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Research Article

Theme: Team Science and Education for Pharmaceuticals: the NIPTE Model
Guest Editors: Ajaz S. Hussain, Kenneth Morris, and Vadim J. Gurvich

# Sensitive Determination of Fentanyl in Low-Volume Serum Samples by LC-MS/MS

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***Abstract.*** Fentanyl is a widely used drug in the management of pain. Present LC-MS/MS methods for analysis of fentanyl require a large volume of serum, but yet the sensitivity was at about 50 pg/mL. Here, we report a modified liquid-liquid extraction method for the analysis of fentanyl in serum. The method is very sensitive with a LLOQ of 5 pg/mL while using only 0.175 mL of serum for analysis. The separation was performed on a Zorbax XDB-C18 column (4.6 × 50 mm, 1.8 μm, 600 bar) using a mobile phase of water: acetonitrile (70:30 *v*/*v*) with 0.1% formic acid that was pumped isocratically at a flow rate of 0.5 mL per minute. The calibration curve was found to be linear over a range of 5–10,000 pg/mL. The inter-day and intra-day accuracy and precision were tested using low (20 pg/mL), medium (1000 pg/mL), and high (5000 pg/mL) quality control samples of fentanyl prepared in blank human serum and were within ± 15% of the nominal value. Fentanyl was also found to be stable in various storage and sample preparation conditions, including short-term bench-top storage **(**for 5 h**)**, freeze-thaw cycling (three cycles), long-term frozen condition (4.5 months at -70°C), and post-preparative storage (for 48 h).

**KEY WORDS:** fentanyl; LC-MS/MS; validation; analytical method; human serum.

## INTRODUCTION

Fentanyl (N-phenyl-N-[1-(2-phenylethyl)piperidin-4yl]propanamide) is a synthetic opioid that is commonly used in pain management. Fentanyl is available in many drug products and can be administered *via* dermal, buccal, sublingual, nasal, and parenteral routes (1). In pre-clinical and clinical studies conducted for development and approval of drug products, accurate and sensitive methods to assay fentanyl in biological fluids are needed. This is particularly important for serum and plasma samples, because fentanyl is a relatively potent drug and generates a therapeutic response at very low concentrations ranging between 0.2 and 1.2 ng/mL (2). Detection methods based on gas chromatography (GC) coupled with mass spectrometry (3), high-performance liquid chromatography (HPLC) (4–6), liquid chromatography coupled with mass spectrometry (LC-MS/MS) (7–11), and immunoassays (12–14) are currently employed to determine fentanyl levels in plasma. However, LC-MS/MS-based methods are generally preferred as they allow rapid and sensitive drug analysis in biological samples (15–17). These methods often require large sample volumes of 0.25–1 mL (14,18–20). Here, we report a validated LC-MS/MS-based method that allows determination of fentanyl in low volume, serum samples (0.175 mL) with very high accuracy, precision, specificity, and sensitivity (LLOQ of 5 pg/mL).

## MATERIALS AND METHODS

### Materials

#### *Chemicals and Reagents*

Fentanyl reference standard and deuterated fentanyl (fentanyl $D_5$) were purchased from the Cerilliant Corporation (Round Rock, TX). Human serum was purchased from Biological Specialty Corporation, Colmar, PA. Sodium hydroxide and 2-butanol were purchased from Sigma-Aldrich, (Saint Louis, MO). Methanol, acetonitrile, n-heptane, and

