Article

Analysis of Fentanyl in Urine by DLLME–GC-MS

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Fentanyl is a synthetic narcotic anesthetic ∼80–100 times more potent than morphine. Owing to the potential for its abuse, the drug may be included in a forensic toxicology work-up, which requires fast, precise and accurate measurements. Here, the stability of fentanyl was assessed when stored at three different temperatures (−20, 4 and 25°C) in synthetic urine. Stability at those three temperatures was demonstrated over 12 weeks upon analysis by gas chromatography–mass spectrometry with a deuterated internal standard (fentanyl-D$_5$) utilizing three different extraction techniques: liquid–liquid extraction (LLE), solid-phase extraction and dispersed liquid–liquid microextraction (DLLME). The DLLME method was then optimized before use in the analysis of fentanyl in urine samples obtained from autopsy cases at the El Paso County Coroner's Office. Accuracy of the DLLME method was assessed by completing spike and recovery studies at three different fortification levels (10, 100 and 250 ng/mL) with excellent recovery (89.9–102.6%). The excellent comparability between DLLME and LLE is demonstrated (Bland–Altman difference plot with a mean difference of 4.9 ng/mL) and the use of this methodology in the analysis of forensically relevant samples is discussed.

Introduction

Fentanyl, a Schedule II drug, is commonly used as an analgesic and anesthetic and may be available for oral, transdermal, epidural, transmucosal or intravenous administration (1, 2). It is a highly potent synthetic narcotic (80–100 times the potency of morphine) and has a rapid onset of action, a moderate volume of distribution ($V_d = 3–8$ L/kg) and a short duration of action (3). Prescriptions for fentanyl jumped from 0.5 million (1994) to 7.04 million (2006) (4). Use of fentanyl patches for transdermal administration is prescribed for management of chronic pain (2). There is slow systemic absorption and prolonged effects of fentanyl when administered by patch (2), so there may a potential for abuse, including inadvertent abuse (3), by this mode of administration such as whole patch ingestion (4). Illicit use of fentanyl peaked in June 2006 with many resulting deaths (3) at which point a plant that manufactured fentanyl in Toluca, Mexico, closed (1). Adulteration of illicit drugs, such as heroin, by fentanyl has been reported to perhaps increase potency or potentiate the euphoric effects of the illicit drugs (1) and may have contributed to the 1,000 reported deaths associated with non-pharmaceutical use of fentanyl from 2005 to 2007 (5).

In a recent survey of 92 deaths in which one or more fentanyl patches were found to be used by the decedent, there were higher blood levels of fentanyl (26.4 ng/mL; range 1–102.2 ng/mL) where accidental intoxication was ruled as cause of death versus the group where cause of death was deemed natural (11.8 ng/mL; 1–78 ng/mL) (2). These data ranges compare well with those reported in a recent review (3). Postmortem redistribution also may significantly affect fentanyl concentrations depending on collection site of a sample at autopsy (2, 6), so it is difficult to correlate the transdermal patch dose and expected postmortem concentration, especially if there is no information regarding degree of opioid tolerance for the decedent (2). Others suggest that there may only be a slight postmortem redistribution of fentanyl (3), and data from a recent case report suggested that postmortem redistribution is not a significant phenomenon for fentanyl after an ∼13 h delay between death and autopsy (7). Peripheral blood concentrations, however, of fentanyl ≥25 ng/mL were indicative of fentanyl as probable cause of death and levels ≥10 ng/mL were strongly correlated with fentanyl as a possible cause of death (6).

