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# Simultaneous extraction and quantitation of fentanyl and norfentanyl from primate plasma with LC/MS detection

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**Abstract**

The quantitation of both fentanyl and its desalkyl metabolite, norfentanyl, in plasma using LC/MS has not been previously described. The detection and quantitation of fentanyl and norfentanyl was achieved using LC/MS detection. The liquid–liquid extraction used toluene as the organic phase. Chromatography was carried out using a Zirchrom-PBD (50 mm × 2.1 mm, 3 μm) column with a mobile phase of acetonitrile–ammonium acetate (10 mM), citrate (0.1 mM, pH 4.4) (45:55, v/v) with a flow rate of 0.3 ml/min. Mass spectroscopy detection was performed using ESI in the positive mode. The LOQ for fentanyl was 25 pg/ml and norfentanyl was 50 pg/ml. For the concentrations of 75, 250, and 750 pg/ml, respectively, fentanyl had inter-day precisions of 6.6, 7.2, and 6.6% with accuracies of 4.0, 5.1, and 5.1% and intra-day precisions of 1.6, 1.9, and 1.9% with accuracies of 11.6, 9.4, and 8.4%, and norfentanyl had inter-day precisions of 7.4, 0.3, and 0.7% with accuracies of 9.1, 8.8, and 12.3% and intra-day precisions of 5.3, 1.4, and 0.1% with accuracies of 10.9, 8.9, and 12.8%. The recoveries of fentanyl were 85, 92, and 75% and of norfentanyl were 40, 49, and 46% at the 75, 250, and 750 pg/ml concentrations, respectively.


*Keywords:* Fentanyl; Norfentanyl; LC/MS; Chimpanzee; Orangutan; Gorilla

## 1. Introduction

Transmucosal fentanyl is an analgesic agent used in the control of cancer pain in humans and as a presurgical sedative for children [1,2]. In great apes, darting or squeeze cages are frequently used to induce anesthesia. These procedures are frequently stressful for the animals and increases the likelihood that they will harm themselves or the veterinary staff. This method was developed to support a pharmacokinetic/pharmacodynamic study of transmucosal fentanyl as a preanesthetic in chimpanzees, orangutans, and gorillas. Along with obtaining data on fentanyl plasma concentrations, it was also desirable to have

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information on the metabolism of fentanyl in these three species of primates.

One of the major human metabolites of fentanyl is norfentanyl [3,4]. Analytical standards of norfentanyl are commercially available, and thus, with the appropriate sensitivity and selectivity, it should be possible to quantify norfentanyl in plasma to obtain metabolism information. There are currently no published extraction and detection procedures that quantitate both fentanyl and norfentanyl from plasma using LC/MS. One published report used GC to quantitate fentanyl and norfentanyl together in urine [5] and several for norfentanyl only in urine using GC/MS [6–8]. Another method used LC to identify metabolites of fentanyl in urine [9]. Fentanyl in plasma has been quantitated using LC [10] and radioimmunoassay [2,11–13]. Furthermore, the lowest level of detection for fentanyl in plasma was 100 pg/ml, the assay reported here allowed quantitation to 25 pg/ml.

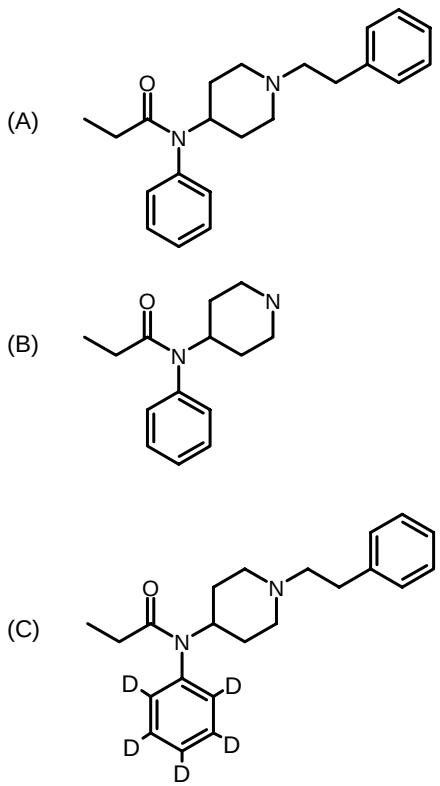


Fig. 1. The molecular structures of (A) fentanyl; (B) norfentanyl; and (C) fentanyl-D5.

