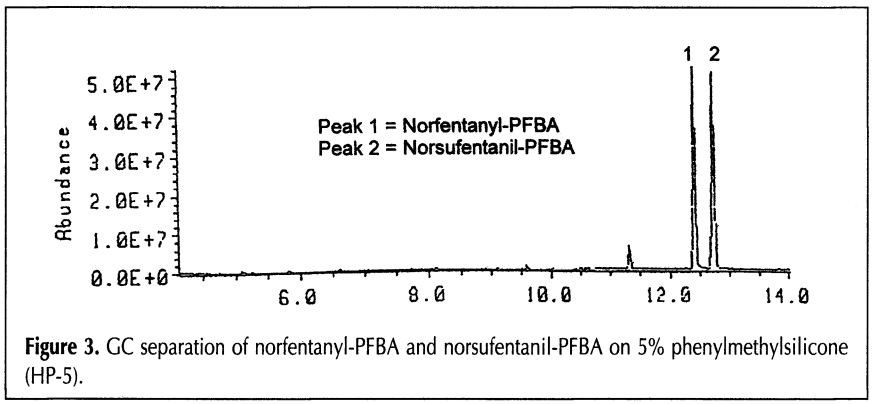

Figure 3. GC separation of norfentanyl-PFBA and norsufentanil-PFBA on 5% phenylmethylsilicone (HP-5).

The mass spectrum in Figure 4C was obtained for the PFBA derivative of norsufentanil–noralfentanil. The molecular weight of the derivative was at mass 470, and the spectrum shows only a trace of molecular ion. The significant peak at highest mass, *m/z* 425, is 45 mass units lower than the molecular ion and is the loss of the methoxymethylene substituent at the 4-position of the piperidine ring (Figure 7). The resulting acylated imine (*m/z* 425) lost the acyl group through a hydrogen rearrangement to yield the major imine fragment at *m/z* 369. The elimination of pentafluorobenzamide from the *m/z* 369 ion yielded the *m/z* 158 fragment. Additional support for the structure of the *m/z* 158 ion in Figure 7 was gained by evaluation of the mass spectrum for the tetrafluorobenzamide of norsufentanil (Figure 6C). This spectrum shows the *m/z* 158 ion as the base peak, whereas the mass of all other major fragments were shifted by 18 mass units (*m/z* 407, 351, and 177). The results of these experiments show that all the major fragment ions in the mass spectrum of norsufentanil-PFBA with the exception of the *m/z* 158 ion contain the benzamide portion of the molecule. Other than the trace level of molecular ion for norsufentanil-PFBA, the only ion showing the intact 4-methoxymethylene group was the *m/z* 321 ion. This ion represents the loss of the propionanilido group from the 4-position of the molecular ion and is simply the methoxymethylene substituted *m/z* 277 ion observed for norfentanyl-PFBA. The *m/z* 289 ion represents the loss of methanol from the 321 ion and may occur as the ring expanded azepine or the fused piperidine-cyclopropyl (3-azabicyclo[4.1.0]heptane) radical cation. These ions were shifted lower by 18 mass units in the tetrafluorobenzamide of norsufentanil (*m/z* 303 and 271), showing that the benzamide moiety remained a portion of the structure of these ions.