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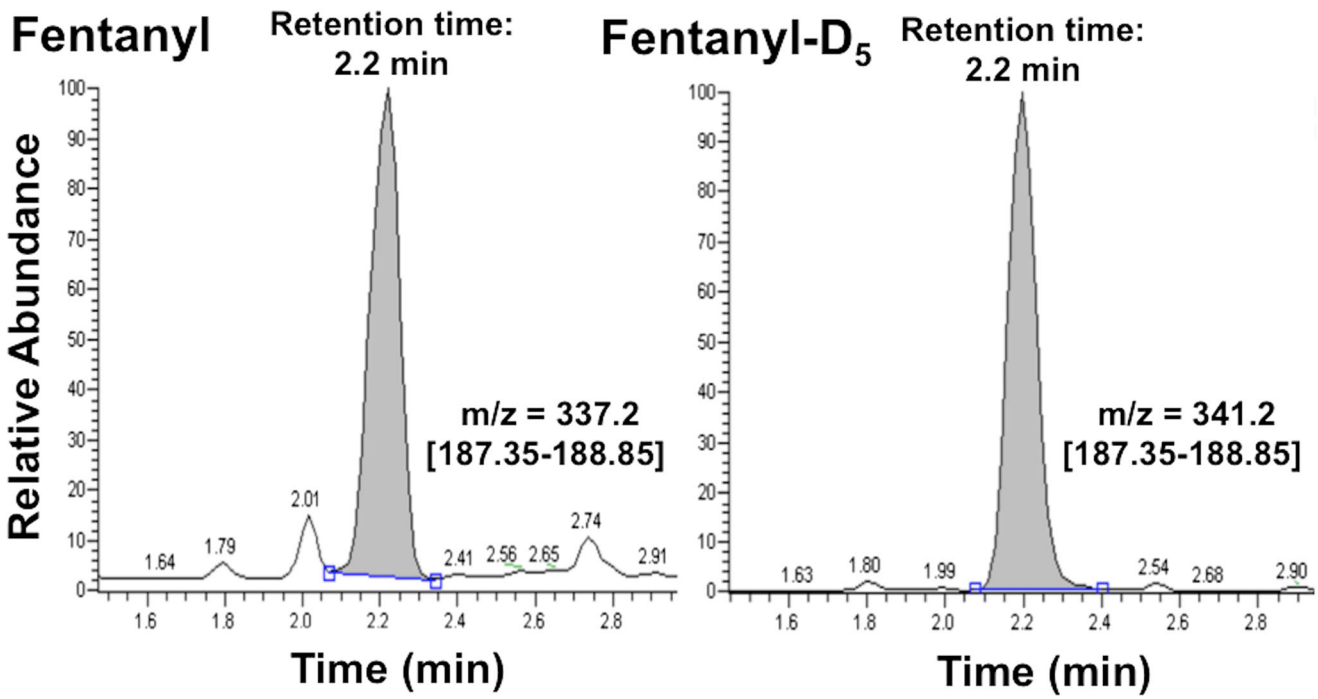


**Fig. 1.** Representative chromatograms of fentanyl analyte (at 5 pg/mL) and fentanyl $D_5$ (internal standard)

formic acid were purchased from Fisher Scientific (Pittsburgh, PA).

*Supplies*

The Zorbax XDB-C18 column (4.6 × 50 mm, 1.8 μm, 600 bar) and flat bottom HPLC vial inserts were purchased from Agilent Technologies (Santa Clara, CA). All other consumables were purchased from Fisher Scientific (Pittsburgh, PA).

*Equipment*

A TSQ Quantum triple-stage quadrupole mass spectrometer (Thermo Electron, San Jose, CA) coupled with 1200 series HPLC (Agilent, Santa Clara, CA) was used in the sample analysis. The OA-SYS™ heating system (Organomation Associates, Berlin, MA) was used for drying the organic phase used for sample extraction. Centrifuges - accuSpin Micro and Thermo electron IEC CL40 (Fisher Scientific, Pittsburgh, PA) were used in the sample preparation.

## Methods

*Preparation of Standard Solutions*

Various sub-stock solutions of fentanyl at concentrations 10, 1, 0.1, 0.01, and 0.001 μg/mL were prepared in methanol from the fentanyl reference standard. These solutions were further diluted in methanol to prepare calibration standard solutions at 70, 35, 17.5, 7, 3.5, 0.7, 0.4, 0.1, 0.07, and 0.035 ng/mL levels. These solutions were stored in screw-capped glass vials at - 20°C. Adequate care was taken to return the fentanyl calibration standard solutions to freezer immediately after use. Deuterated fentanyl (fentanyl $D_5$) was used as the internal standard (ISTD). A working stock solution of 100 ng/mL was prepared in methanol and stored at - 20°C.

*Quality Control Samples*

Quality control samples of fentanyl were prepared in blank human serum. High (5000 pg/mL), medium (1000 pg/mL), and low (20 pg/mL) quality control (QC) samples were prepared as single-use aliquots and then stored at - 70°C until use.

*Extraction Procedure*

The extraction of fentanyl from serum was based on a liquid-liquid extraction method by Huynh *et al.* (7) with several modifications. Importantly, the extraction method was further optimized to reduce the required serum volume to 0.175 mL.