Fentanyl has been previously extracted from forensically or toxicologically relevant samples such as urine (8–13), serum (14) or plasma (9, 11, 15), blood (7) and hair (16). Fentanyl is primarily excreted through urine (up to 85% of intravenously administered parent drug is excreted in this mode) as metabolites (3). Common clean-up procedures include solid-phase extraction (SPE) (8) and liquid–liquid extraction (LLE) (7, 8, 12). Other techniques that have been utilized for extraction of fentanyl include solid-phase microextraction (SPME) (8, 10, 15), single-drop liquid–liquid–liquid microextraction (LLLME) (9, 11), single-drop microextraction (SDME) (17) and dispersed liquid–liquid microextraction (DLLME) (11, 18) prior to analysis by gas chromatography–mass spectrometry (GC–MS) (7, 12, 13, 15, 17) or high-performance liquid chromatography (HPLC) with UV–Vis (9, 11) or tandem mass spectrometric detection (LC–MS-MS) (8, 14, 16). Typical limits of detection range from 0.08– to 5 ng/mL (9–15, 18) while limits of quantitation range from 0.3 to 10 ng/mL (8, 9, 11, 12, 14) depending upon sample preparation methods and analytical techniques employed.

In particular, use of microextraction procedures is becoming more popular (19–21), including DLLME, for sample preparation of toxicologically relevant analytes (22). The DLLME technique was first introduced in 2006 by Rezaee et al. (23) and involves the use of a ternary solvent system: aqueous sample matrix, water-miscible dispersive solvent and water-immiscible extraction solvent. The technique has been widely applicable because of its simplicity, low cost and ease of use, though modifications to the original technique have been required (24). One particular difficulty in adopting DLLME is addressing matrix complexity (25) because of the pelleting of matrix components with the dense extraction solvent at the bottom of the centrifuge tube, thus making it difficult to remove the extraction solvent for instrumental analysis. Kohler et al. (18) recently applied the DLLME technique for extraction of 30 toxicological compounds, including fentanyl, from urine prior to analysis by capillary

electrophoresis–MS. Saraji *et al.* (11) also optimized a DLLME protocol for extraction of three narcotics, including fentanyl, from urine and plasma.

Given the interest in the analysis of fentanyl, especially by microextraction techniques, the objectives of the work presented here were as follows: (i) to assess stability of fentanyl over 12 weeks when spiked into synthetic urine; (ii) to compare three extraction protocols: SPE, LLE and DLLME; (iii) to optimize a DLLME protocol for urine sample volumes below 800 µL (forensic samples are often volume-limited) and finally (iv) to compare fentanyl concentrations in forensic cases in which fentanyl had been a significant toxicological finding by LLE to the optimized DLLME protocol, with comparisons demonstrated by correlation and Bland–Altman difference plots.

Experimental

Standards and reagents

The reference material fentanyl (1 mg/mL in methanol; Supplementary data, Figure S1A) and the internal standard (IS) fentanyl-D_5 (100 µg/mL in methanol; Supplementary data, Figure S1B) were obtained from Cerilliant (Round Rock, TX, USA) and stored at $4^{\circ}C$ until use. Simulated blank urine was obtained from Immunalysis (Pomona, CA, USA). Certified controls (Pain Management 100) were obtained from UTAK Laboratories (Valencia, CA, USA) and contained 10 ng/mL fentanyl (lot number 6639 with expiration date 11/13). Liquichek^{TM} Qualitative Urine Toxicology Control (Positive Level, #455) was obtained from Bio-Rad (Hercules, CA, USA) and analytes are included in the 'Supplementary data'. Only HPLC-grade water (18 MΩ) from Barnstead E-pure system (Thermo Scientific, Asheville, NC, USA) was used. Chemicals 1-chlorobutane, ammonium hydroxide, sodium hydroxide, chlorobenzene, ethyl acetate, isopropanol and methanol were obtained from Fisher Scientific (Fairlawn, NJ, USA) as were all other chemicals unless specified otherwise. All chemicals were of analytical grade.

Preparation of standard solutions

Standard solutions of fentanyl and fentanyl-D_5 were prepared by diluting the purchased standards in methanol with 10 mL volumetric flasks to produce individual stock solutions with concentrations of 10,000 ng/mL for fentanyl and fentanyl-D_5. Using these stock solutions, three standards were created to contain both fentanyl and fentanyl-D_5 at 1,000 ng/mL in methanol. The three standards were kept in the dark at each of the three temperatures for the 12-week duration of the stability study: one at room temperature ($25^{\circ}C$), one in the refrigerator ($4^{\circ}C$) and one in the freezer ($-20^{\circ}C$).