The chemical structures of fentanyl, norfentanyl, and fentanyl-D5 are shown in Fig. 1. The molecular weights (MW) of fentanyl and fentanyl-D5 were 336 and 341, respectively. Norfentanyl is an N-dealkylated metabolite of fentanyl that has a MW of 232. The p$K_a$ of fentanyl was reported as 8.4 in water [10].

In this paper, a sensitive and specific assay for fentanyl and norfentanyl is reported. The method describes a relatively simple liquid–liquid extraction, adapted in part from a sensitive sufentanil extraction method [14]. The mobile phase and LC column were acceptable for use with MS. The detection was performed by a MS with an ESI interface and an ion trap, which gave good sensitivity and specificity. Fentanyl and norfentanyl concentrations were quantifiable at 25 and 50 pg/ml, respectively.

## 2. Experimental

### 2.1. Chemicals and reagents

Fentanyl was obtained from Sigma Aldrich (St. Louis, MO). Norfentanyl (1 mg/ml in acetonitrile) and fentanyl-D5 (100 μg/ml in methanol) solutions were obtained from Cerilliant (Round Rock, TX). The acetonitrile, acetic acid, and ammonium acetate were HPLC grade and obtained from Fisher Science (Pittsburgh, PA). Water was deionized using a Nanopure (Barnstead/Thermolyne, Dubuque, IA) system.

### 2.2. Calibration standards and quality control (QC) samples

Standard and quality control samples were made by spiking fentanyl and norfentanyl naïve plasma with stock solutions of fentanyl and norfentanyl. A fentanyl stock solution was made by dissolving fentanyl in mobile phase. Norfentanyl and fentanyl stock solutions were combined and serially diluted to make the solutions used to spike the standard and QC samples. The resulting solutions were equivalent to 10× the final concentration in plasma. To make plasma standards, 100 μl of solution was added to 900 μl of plasma to make a 1 ml plasma solution. Standards and their spike solutions were made fresh each day of analysis. The QC samples were made in bulk and aliquoted into 1 ml aliquots and stored in a −20 °C freezer until used. The

standards and QC samples were extracted in the same manner as the samples. The fentanyl and norfentanyl concentrations in the standard curve were: 25 (fentanyl only), 50, 100, 500, 1000, and 2500 pg/ml. The concentrations of the QCs for this assay were: 75, 250, and 750 pg/ml. A standard curve was generated using height ratios and linear regression. Height ratios for the QCs and samples were compared to the standard curve to obtain their values.

### 2.3. Chromatography

The LC system and the software used to operate the LC/MS system were manufactured by ThermoFinnigan (San Jose, CA). The LC system was equipped with a SCM1000 vacuum degasser, P4000 pump, and AS3000 autosampler. Separation was performed using a Zirchrom-PBD (50 mm × 2.1 mm, 3 μm) column (Zirchrom Separations Inc., Anoka, MN). The mobile phase consisted of acetonitrile–ammonium acetate (10 mM), citrate (0.1 mM, pH 4.4) (45:55, v/v) with a flow rate of 0.3 ml/min. Injections were 50 μl.

### 2.4. Mass spectrometry

The LC/MS system consisted of a $LCQ_{DUO}$ manufactured by ThermoFinnigan and used an electrospray ionization (ESI) source with positive ionization. For the detection of fentanyl the instrument was optimized using fentanyl and set as follows: spray voltage, 4.0 kV; sheath gas flow rate, 46 A.U.; auxiliary gas flow rate, 42 A.U.; capillary voltage, 9 V; capillary temperature, 235 °C; lens voltage, −15 V; multipole 1 offset, −2 V; and multipole 2 offset, −4.5 V. For the detection of norfentanyl, the instrument was optimized using norfentanyl and set as follows: spray voltage, 4.0 kV; sheath gas flow rate, 85 A.U.; auxiliary gas flow rate, 36 A.U.; capillary voltage, 18 V; capillary temperature, 235 °C; lens voltage, −19 V; multipole 1 offset, −4.5 V; and multipole 2 offset, −5.8 V.

