Technical Note

Analysis of Fentanyl and Norfentanyl in Human Plasma by Liquid Chromatography–Tandem Mass Spectrometry Using Electrospray Ionization

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Abstract

This report describes a sensitive and specific high-performance liquid chromatography (HPLC)–electrospray ionization-tandem mass spectrometry (MS–MS) method for the detection of subnanogram concentrations of fentanyl and its metabolite norfentanyl in human plasma. The assay was based on a liquid–liquid extraction of 0.5 mL of human plasma, with a lower limit of quantitation (LLOQ) of 0.05 ng/mL. Sample extracts were analyzed using a ThermoQuest TSQ® MS–MS interfaced with a Hewlett-Packard series 1100 HPLC and a Phenomenex® (30 × 2.00-mm, 5 μ Luna C18(2)) column. The intra-assay precision and accuracy ranged from 2.1 to 12.5% for both analytes at concentrations of 0.1, 0.5, 1.0, and 10 ng/mL. The interassay accuracy and precision ranged from 7.34 to 10.95%.

Introduction

Fentanyl, N-(1-phenethyl-4 piperidyl) propionanilide, is an opiate agonist used in medical procedures as a narcotic analgesic (1). Analysis of fentanyl and its metabolite norfentanyl in specimens with limited sample volume has become more feasible with liquid chromatography (LC) and atmospheric-pressure ionization mass spectrometry (API-MS). Previous methods of fentanyl and norfentanyl analytes employed gas chromatography (GC), GC–MS, and radioimmunoassay (RIA) (2–10); however, some GC and GC–MS methods can be time consuming because of the need for possible sample hydrolysis (urine) and derivatization of the analytes to achieve comparable levels of sensitivity. RIA methods are not as specific as MS and are more commonly used for higher concentrations of fentanyl. The coupling of LC with MS and API offers a much more sensitive and specific analytical technique for the detection and quantitation of subnanogram-per-milliliter concentrations of fentanyl and its metabolite norfentanyl in specimens with limited sample volume. Tandem mass spectrometry (MS–MS) affords an even greater specificity than single-stage MS. MS–MS provides im-

proved signal-to-noise ratios and lower limits of detection (low picogram-per-milliliter range) for many commonly detected drugs, such as fentanyl. Solid-phase extractions coupled with LC–MS–MS achieve similar levels of detection compared with GC–MS (11,12). However, these methods are often less cost effective and more labor intensive than a liquid–liquid extraction procedure. This paper describes a rapid and effective procedure to detect lower concentrations of fentanyl and its metabolite norfentanyl using small sample volumes and a less intensive extraction procedure.

Materials and Methods

Chemicals and reagents

Fentanyl (1 mg/mL in methanol), fentanyl-d_5 (100 μg/mL in methanol), norfentanyl (1 mg/mL in methanol), and norfentanyl-d_5 (100 μg/mL in acetonitrile) reference materials were obtained from Cerilliant™ (Austin, TX), with a stated minimum purity of 99%. High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were obtained from Burdick and Jackson Brand (Muskegon, MI); n-butyl chloride, ammonium hydroxide and concentrated formic acid from Fisher Scientific (Pittsburgh, PA). All solvents were 99.9+% purity as stated by manufacturer. Water for the preparation of the mobile phase was drawn from a Milli Q™ filter (Millipore, Bedford, MA) apparatus.

Standards and quality control samples

Working solutions were prepared in methanol at 10, 1.0, 0.1, 0.01, and 0.001 ng/μL of fentanyl and norfentanyl from purchased reference material. Working solutions were then used to prepare the calibration curve. Calibration curves were prepared daily in drug-free human plasma containing 1% NaF. The calibration curve was fortified with fentanyl and norfentanyl at 0.05, 0.075, 0.1, 0.25, 0.5, 0.75, 1.0, 5.0, 10.0, and 50 ng/mL. Each calibrator was analyzed in duplicate and quality control specimens were analyzed in triplicate. A second set of working solutions was prepared from separate lot numbers of reference material and used fortify quality control samples. Quality control samples were prepared at 0.1-, 0.5-, 1.0-, and 10-ng/mL con-

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centrations. Drug-free plasma was also extracted in each batch in duplicate and analyzed as negative controls. Working solutions were stable for up to 6 months at –20°C.