For optimization of the DLLME protocol, a working stock solution of 100,000 ng/mL was created in methanol. This working stock solution was diluted again to prepare solutions of 1,000 ng/mL fentanyl in HPLC-grade water as needed.

Urine samples

For method development studies, only simulated blank urine (Immunalysis) was used. Upon completion of method development, urine samples that were collected at autopsy from forensic cases collected in 2012 ($n = 5$) or 2013 ($n = 7$) were analyzed. Samples were given a unique seven digit identification code and

were dissociated from the deceased's name or any other personal identifiers. Per the El Paso County Coroner's Office documentation, stored samples collected at autopsy in a completed case may also be used to research new analytical techniques for legitimate diagnostic purposes by parallel testing of the specimens with other methods. No implied consent document is generated for these uses because of Institutional Review Board exemption (26).

Stability study

For the stability study, 50 mL of the control synthetic urine was pooled from 10 separate vials each containing 5 mL each of the synthetic urine. Fentanyl and the IS, fentanyl-D_5, were added to the pooled synthetic urine for a final concentration of 1,000 ng/mL. Five hundred microliters of the spiked urine were added to 72 individual clean borosilicate glass test tubes and sealed with Parafilm. Twenty-four samples were each stored in the dark at the three storage temperatures of interest: $-20^{\circ}C$ (freezer), $4^{\circ}C$ (refrigerator) and $25^{\circ}C$ (room temperature). At weeks 2, 3, 4, 5, 6, 7, 8 and 12, the samples were prepared for analysis by GC–MS by LLE, SPE or DLLME (Supplementary data, Figure S2) as described below such that there was one sample per time point, per extraction protocol and per storage temperature.

Sample preparation for stability study

LLE

1-Chlorobutane (3.5 mL) was added to 500 µL of the synthetic urine (containing 1,000 ng/mL each of fentanyl and fentanyl-D_5) adjusted to pH 11 with 1 M sodium hydroxide in a 15 mL polypropylene centrifuge tube. The solution was sonicated for 5 min using a Fisher Scientific 20SD sonicator to improve mixing of the synthetic urine and 1-chlorobutane. The samples were centrifuged at $1,380 \times g$ (Fisher Scientific Centrific Model 228 centrifuge). The top layer (1-chlorobutane) was carefully transferred to a clean borosilicate glass test tube and carefully evaporated just to dryness in a heating block with a gentle air stream (Reacti-Therm II, Perkin Elmer) at $70^{\circ}C$. The sample was reconstituted in 100 µL methanol and transferred to a 150 µL PolySpring insert prior to GC–MS analysis.

SPE

Fentanyl was also extracted from 500 µL of synthetic urine (containing 1,000 ng/mL each of fentanyl and fentanyl-D_5) by SPE. Here, Oasis HLB columns (60 mg/3 mL, Waters Corporation, Milford, MA, USA) were conditioned with one column volume of methanol followed by one column volume of 18 MΩ deionized (DI) water. The pH-adjusted urine sample (again, at pH 11 with 1 M NaOH) was added to the conditioned column and allowed to elute through by gravity. Fentanyl was eluted with the addition of 500 µL ethyl acetate. This fraction was collected into a clean borosilicate glass test tube containing ~0.5 g anhydrous sodium sulfate. Fifty microliters of the ethyl acetate layer containing the fentanyl and fentanyl-D_5 were transferred to a 150-µL PolySpring insert prior to analysis by GC–MS.