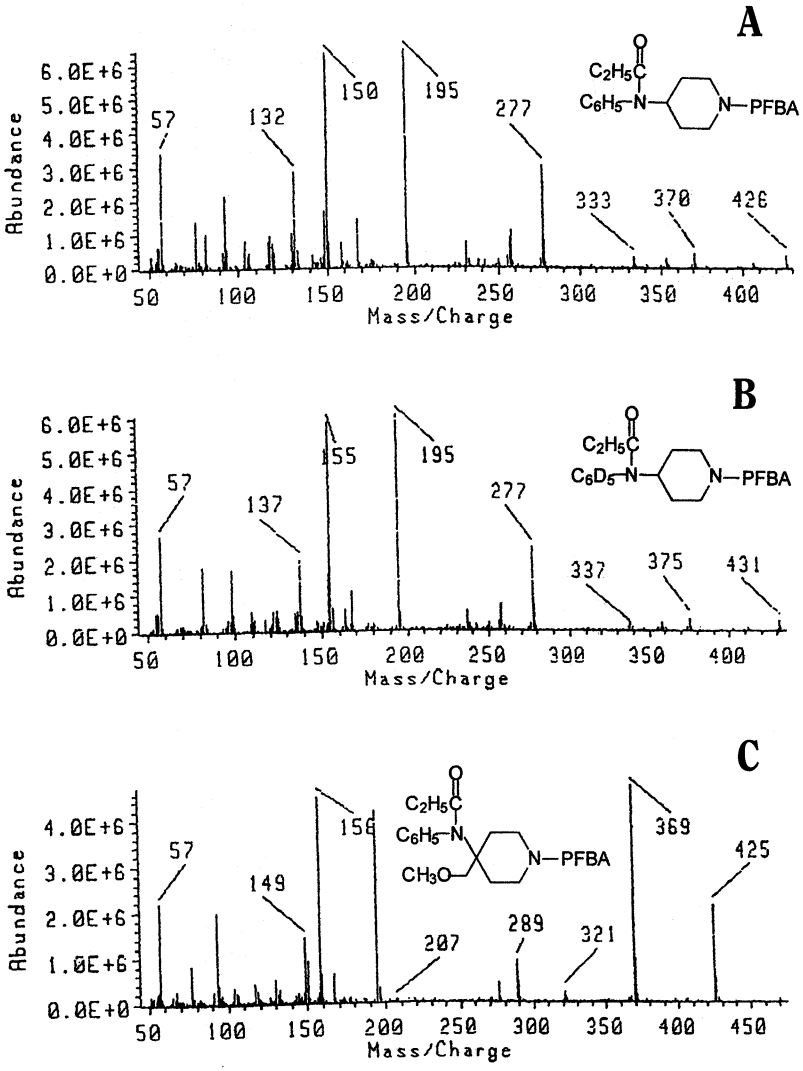

Figure 4. Mass spectra of norfentanyl-PFBA (A), norfentanyl-d_5-PFBA (B), and norsufentanil–noralfentanil-PFBA (C).

The GC–MS data for the derivatized metabolites indicated that the PFBA derivatives are excellent choices for development of a biological detection assay. These derivatives show excellent chromatographic properties and give rise to unique high mass fragments of high relative abundance in the MS. These characteristics should allow for the development of a sensitive, specific method for the identification of these metabolites in biological samples. One of the primary advantages of the PFBA derivatives over the more common pentafluoropropionyl-amides (PFPA) observed in this study was lower background interference. Some of the major fragment ions for norsufentanil-PFPA occurred at the same mass as fragment ions for cholesterol and other background steroids.

A variety of extraction procedures were evaluated for the isolation of norfentanyl and norsufentanil from urine samples. A liquid–liquid extraction procedure was found to be

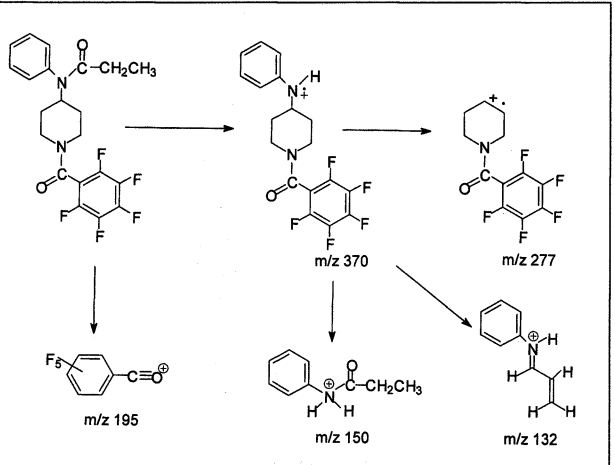

Figure 5. MS fragmentation of norfentanyl-PFBA.

superior to the solid-phase extraction methods evaluated. The urine sample was adjusted to pH 12, saturated with KCl, and extracted with ethyl acetate–*n*-butanol (4:1). The target compounds were then extracted into HCl solution, followed by basification and extraction into *n*-butyl chloride. Following evaporation, the residue was derivatized with pentafluorobenzoyl chloride and analyzed by GC–MS. Preliminary studies indicated that the extraction recovery was greater than 80% for both metabolites and the internal standard.