Sample preparation and extraction

Internal standard (0.5 ng/mL of fentanyl-d_5/norfentanyl-d_5; 25 mL of a 0.01-ng/µL stock solution) was added to 0.5 mL of each calibrator and control. The samples were vortex mixed and allowed to equilibrate for 30 min. Concentrated ammonium hydroxide (50 µL) was added to each tube to adjust the pH to greater than 9 and mixed well. The fentanyl and norfentanyl were extracted from the samples by the addition of 4 mL of *n*-butyl chloride/acetonitrile (4:1, v/v). The samples were then mixed by rocking for 20 min. After mixing the samples were centrifuged at 2250 rpm for 20 min. The organic layer of each was transferred to clean silanized tubes and evaporated to dryness under a stream of air in a turbo vap at 40°C. The residues were reconstituted in 50 µL of 0.1% formic acid in water/methanol (90:10, v/v) and transferred to 0.7-mL conical bottom autosampler vials.

HPLC–ESI–MS–MS analysis

HPLC–ESI–MS–MS analysis of the sample extracts was performed using a ThermoQuest TSQ tandem MS (ThermoQuest Instruments, San Jose, CA) interfaced with a Hewlett-Packard series 1100 HPLC (Agilent Technologies, Palo Alto, CA). The HPLC mobile phase consisted of 0.1% formic acid in water and methanol (90:10, v/v). The pump was operated isocratically at 0.20 mL/min and ambient temperature. Chromatographic separation of analytes was achieved on a Phenomenex 30-mm × 2.00-mm Luna 5µ C18(2) column (Phenomenex USA, Torrance, CA).

The ESI source was operated with the spray voltage at 4.5 kV with 80 psi of sheath gas (high purity N_2). The heated capillary was maintained at 250°C. Positive precursor ions for fentanyl (*m/z* 337), fentanyl-d_5 (*m/z* 342), norfentanyl (*m/z* 233), and norfentanyl-d_5 (*m/z* 238) were selected to pass through the first quadrupole. In the second quadrupole, collision-induced dissociation (CID) was achieved using argon as the collision gas (at 3mTorr) and collision voltages of –30 volts for fentanyl and –25 volts for norfentanyl. Product ions monitored in the third quadrupole were *m/z* 84.3 (norfentanyl and norfentanyl-d_5) and *m/z* 188.4 (fentanyl and fentanyl-d_5). The scan time was 0.2 s/scan.

Quantitative analysis

Quantitative concentrations of fentanyl and its metabolite norfentanyl in plasma were determined by calculating peak-area ratios for the MS–MS product ions of each analyte and its respective deuterated isotopomer internal standard. The sample volume extracted was between 250 µL and 500 µL. Linear curve fits with 1/Y weighting were used to ensure accurate quantitation across the range of the assay. Finnigan LCQuan™ (version 1.2) quantitation software was used to generate calibration curves and to calculate fentanyl, and norfentanyl concentrations in analyzed samples.

Precision and accuracy experiments

Intra-assay (within-run) precision and accuracy were evaluated by analyzing quality control (QC) samples in a single batch

at concentrations of 0.1, 0.5, 1.0, and 10 ng/mL (*n* = 3 each concentration). The mean measured concentration of each analyte was determined and expressed as a percentage of the target concentration to evaluate the quantitative accuracy. The coefficient of variation (%CV) for each analyte at each concentration was also determined to assess precision.

Interassay (between-day) precision and accuracy was accumulated from eight analytical batches for fentanyl and norfentanyl. Of these batches, seven consisted of QC material analyzed in triplicate, whereas the eighth included QC material in duplicate. A total of 23 QC samples across the eight analytical runs were evaluated. The mean measured concentration of each analyte was determined and expressed as a percentage of target concentration to evaluate the interassay quantitative accuracy over the eight analytical runs. The coefficient of variation (%CV) for each analyte at each concentration was also determined to assess precision.

Recovery experiments

In a separate experiment, a calibration curve was extracted concurrently with two separate sets of QCs to determine the recovery of fentanyl and norfentanyl. Analyte recovery was determined at 0.1, 0.5, 1.0, and 10 ng/mL. One set of drug-free plasma samples (*n* = 5 at each concentration) was fortified with analyte and internal standard and processed through the extraction procedure. A second set (*n* = 5 at each concentration) was only fortified with non-deuterated analyte. This second set was then extracted (see Sample preparation). After extraction internal standard was added to extract residues followed by evaporation to dryness under stream of air.

Percent recovery was determined at each concentration by dividing the average peak area at each concentration from the extracted samples with IS added after extraction by the average peak area for each concentration from the extracted samples with IS added initially and multiplying by 100. Carryover was estimated by evaluation of a negative calibrator fortified with internal standard injected after highest calibrator.