DLLME

There were few reported methods in the literature describing the extraction of fentanyl from urine (real or synthetic) using

the DLLME approach and these methods utilized large volumes of urine (11, 18). Thus, the initial conditions, prior to the optimization of this method, were as follows: to a 1.5-mL polypropylene microcentrifuge tube, 500 μL of the pH-adjusted (pH 11) synthetic urine sample containing the analyte and IS were added. Then, 200 μL of methanol (as dispersive solvent) and 30 μL chlorobenzene (for samples from weeks 2 to 3) or 200 μL chlorobenzene (for samples from weeks 4 to 12) as extraction solvent were added. The samples were sonicated for 5 min using a Fisher Scientific FS20D sonicator prior to centrifugation at $10,500 \times g$ for 3 min with an Abbott Laboratories TDX centrifuge (model LN9527-01; Abbott Park, IL, USA). Five microliters of the bottom chlorobenzene layer were removed using a 10-μL SGE gas-tight microsyringe and transferred to a PolySpring insert. Forty-five microliters of ethyl acetate were added prior to GC−MS analysis. Given the success of the DLLME method for the extraction of fentanyl and the IS, this approach was further optimized.

Optimization of the DLLME protocol

To optimize the DLLME protocol, 800 μL of the aqueous 1,000 ng/mL fentanyl solution in HPLC-grade water was transferred to a 1.5-mL polypropylene microcentrifuge tube. The pH of the sample was adjusted to 10 by the addition of ammonium hydroxide (50 μL). Dispersive solvent (200 μL) and extraction solvent (30 μL) were added to the microcentrifuge tube before sonicating (Fisher Scientific 20SD) for 5 min followed by centrifugation ($10,500 \times g$) for 3 min with an Abbott Laboratories TDX centrifuge. Ten microliters of the extraction solvent were removed with a GC syringe and transferred to a PolySpring insert along with 40 μL of ethyl acetate to bring the sample up to enough volume for GC−MS analysis.

For all optimization experiments, high peak area and low precision values (as measured by % relative standard deviation, RSD) were used for data evaluation. Dispersive solvents (added at 200 μL in the initial trials) evaluated included: methanol, acetonitrile, acetone, dimethyl sulfoxide, tetrahydrofuran, ethanol and 2-propanol ($n = 3$ for each solvent). After selecting the dispersive solvent with the highest peak area (2-propanol), the optimal volume of dispersive solvent was evaluated: 50, 100, 150, 200 or 500 μL. Two possible extraction solvents were selected for evaluation in the method: chlorobenzene ($\rho = 1.106$ g/mL at 25°C) and chloroform ($\rho = 1.48$ g/mL at 25°C). In the initial evaluation of these two possible extraction solvents, 30 μL were added to the aqueous sample (800 μL) containing dispersive solvent (100 μL was optimal). Extraction solvent volumes of 10, 20, 30, 40 and 50 μL were also evaluated after determining that sonication time and temperatures were important parameters to investigate. In the trials completed to investigate sonication time (2, 5, 8, 11 and 14 min) and temperature (24 and 45°C), it was determined that the higher temperature was important for improving the extraction of fentanyl into the extraction layer. For future analyses, samples were sonicated for 11 min at 45°C to improve extraction efficiency. These data are further discussed in the 'Results and Discussion' section.

Finally, it was determined that the removal of the complex matrix (either real or synthetic urine) after the centrifugation step was necessary. Upon removal of the top aqueous urine layer, an additional 20 μL of chlorobenzene were added (for a total of 50 μL of added extraction solvent), the sample was re-mixed and re-centrifuged. Fifteen microliters of the extraction solvent were removed with a LC syringe, transferred to a PolySpring insert and 35 μL of ethyl acetate added to bring the final volume to 50 μL.

Preparation of calibration curves

For the LLE approach, calibration curves at the El Paso County Coroner's Office were created by first creating a working stock solution (10,000 ng/mL) of fentanyl in methanol from the 100 μg/mL stock from Cerilliant. Standards at 2.5, 5, 10, 25, 50 and 100 ng/mL were created using the working stock solution in blank blood. The IS, fentanyl-D₅, was diluted from its original concentration (100 μg/mL; Cerilliant) to an intermediate working stock solution of 1,000 ng/mL. One hundred microliters of this working stock were added to all standards, controls and test aliquots for an IS concentration of 100 ng/mL. To prepare these standards for GC−MS analysis, 1 mL of each standard (in blank blood) was made basic by the addition of five drops of concentrated ammonium hydroxide. Eight milliliters of chloroform were added and the standards mixed well (by a mechanical shaker) for 10 min. After 5 min centrifugation, the upper aqueous layer was removed and the bottom organic layer was retained. This bottom organic layer was filtered into a second glass extraction tube and solvent slowly evaporated just to dryness under air while the tube contents were heated in a water bath (60−70°C). The residue was reconstituted in 50 μL methanol prior to GC−MS analysis.