For the first 2.5 min the MS was set to monitor in SIM mode at *m*/*z* 337 for fentanyl and *m*/*z* 342 for fentanyl-D5. The fentanyl ions had MS/MS performed at a normalized collision energy of 35%. At 2.5 min the mode was changed to scan for norfentanyl at *m*/*z* 233. The norfentanyl ion also had MS/MS performed at a normalized collision energy of 35% to confirm its identity and for quantitation.

### 2.5. Sample preparation and extraction

Samples were removed from the freezer and allowed to thaw at room temperature (∼25 °C). Once thawed, a 1 ml aliquot was transferred to a 15 ml centrifuge tube. To the 1 ml of plasma, 100 μl of the internal standard solution (5 ng/ml fentanyl D-5) was added. The resulting solution was mixed and then 100 μl of 1 M NaOH was added. The samples were mixed again, and 200 μl of 2-propanol and 2 ml of toluene, in that order, were added. This mixture was vortexed 2× for 1 min each. The extraction mixture was allowed to sit for 20 min and then centrifuged at 2000 × *g* for 10 min. The supernatant was transferred to a fresh tube and another 200 μl of 2-propanol and 2 ml of toluene were added to the remaining plasma, vortexed, and centrifuged again as above. The supernatant was added to the supernatant from the first extraction. The combined supernatants were dried under $N_2$ in a 40 °C water bath, then reconstituted using 100 μl of mobile phase. The reconstituted sample was vortexed for 1 min then transferred to a vial and 50 μl injected onto the LC/MS.

### 2.6. Validation

For the validation assay, two standards were prepared and five QC samples were selected at random from each concentration. These were extracted and tested using the conditions described above. For each subsequent assay, standards were prepared in duplicate and duplicate QC samples were selected at random and extracted with the unknown samples. To be a valid run, the assay had to have both precision and accuracy of the QCs as quantitated by the generated standard curve line equation ≤15%. Precision was measured as (([intended] − [measured])/[intended]) × 100 and accuracy was measured as the coefficient of variation (S.D./average) × 100. For each assay run, no more than 2 QC samples could be >15% from their expected values. If so, the samples were re-extracted and quantitated.

Recovery was measured with the validation run using the same concentrations as the QCs; recovery samples consisted of 100 μl aliquots of the standard spike

solution (equivalent to 100 μl spike, then extraction and reconstituted to 100 μl).

## 3. Results and discussion

### 3.1. Chromatography

Chromatograms of an analytical standard are shown in Fig. 2. The retention times of fentanyl and fentanyl-D5 were the same, as expected. The identity of fentanyl was confirmed by using MS/MS and monitoring for the major daughter ion of *m/z* 188. Norfentanyl was detected approximately 1 min later and the daughter ions of *m/z* 84, 150, and 216 were used for quantitation. Representative chromatograms from each of the three species evaluated are shown in Fig. 3. Only the results from the daughter ions for fentanyl and norfentanyl are shown. There was little background noise from the daughter ion monitoring of fentanyl and norfentanyl.

The mobile phase pH was set at 4.4 because this was a good pH for the ionization of fentanyl and it was relatively easy to maintain using acetate, a