The serum samples (0.175 mL) were spiked with 20 μL of the ISTD and then vortex mixed. The samples were combined with 1 M aqueous sodium hydroxide (0.053 mL), reverse osmosis purified water (0.18 mL), and n-heptane containing 3% 2-butanol (1.23 mL). Then, the samples were mixed by vortexing for 5 min followed by centrifugation at 15,000×*g* for 10 min. The organic phase was transferred to 100 × 12-mm glass tubes, and then the solvent was completely evaporated under nitrogen using an OA-SYS™ heating system maintained at 37°C. The residual content was reconstituted in mobile phase (100 μL), centrifuged (9600×*g*) for 5 min at 4°C, and the clear supernatant was analyzed by LC-MS/MS.

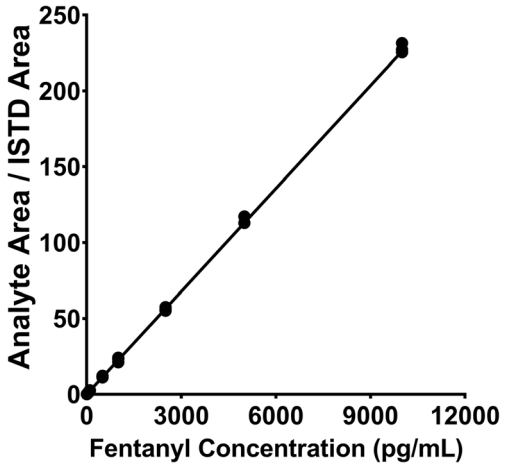


**Fig. 2.** Standard curve of fentanyl in human serum. Linear regression model with a weighting scheme of $1 \quad x^2$ was used to analyze the fentanyl/fentanyl $D_5$ peak area ratio *versus* fentanyl concentration

*Liquid Chromatography Conditions*

The samples (15 μL) were injected onto a Zorbax XDB-C18 column and separated using a mobile phase composed of water/acetonitrile (70:30 *v*/*v*) containing 0.1% formic acid at a flow rate of 0.5 mL/min. Column temperature was set at 30°C.

*Mass Spectrometer Conditions*

The detector settings of the TSQ Quantum were as follows. A stainless steel spray needle was used for electron spray ionization (ESI) in positive polarity mode, selective reaction monitoring mode (SRM); spray voltage, 5000 V; capillary temperature, 400°C; nitrogen sheath gas pressure, 50 psig; nitrogen aux gas, 16 units; argon collision gas pressure, 1.5 mTorr; unit resolution for Q1 and Q3, 0.7 u (FWHM); and ions detected (*m*/*z*), fentanyl $D_5$ (IS) precursor 341, product 188, and fentanyl precursor 337, product 188. The collision energy was 5 eV for both fentanyl and the ISTD. *Validation of the Method.* The developed method was validated for linearity, accuracy, precision, recovery, and stability.

1. Linearity: The linearity of the method was determined using 10 non-zero fentanyl standards spanning the concentration range of 5–10,000 pg/mL. The correlation coefficient obtained by plotting peak area ratio of fentanyl/fentanyl $D_5$ ISTD against fentanyl concentration and fitting it to a linear regression model with a weighting scheme of $1 \quad x^2$.

**Table I.** Parameters of the Calibration Curves of Fentanyl Standards in Human Serum

| | Mean | S.D. |
|---|---|---|
| Slope | 0.0227 | 0.001 |
| Intercept | −0.0362 | 0.012 |
| $r^2$ | 0.9932 | 0.002 |

**Table II.** Accuracy and Precision of Fentanyl Calibration Standards in Human Serum

| Calibrator concentration (pg/mL) | Accuracy (%RE) | Precision (%RSD) |
|---|---|---|
| 5 | −4.8 | 15 |
| 10 | −2.6 | 19 |
| 20 | −6.4 | 8 |
| 50 | −6 | 8 |
| 100 | 6.9 | 5 |
| 500 | 2.5 | 3 |
| 1000 | 0.6 | 5 |
| 2500 | −0.8 | 4 |
| 5000 | 2.0 | 5 |
| 10,000 | 0.4 | 4 |

2. Inter-day and intra-day assay accuracy and precision: The inter-day and intra-day accuracy as well as precision of the method was investigated on three separate days. On each run, blank samples, calibrators, and QCs were assayed as described below.

Blanks: Serum blank (without drug or ISTD) and serum spiked with ISTD were assayed to ensure that the method could specifically detect fentanyl in the samples. Mobile-phase blanks were included in the assay to capture any carryover between the sample injections.

Calibrators: Fentanyl standards at concentrations of 5, 10, 20, 50, 100, 500, 1000, 2500, 5000, and 10,000 pg/mL were used. For the calibrators at lower concentration, 5, 10, 20, and 50 pg/mL, two replicates were employed to enhance the assay precision.