For the DLLME approach, calibration curves were created at levels of 10, 50, 100, 500 and 1,000 ng/mL fentanyl in 800 μL synthetic urine with the IS at a concentration of 100 ng/mL in all standards. Five replicates were included for each standard and the urine samples were prepared for GC−MS analysis using the optimized DLLME method described previously (Supplementary data, Figure S3), where 800 μL of solvent was mixed with 100 μL 2-propanol and 50 μL of chlorobenzene (the latter being added in two increments: 30 μL followed by sonication and centrifugation with subsequent removal of the aqueous layer and the remaining 20 μL then added). Fifteen microliters of the chlorobenzene layer were removed and mixed with 35 μL ethyl acetate prior to GC−MS analysis.

Calibration curves were also created at levels of 10, 50, 100, 250, 500, 1,000 and 2,500 ng/mL fentanyl with 100 ng/mL IS in methanol. These standards were utilized during the spike and recovery studies for assessment of method accuracy, as well as for the determination of the instrument limit of detection (LOD) and limit of quantitation (LOQ).

Sample preparation for real urine samples from case studies

LLE

To compare the quantitative results obtained by the DLLME method, the following LLE method was utilized: 1 mL of urine (with added IS) was made alkaline with the addition of five drops of ammonium hydroxide and extracted with 8 mL of 1-chlorobutane. The sample was mixed (using a shaker table) for 10 min and centrifuged for 5 min. The 1-chlorobutane layer was transferred to a clean borosilicate glass test tube and evaporated just to dryness using a steam bath. The analytes were reconstituted in 50 μL ethyl acetate, transferred to a PolySpring insert and analyzed by GC−MS.

Optimized DLLME method

Here, 800 µL of urine (with added IS) was transferred to a borosilicate glass test tube (13 × 125 mm) and adjusted to pH 10 with the addition of 50 µL of ammonium hydroxide or 10 µL 2.5 M NaOH. Dispersive solvent (100 µL of 2-propanol) and extraction solvent (30 µL of chlorobenzene) were added and the samples sonicated at 45°C for 11 min. After sonication, the ternary mixture was transferred to a 1.5-mL microcentrifuge tube made of polypropylene (note: the sonication step was performed in glass to avoid extracting polymer components from the microcentrifuge tube). The microcentrifuge tube was centrifuged for 3 min and the aqueous urine layer removed. An additional 20 µL of chlorobenzene were added. The tube contents were re-mixed and centrifuged before removing 15 µL to a PolySpring insert along with 35 µL of ethyl acetate for GC–MS analysis. For the spike and recovery study samples only, the IS was added (5 µL of a 1,000 ng/mL solution) just before GC–MS analysis to obtain absolute recovery data.

Analysis by GC–MS

Samples were analyzed at two locations: at the University of Colorado Colorado Springs (UCCS, for method development and optimization) and at the Analytical Toxicology Laboratory of the El Paso County Coroner's Office (for analysis of urine samples from forensic cases).