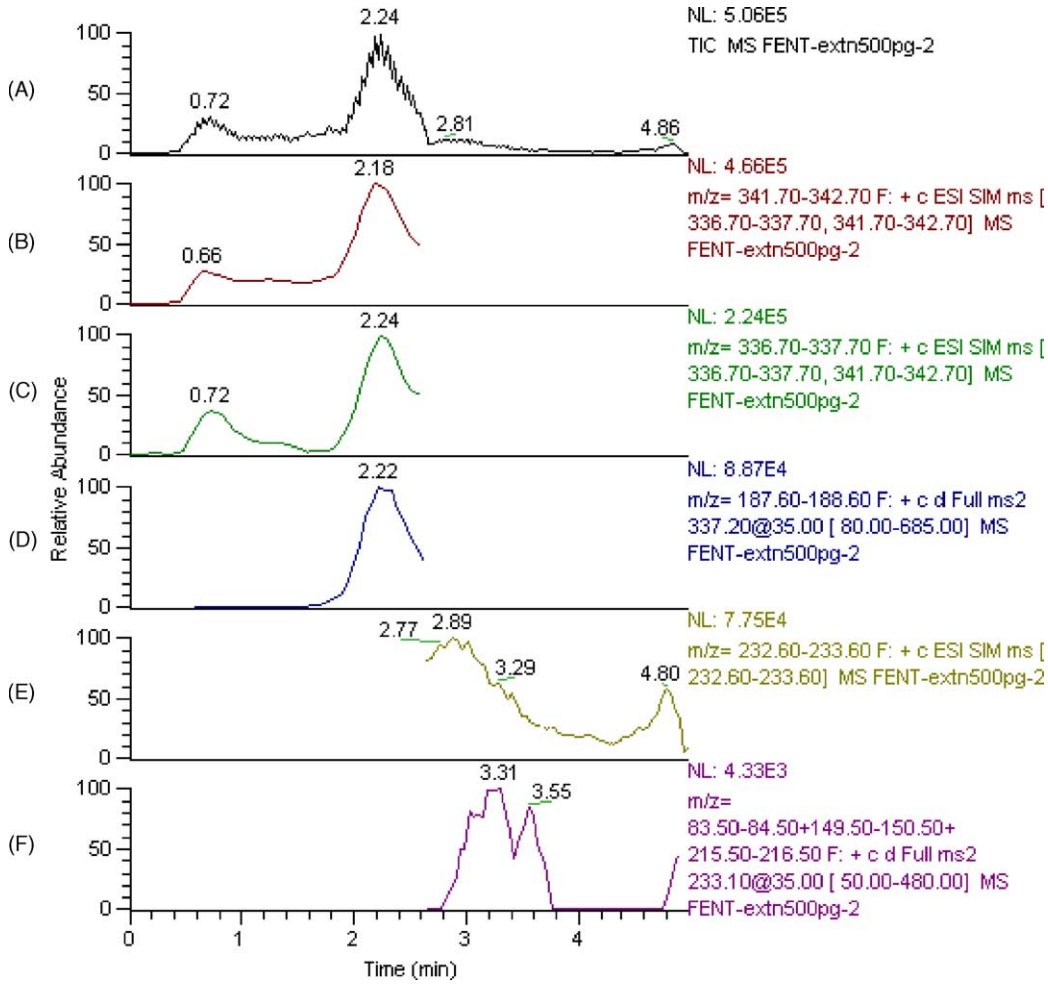


Fig. 2. Picture of chromatogram for fentanyl and norfentanyl taken from a mid-range standard of 500 pg/ml: (A) total ion count for whole chromatogram; (B) fentanyl-D5, *m/z* 342 only; (C) fentanyl, *m/z* 337, prior to MS/MS; (D) main daughter ion, *m/z* 188, from MS/MS of fentanyl; (E) norfentanyl, *m/z* 232 only; and (F) the daughter ions, *m/z* 84, 150, 216, of norfentanyl.