QC samples: Five replicates each of low, medium, and high QC samples were assessed. Fentanyl levels in the QCs were back calculated from the calibration curve. Accuracy was determined by calculating the percentage of relative error (% RE) as in (21). Precision of the method was estimated by calculating the percentage of relative standard deviation (%RSD). Intra- and inter-assay variability (expressed as $\%RSD_{intra}$ and $\%RSD_{inter}$, respectively) was analyzed using single-factor ANOVA and a statistical model initially described by Robard *et al.* (22).

3. Extraction efficiency: Extraction efficiency was tested by using 10 non-zero fentanyl standards spanning 5–10,000 pg/mL. Fentanyl concentrations estimated from the calibration curve of fentanyl that was added to and extracted from serum were compared against fentanyl concentrations estimated from the calibration curve of fentanyl analyte in methanol.
4. Stability studies: The bench-top, freeze-thaw, long-term, and shipping stability of fentanyl samples was assessed using five replicates each of low, medium, and high QC samples. For bench-top stability studies, QC samples prepared and frozen at −70°C were thawed unassisted and then left undisturbed for 5 h at room temperature prior to sample extraction. For freeze-thaw stability studies, QC samples prepared and frozen at −70°C were thawed unassisted at room temperature and then re-frozen for 24 h. The samples were then subjected to two more free-

**Table III.** Accuracy and Intra-assay ($RSD_{intra}$) and Inter-assay ($RSD_{inter}$) Precision of Fentanyl Quality Controls in Human Serum

| Quality control concentration (pg/mL) | Accuracy (%RE) | $\%RSD_{intra}$ | $\%RSD_{inter}$ |
|---|---|---|---|
| 20 | −2.9 | 13.7 | 5.1 |
| 1000 | −5.5 | 5.2 | 0.1 |
| 5000 | 0.1 | 4 | 2.1 |

**Table IV.** Stability Studies of Fentanyl in Human Serum

| Quality control samples (pg/mL) | Bench-top | Freeze-thaw | Long-term stability | Shipping and handling stability |
|---|---|---|---|---|
| 20 (low, $n = 5$) | 105.9 | 106.4 | 97.6 | 96.5 |
| 1000 (medium, $n = 5$) | 94.7 | 99.7 | 108.4 | 93.8 |
| 5000 (high, $n = 5$) | 97.4 | 97.9 | 109.6 | 98.1 |

Data shown is accuracy %

thaw cycles and then extracted on the third day. For long-term stability studies, QC samples stored at −70°C for about 145 days (about 4.5 months) were extracted and assayed as described. Routinely, serum samples containing fentanyl are shipped to the reference laboratories for analysis. Hence, we validated the shipping stability of the fentanyl samples. QC samples stored at −70°C were transferred to a Styrofoam box filled with dry ice and then shipped by overnight mail to one of our research collaborators based in Chicago. Upon receiving the shipment, our collaborator independently authenticated the contents and then sent us back with the shipment by overnight mail. Once the samples were received, they were stored at −70°C prior to subsequent processing. For post-preparative stability studies, fentanyl standards were extracted from human serum samples and then transferred to autosampler vials. Samples from the same vials were injected after 24 and 48 h. In between the runs, the samples remained refrigerated in the sample tray.

**Table V.** Post-preparative Stability of Fentanyl Calibration Standards in Human Serum

| Fentanyl calibrator concentrations (pg/mL) | Day 0 | Day 1 | Day 2 |
|---|---|---|---|
| 5 | 114.6 | 102.0 | 121.9 |
| 5 | 92.6 | 96.5 | 100.1 |
| 10 | 97.3 | 102.9 | 97.0 |
| 10 | 92.2 | 101.0 | 94.3 |
| 20 | 94.5 | 95.3 | 94.1 |
| 20 | 88.8 | 100.8 | 89.4 |
| 50 | 98.5 | 100.4 | 99.3 |
| 100 | 108.8 | 106.7 | 109.4 |
| 500 | 112.8 | 103.5 | 111.7 |
| 1000 | 96.0 | 104.6 | 96.3 |
| 2500 | 106.3 | 102.5 | 102.1 |
| 5000 | 95.8 | 96.9 | 99.0 |
| 10,000 | 100.9 | 86.8 | 92.5 |
| Mean accuracy % | 99.93 | 100 | 100.5 |

Data shown is accuracy %

## RESULTS AND DISCUSSION

*LLOQ.* The fentanyl assay developed here uses low volume of serum (0.175 mL). The lower limit of quantification (LLOQ) of the assay with acceptable accuracy and precision as per the FDA guidance for bioanalytical method validation is 5 pg/mL (RSD of 15%). An example chromatogram of 5 pg/mL standard is presented in Fig. 1. It exhibited a very high signal to noise ratio of 1220.