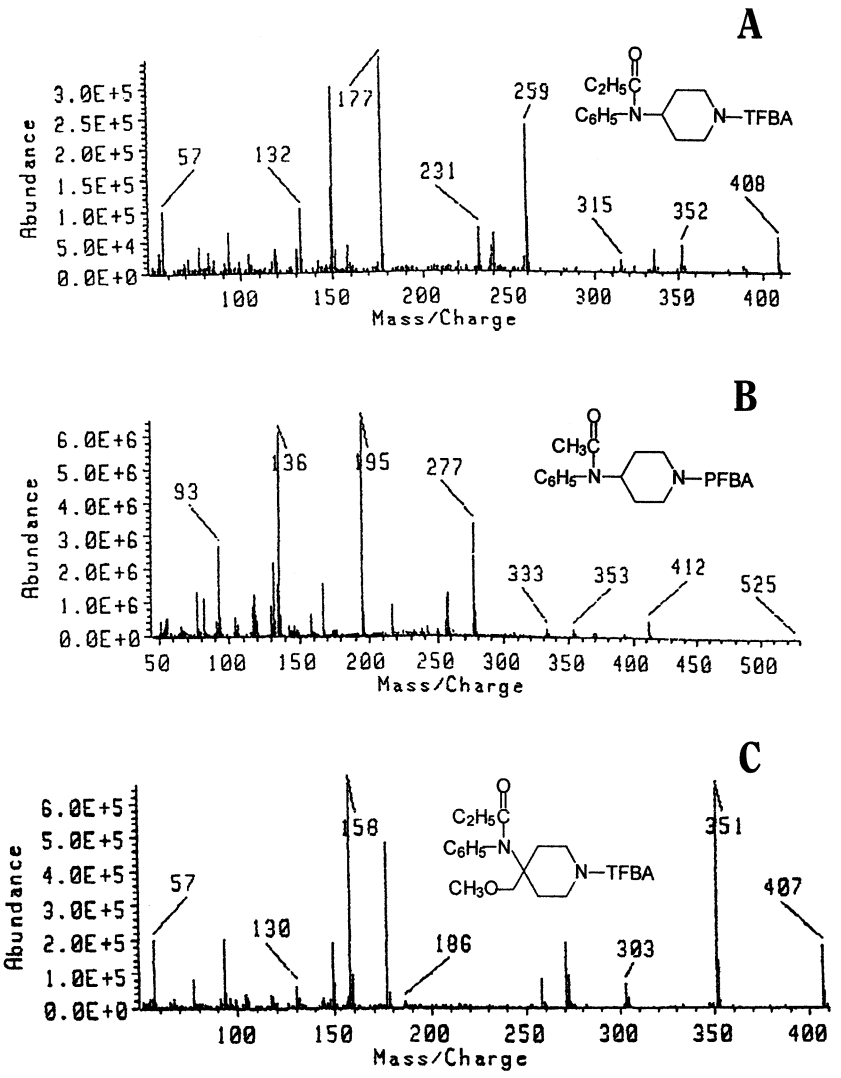

Figure 6. Mass spectra of norfentanyl-TFBA (A), 4-acetanidlidopiperdine-PFBA (B), and norsufentanil-TFBA (C).

The chromatogram in Figure 8 was obtained from the analysis of a urine sample containing 3.0 ng/mL of norsufentanil. A 10-mL portion of urine was extracted and derivatized with pentafluorobenzoyl chloride. The resulting norsufentanil-PFBA derivative was analyzed by GC–MS using selective ion monitoring. The unique ions providing selectivity for norsufentanil-PFBA are *m/z* 425, 369, and 289. The ions monitored for norfentanyl-PFBA are *m/z* 426, 277, and 150. Two unique ions for the internal standard $^2H_5$-norfentanyl-PFBA were monitored at *m/z* 431 and 155. Initial results indicated that norfentanyl and norsufentanil could be detected in urine samples containing concentrations in the 0.3 ng/mL range using the assay described. This level of sensitivity coupled with the high levels of these metabolites excreted in human urine (compared with the parent drugs) should provide significant enhancement to drug screening capabilities for these drugs.

Conclusion

In summary, reference samples of norfentanyl, $^2H_5$-norfentanyl, and norsufentanil–noralfentanil were synthesized and characterized through standard analytical procedures. These secondary amines formed pentafluorobenzamides with good GC properties and unique high-mass fragments. The metabolites could be isolated from human urine by liquid–liquid extraction, derivatized, and monitored by GC–MS using selected ion monitoring. This method will allow maximum sensitivity and specificity for the screening of urine samples for fentanyl, sufentanil, and alfentanil metabolites.

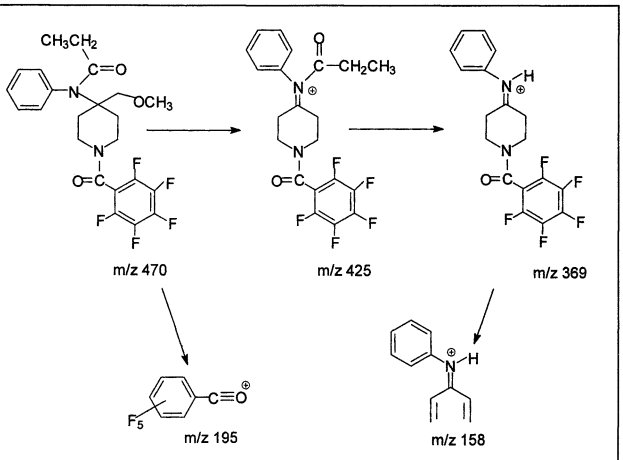

Figure 7. MS fragmentation of norsufentanil-PFBA.

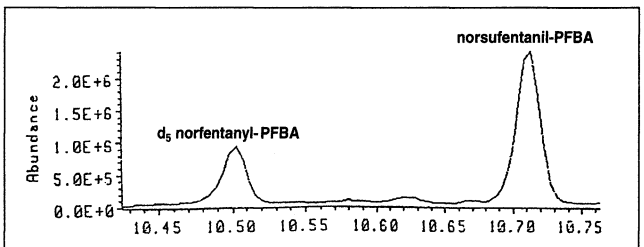

Figure 8. GC–MS ion chromatogram of a urine sample containing norsufentanil.

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