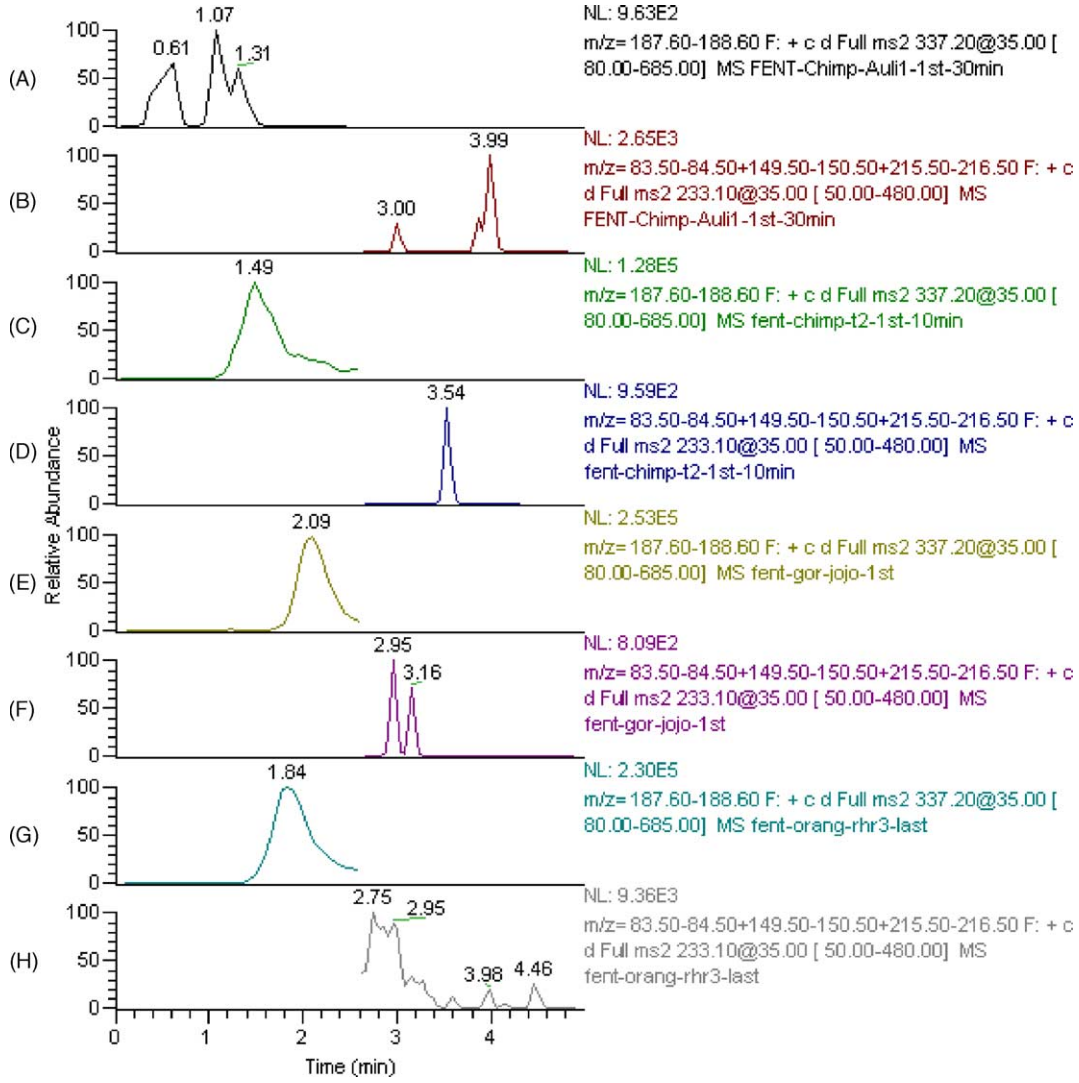


Fig. 3. Chromatograms showing extractions for fentanyl and norfentanyl from chimpanzee, gorilla, and orangutan plasma: (A) absence of fentanyl signal from a chimpanzee that did not receive fentanyl; (B) absence of norfentanyl from the same animal; (C) fentanyl signal from a chimpanzee that received fentanyl; and (D) norfentanyl not apparent in that chimpanzee; (E) fentanyl in a gorilla that received fentanyl; and (F) norfentanyl not detected in that gorilla; (G) fentanyl in an orangutan who received fentanyl; and (H) norfentanyl was present in this animal.

volatile, MS friendly buffer. Changes in the mobile phase constituents had the expected results; increasing the organic phase content resulted in less retention at the expense of peak separation and increasing the aqueous phase allowed for better resolution of the peaks with lower peak height and less sensitivity. The citrate was added to the mobile phase to reduce tailing.

### 3.2. Mass spectrometry

The mass spectra obtained from fentanyl is shown in Fig. 4 and the spectra for norfentanyl is shown in Fig. 5. Both molecules showed similar fragmentation patterns. The fragments are consistent with others previously reported. Specifically, fragmentation around the nitrogen atoms and some dehydrogenation.