Analysis of pediatric plasma specimens

The performance of the developed assay was evaluated in plasma from five children who were receiving fentanyl by continuous infusion. These patients were part of a larger on-going study of fentanyl pharmacokinetics in critically ill children in the pediatric intensive care unit. The study was approved by the Institutional Review Board of the University of Utah and informed consent was obtained from the parents prior to study enrollment.
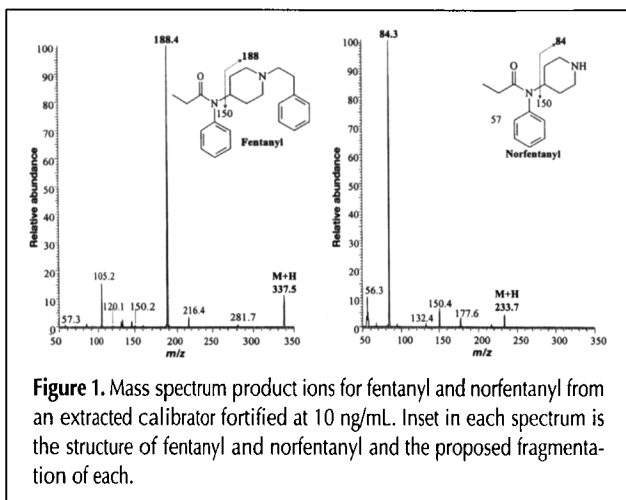
Statistical analysis

Analysis of data for statistical evaluation was processed using Microsoft Excel for Windows® (version 9.0).

Results and Discussion

Analytical method

The chromatograms exhibited Gaussian peak shape and signal-to-noise ratios for fentanyl and norfentanyl were 23:1

Figure 1. Mass spectrum product ions for fentanyl and norfentanyl from an extracted calibrator fortified at 10 ng/mL. Inset in each spectrum is the structure of fentanyl and norfentanyl and the proposed fragmentation of each.
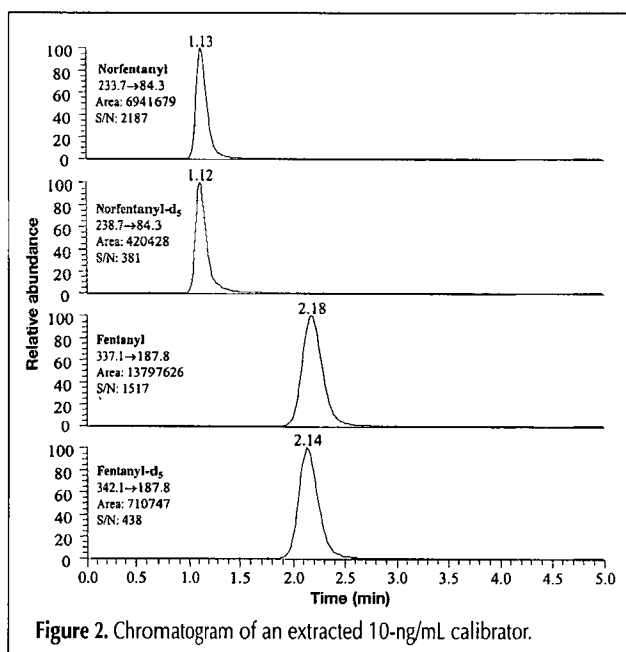
Figure 2. Chromatogram of an extracted 10-ng/mL calibrator.

and 3:1, respectively, at 0.05 ng/mL (LOQ). The product ion spectra for fentanyl and norfentanyl are shown in Figure 1. A chromatogram obtained from the analysis of an extracted plasma calibrator fortified at 10.0 ng/mL is shown in Figure 2.

Precision and accuracy

Intra-assay and interassay precision and accuracy are summarized in Table I. The intra-assay accuracy of fentanyl ranged from 82 to 95% of the theoretical target concentration. The interassay accuracy of fentanyl ranged from 93 to 97%. The intra-assay accuracy of norfentanyl ranged from 97 to 110% of the theoretical target concentration, and the interassay accuracy ranged from 93 to 102%.

The intra-assay precision for fentanyl ranged from 2.1 to 12.5% CV across three concentrations. The interassay precision ranged from 7.7 to 13.2% CV across 23 QC samples. The intra-assay precision of norfentanyl ranged from 4.1 to 11% CV, and the interassay precision ranged from 10.5 to 11.8%. CV%s presented are comparable to accepted ranges for LC–MS–MS methods for analysis of biological samples.

No carryover was observed (no peaks were found with signal-to-noise ratios higher than 3:1) in blank specimens following highest calibrator.

Pediatric samples

Five children received fentanyl by continuous infusion ranging from 4 to 10 µL/kg/h (see Table II). Steady-state plasma fentanyl concentrations ranged from 1.83 to 4.63 ng/mL and norfentanyl concentrations ranged from 0.68 to 3.06 ng/mL. An example of a typical chromatogram is shown in Figure 3. This demonstrates that the method is sufficiently sensitive and specific for the detection of therapeutic concentrations of fentanyl and norfentanyl in pediatric specimens.