*Linearity.* The assay was found to be linear over the tested calibration range (5–10,000 pg/mL) in human serum samples (Fig. 2). A weighting scheme of $1 \quad x^2$ was employed based on the best estimation of back-calculated concentration of the calibrators and the coefficient of determination ($r^2$), which was found to be 0.99. In addition, $1 \quad x^2$ weighting is recommended for LC-MS/MS bioanalytical assays (21). Parameters of the calibration curves obtained over three validation assays are presented in Table I. Accuracy of the calibration standards ranged between −6.4 and 6.9% RE and precision ranged from 4 to 19% RSD (Table II).

*Accuracy and Precision.* The inter-day and intra-day accuracy and precision were tested using low (20 pg/mL), medium (1000 pg/mL), and high QCs (5000 pg/mL). The assay was conducted on three separate days using five replicates of each QC sample. The results obtained in these studies are presented in Table III. Accuracy of the quality control samples ranged from −5.5 to 0.1% RE. The inter-assay precision ($RSD_{inter}$) and intra-assay precision ($RSD_{intra}$) were calculated by single-factor ANOVA and using a statistical model initially described by Rodbard *et al.* (22). The $RSD_{inter}$ and $RSD_{intra}$ estimates were within ± 15%.

**Table VI.** Comparison of the Current Method with the Best LC-MS/MS Published Methods for Quantification of Fentanyl

| Extraction procedure | Matrix type | Volume of matrix (μL) | Column | Calibration curves (pg/mL) | LLOQ (pg/mL) | Reference |
|---|---|---|---|---|---|---|
| LLE | Serum | 200 | Phenomenex Luna® HILIC, 150 × 3.00 mm | 10–10,000 | 10 | (11) |
| SPE | Plasma | 200 | Intersil ODS3, 50 × 3 mm | 50–100,000 | 50 | (23) |
| LLE | Serum | 200 | Agilent Zorbax SB-Aq, 50 × 2.1 mm | 50–5000 | 50 | (24) |
| LLE | Serum | 175 | Zorbax XDB-C18 column, 4.6 × 50 mm | 5–10,000 | 5 | Current method |

*Extraction Efficiency.* Extraction efficiency of the analyte was found to be 58.18% (RSD 5.7%, $n = 12$) and the ISTD, fentanyl $D_5$, was found to be 53.44% (RSD 8.4%, $n = 12$). Although the method did not recover the analyte and ISTD completely, the method was able to recover them with very high precision. Further, the extraction efficiencies of the analyte and ISTD were found to be similar. It is to be noted that during the extraction, we intentionally left behind about 15% of organic phase in contact with the aqueous phase in order to minimize the loading of residues prevalent at the interface on the HPLC column. Although, this practice contributes to the loss of analyte recovery, it helps ensure the column performance.

*Stability Studies.* We performed a suite of stability studies to ensure that low volume of the serum being used in the assay does not compromise the assay performance. Fentanyl was found to be stable under all the conditions investigated, which was affirmed by the studies conducted to test bench-top stability, freeze-thaw stability, and long-term stability under frozen conditions (Table IV). We also observed that the samples exhibited excellent post-preparative stability for up to 48 h. The results are described in Table V.

*Conclusions.* Many published LC-MS/MS methods for fentanyl require at least 1 mL of human serum or plasma to ensure fentanyl assay with high accuracy and precision. In this study, we have developed and validated a robust and sensitive LC-MS/MS method to assay fentanyl in human serum, which requires only 0.175 mL serum. A comparison of this method with a selected list of best available methods for fentanyl assay is presented in Table VI. Due to the low volume of serum required, multiple investigations of valuable serum samples can be easily accomplished. Further, amount of blood drawn from human subjects can be minimized. This will be very useful in pharmacokinetic studies that often involve collecting multiple blood samples from human subjects over an extended period of investigation. Further, the smaller sample volume allows for less reagents and the use of disposable microcentrifuge tubes over glass, simplifies the extraction procedure, and thus facilitates rapid sample processing. In summary, our method for fentanyl analysis permits better throughput at lower costs with improved detection limits. This streamlined and more sensitive method is better suited for processing large numbers of samples for PK studies or therapeutic drug monitoring.

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