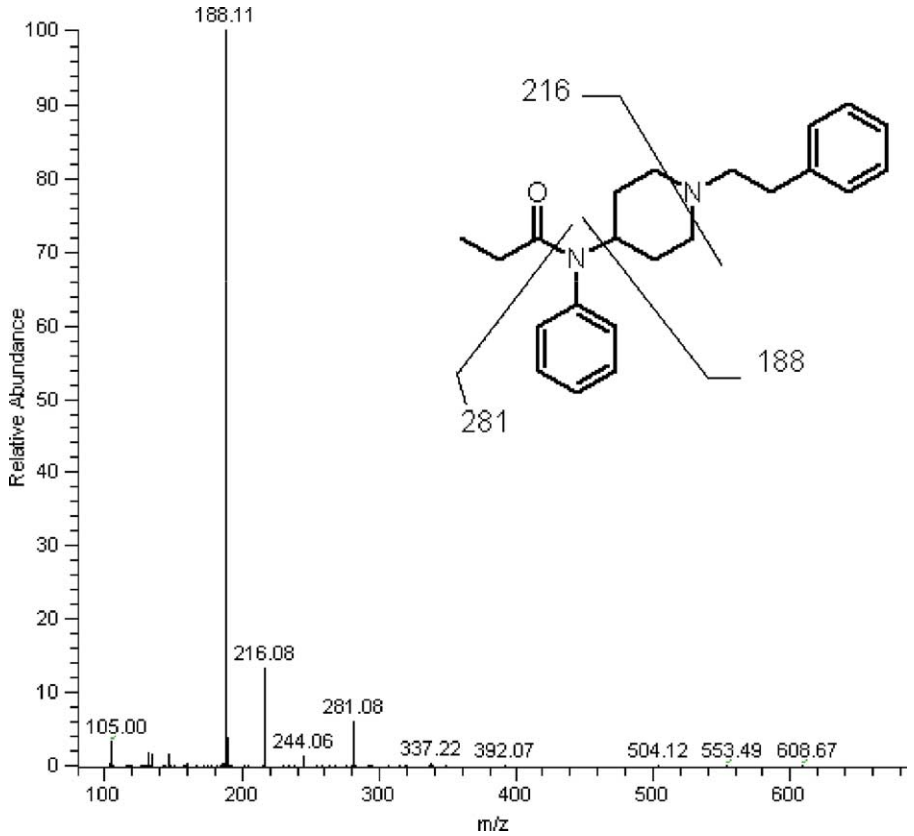


Fig. 4. Spectra of fentanyl obtained from MS/MS. Proposed fragmentation patterns are shown in the molecular diagram.

In this case, for both fentanyl and norfentanyl the production of the daughter ion at *m*/*z* 216 could be caused by the loss of the indicated groups along with the formation of two double bonds in the remaining atoms of the original ring, probably as some type of resonance structure. The production of the other fragments was more straightforward and was the result of simple losses of alkyl groups of the nitrogen.

### *3.3. Validation and recovery*

The intra-day and inter-day precision and accuracy data are shown in Table 1. All values were within the 15% value required for this laboratory. Norfentanyl was quantitated using the daughter ions and was therefore expected to be less accurate. The linear regression equation (mean ± S.D.) for fentanyl was $Y =$

Table 1
The inter-day and intra-day precision and accuracy data for fentanyl and norfentanyl

| | Concentration (pg/ml) | | |
|---|---|---|---|
| | 75 | 250 | 750 |
| Precision | | | |
| Fentanyl | | | |
| Inter-day | 6.6 | 7.2 | 6.6 |
| Intra-day | 1.6 | 1.9 | 1.9 |
| Norfentanyl | | | |
| Inter-day | 7.4 | 0.3 | 0.7 |
| Intra-day | 5.3 | 1.4 | 0.1 |
| Accuracy | | | |
| Fentanyl | | | |
| Inter-day | 4.0 | 5.1 | 5.1 |
| Intra-day | 11.6 | 9.4 | 8.4 |
| Norfentanyl | | | |
| Inter-day | 9.1 | 8.8 | 12.3 |
| Intra-day | 10.9 | 8.9 | 12.8 |

All values are expressed as percent.