Recovery

Recoveries of fentanyl and norfentanyl were from 67% to 84% and from 72% to 90%, respectively. Data are summarized in Table III.

Table I. Precision and Accuracy of Fentanyl and Norfentanyl in Plasma

	Fentanyl*					Norfentanyl*				
	Target Conc. (ng/mL)	n	Mean Conc. (ng/mL)	% of Target	CV(%)	Target Conc. (ng/mL)	n	Mean Conc. (ng/mL)	% of Target	CV(%)
Intra-assay[†]	0.1	3	0.10	95	12.5	0.1	3	0.11	110	11.0
	0.5	3	0.44	88	2.3	0.5	3	0.50	100	4.1
	1	3	0.82	82	2.1	1.0	3	0.97	97	5.4
	10	3	9.07	91	4.2	10.0	3	10.01	100	8.8
Interassay[‡]	0.1	23	0.10	97	13.2	0.1	23	0.10	102	11.0
	0.5	23	0.47	95	7.7	0.5	23	0.46	93	11.6
	1	23	0.93	93	10.9	1.0	23	0.96	96	10.5
	10	23	9.74	97	10.2	10.0	23	9.94	99	11.4

* Mean values at each concentration were used to calculate the %target and the %CV.
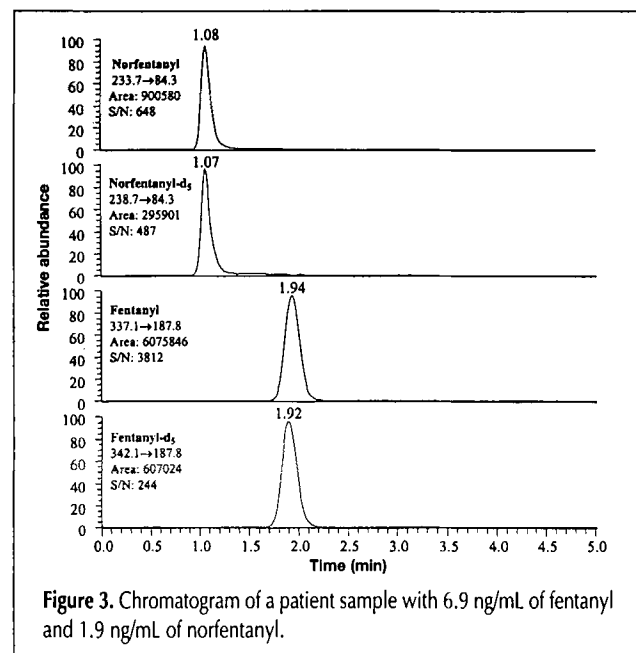[†] Intra-assay precision analysis performed on a single day of analysis.
[‡] Interassay precision performed across eight batches for both fentanyl and norfentanyl of which seven batches were run in triplicate, while the eighth was run in duplicate. All batches were run on separate days.

Conclusions

This report describes a sensitive and specific method for the analysis of fentanyl and its metabolite norfentanyl in plasma using HPLC-ESI-MS-MS. The assay has an LLOQ of 0.05 ng/mL for both drugs. The assay was shown to have good quantitative precision and accuracy after storage at -20°C and processing conditions during experimentation that are considered normal sample handling situations.

Table II. Steady-State Plasma Fentanyl and Norfentanyl Concentrations in Five Children Receiving Fentanyl by Continuous Infusion

Age (years)	Weight (kg)	Fentanyl Infusion Rate (μg/kg/h)	Fentanyl Conc. (ng/mL)	Norfentanyl Conc. (ng/mL)
8.6	25.0	4	2.52	0.68
17.7	52.0	5	4.17	0.99
1.1	6.0	5	1.83	2.29
2.2	12.0	10	4.36	3.06
1.8	7.6	4	2.17	0.96

Figure 3. Chromatogram of a patient sample with 6.9 ng/mL of fentanyl and 1.9 ng/mL of norfentanyl.

Table III. Estimated Recovery of Fentanyl and Norfentanyl Extracted from Human Plasma*

	Concentration Tested (ng/mL)			
	Non-deuterated Analytes			
	0.1	0.5	1	10
Fentanyl	83.77%	83.81%	79.69%	67.28%
		mean: **78.64%**		
Norfentanyl	88.66%	72.20%	75.09%	89.78%
		mean: **81.43%**		

* Each concentration was at (n = 5). Values were calculated by dividing the mean peak area at each concentration for standards which had internal standard added after extraction by those where the internal standard was added prior to extraction and multiplying by 100.

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