UCCS samples were analyzed on a Hewlett Packard 5890 Series II GC with a 5971A MS. Samples (1.0 µL) were injected at 250°C in splitless mode (with the split vent opening 0.5 min after injection). Analytes were separated on a DB-1MS column (25 m × 0.25 mm i.d., 0.4 µm thickness; Agilent Technologies, Santa Clara, CA, USA) with helium used as the carrier gas (1.2 mL/min). The initial column temperature was 100°C (hold for 2.00 min) with a ramp of 15.0°C/min to a final temperature of 305°C (hold for 3.00 min) for a total run time of 18.67 min. The transfer line was held at 280°C and the MS source at 230°C. Ions were detected in selected ion monitoring (SIM) mode after a 10.00-min solvent delay with a 100-ms dwell time for all ions. Ions m/z 245 (quantitation ion) and m/z 189 and m/z 146 (confirmation ions) were monitored for fentanyl. Ion m/z 250 was monitored for the IS, fentanyl-D$_5$. The instrument LOD was 10 ng/mL and LOQ was 50 ng/mL.

Samples analyzed at the El Paso County Coroner's Officer were injected onto a Hewlett Packard 6890N Series Gas Chromatograph with a 5973A Mass Selective Detector. The column employed was an Agilent HP-5 (15.0 m, 0.25 mm i.d., 0.25 µm thickness; Agilent Technologies). Helium was used as the carrier gas at 1.0 mL/min. The initial column temperature was 100°C (hold for 2.00 min) with a ramp of 25.0°C/min to a final temperature of 280°C (hold for 5.00 min) for a total run time of 14.20 min. A sample volume of 1.0 µL was injected at 250°C in splitless mode (with split vent opening after 2.00 min after injection). The MS detector source was at 230°C (transfer line was at 280°C) and was utilized in SIM mode with a 4.00-min solvent delay. Dwell times for ions (m/z 245, 146, 189 and 250) were 100 ms.

Results and discussion

Stability study

Fentanyl and the IS were spiked into blank synthetic urine such that the final concentration for each was 1,000 ng/mL. Samples were stored in the dark over a 12-week period at three different temperatures (−20, 4 and 25°C) before being prepared for GC–MS analysis via LLE, SPE or DLLME. There was no trend towards a statistically significant difference for the three temperature datasets by single-factor ANOVA ($F = 1.65 < F_{crit} = 3.47$). The mean concentration of fentanyl in the samples over the 12-week study was 881 ng/mL (−20°C storage temperature), 826 ng/mL (4°C) and 841 ng/mL (25°C).

When the three extraction protocols (LLE, SPE and DLLME) were compared for pre-concentration of the fentanyl prior to GC–MS over the 12-week period by single-factor ANOVA, there was no statistically significant difference between the extraction methods ($F = 1.18 < F_{crit} = 3.49$), which was to be expected given that the IS was added to the synthetic urine at the start of the stability study and reported results were corrected by the IS. The mean determined concentration of fentanyl by LLE was 836 ng/mL, 870 ng/mL by SPE and 841 ng/mL for DLLME.

Within-day and between-day precision, as indicated by the % RSD, was also readily determined from these data (Supplementary data, Table S1). When organized by storage temperature, the within-day % RSD values ranged from 1.0% (week 3, 4°C) to 23.1% (week 2, −20°C). Generally, a precision value ≤10% is desired but these samples were prepared for analyses by three different extraction protocols and so there is an interactive effect (temperature × extraction protocol). The between-day precision based on the reported mean sample concentrations ranged from 6.9% (storage at 4°C) to 8.0% (storage at 25°C).

When comparing the extraction protocols (Supplementary data, Table S1), the within-day % RSD values ranged from 1.0% (Week 4, SPE) to 36.3% (Week 2, DLLME). The between-day precision based on the reported mean sample concentrations for weeks 2 through 12 ranged from 4.7% (DLLME) to 6.1% (SPE). These reported precision values are acceptable considering that the reported concentrations of fentanyl in the simulated urine experienced an interactive effect from treatment groups (storage temperature × extraction protocol).

DLLME sample preparation and method development

Optimization of the DLLME protocol began with the use of 800 µL of 1,000 ng/mL fentanyl solution in 18 MΩ DI water. The pH of the sample was adjusted to 10 and the dispersive solvent (200 µL) and extraction solvent, chlorobenzene (30 µL), were added. Dispersive solvents methanol, acetonitrile, acetone, dimethyl sulfoxide, tetrahydrofuran, ethanol and 2-propanol were evaluated ($n = 3$ per solvent). There was significant variability in this initial experiment (Supplementary data, Figure S4) and no statistically significant difference between dispersive solvents (single-factor ANOVA, $F = 2.86 < F_{crit} = 3.00$) though the peak areas were highest when 2-propanol was used as the dispersive solvent. Thus, 2-propanol was selected as the dispersive solvent for future samples prepared through this method.