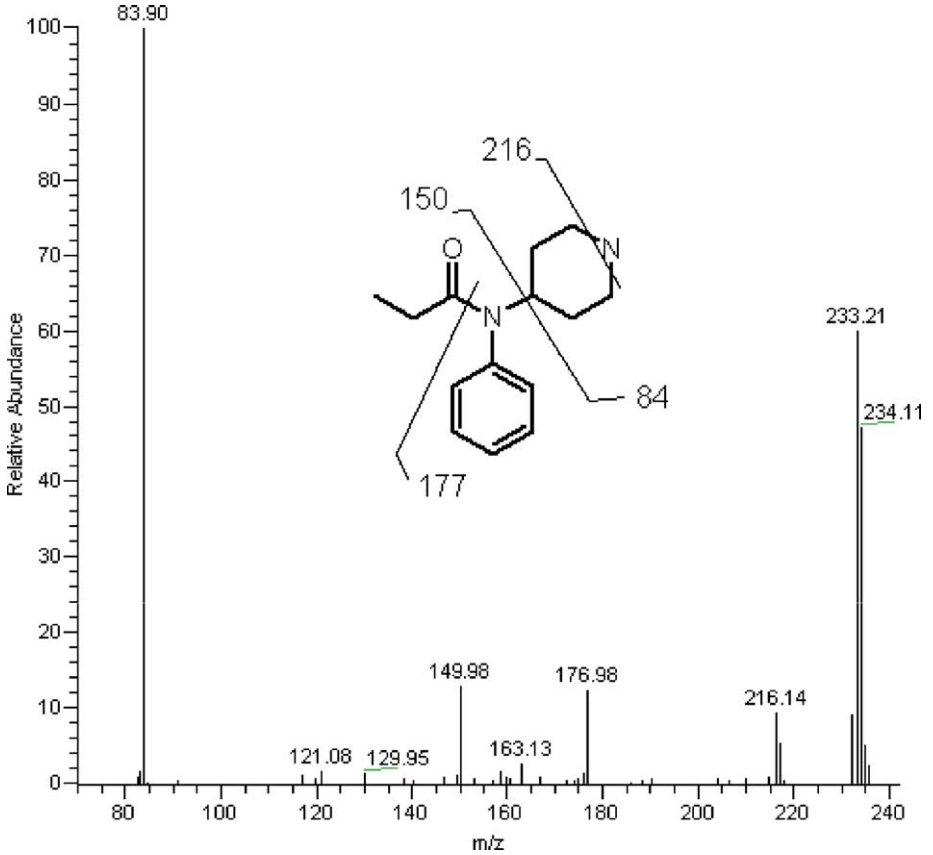


Fig. 5. Spectra of norfentanyl obtained from MS/MS. Proposed fragmentation patterns are shown in the molecular diagram.

5.27E−04(±1.58E−04)$X$ + 4.70E−04(±7.23E−03), $R2 = 0.9983(\pm 0.0022)$; and for norfentanyl was $Y =$ 1.08E−05(±1.59E−06)$X$ + 6.18E−04(±6.59E−04), $R2 = 0.9778(\pm 0.0220)$.

Measurement of the recovery of both compounds was performed. Fentanyl had 85, 92, and 75% recovery at the 75, 250, and 750 pg/ml concentrations, respectively. Norfentanyl extraction resulted in much lower recoveries, 40, 49, and 46% at the 75, 250, and 750 pg/ml concentrations, respectively. It was expected that the lack of the phenethyl group between fentanyl and norfentanyl results in enough of a lipid solubility difference to allow for better extraction of fentanyl over norfentanyl. The primary goal of this method was for the quantitation of fentanyl at low levels. With that in mind, less than optimal extraction efficiency for norfentanyl was acceptable.

## 4. Conclusions

This method results in a sensitive and accurate assay that allows for the quantitation of both fentanyl and norfentanyl from primate plasma. The liquid–liquid extraction combined with the sensitivity of MS detection has allowed lower quantitation concentrations of both compounds than previously reported. Further improvements to this assay would include an extraction that achieves better extraction efficiency for norfentanyl while not sacrificing the extraction efficiency of fentanyl.

## Acknowledgements

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