Several volumes (50, 100, 150, 200 and 500 µL) of the 2-propanol as dispersive solvent were tested and the highest levels were observed for 100, 150 and 200 µL. These results were replicated in a repeated experiment ($n = 3$ per tested volume) with the highest peak areas observed when 100 µL of the 2-propanol were added as the dispersive solvent (Supplementary data, Figure S5) with a statistically significant difference noted between groups (single-factor ANOVA, $F = 15.65 > F_{crit} = 5.14$).

Following selection of the dispersive solvent type and volume, the extraction solvents chlorobenzene and chloroform were evaluated. Both solvents resulted in very high peak areas for the 1,000 ng/mL fentanyl extracted from the 800 μL water (areas were in excess of 8 million counts) and good precision, with % RSD below 2.0. Given these comparable results, chlorobenzene was selected for further use because of its lower toxicity compared with chloroform as reported through material safety data sheets.

In the final optimization experiments for the DLLME protocol, 10, 20, 30, 40 and 50 μL of the extraction solvent chlorobenzene were evaluated for extraction efficiency. There was no statistically significant difference between volumes (single-factor ANOVA, $F = 1.95 < F_{crit} = 3.36$). However, improved precision was observed for the 30 and 50 μL volume (RSD of 5.4 and 3.9%, respectively) and the 50 μL was selected as the volume in future experiments, though a modification was required that this volume be added in two increments (30 and 20 μL) for improved recovery from the synthetic and real urine matrices.

Finally, to ensure optimal mixing of the aqueous sample, dispersive solvent and extraction solvent, the time of sonication and temperature of the sonication bath were evaluated. As shown in Supplementary data, Figure S6, improved peak areas were observed when the sonication bath temperature was 45°C versus the bath temperature at 24°C. A sonication bath time of 11 min was selected for future studies because it had the highest peak area and lowest % RSD (3.9%). There was a statistically significant difference between sonication times at 45°C by single-factor ANOVA ($F = 7.17 > F_{crit} = 3.36$).

In conclusion, the optimized DLLME method required the following conditions: 800 μL of the sample adjusted to pH 10, 100 μL of 2-propanol, 50 μL of chlorobenzene (added in two increments of 30 and 20 μL) with sonication at 45°C for 11 min. This protocol is an improvement in solvent consumption (150 μL total for extraction) when compared with previous reports of DLLME for fentanyl, where 2,000 μL [18] or 2,142 μL [11] of organic solvents were used to complete the extraction procedures. As well, it is a significant improvement in terms of solvent consumption versus the LLE method, which utilized 8,000 μL of 1-chlorobutane.

Method verification

Given that DLLME is a relatively new technique [23] and there are few reports of its use in the forensic setting [11, 18, 22], it was important to complete method verification experiments. To that end, fentanyl and the IS were spiked into synthetic urine and prepared for instrumental analysis using the DLLME approach at five different levels with $n = 5$ at each level. These data were used to construct the calibration curve (Supplementary data, Figure S3), which had precision values (as % RSD) ranged from 14.3% (500 ng/mL) to 5.8% (100 ng/mL). The method LOD was below 1 ng/mL and levels at 1 ng/mL could be quantified. However, when a 1 ng/mL spike was included in synthetic urine and prepared for GC−MS analysis by DLLME, the precision was 30.8%, even after repeated experiments and instrument maintenance. For these reasons, the lowest standard included in the calibration curve for DLLME was 10 ng/mL.

Accuracy was assessed via a spike and recovery study wherein synthetic urine samples (800 μL) were spiked for final concentrations of 10 (the instrument LOD), 100 and 250 ng/mL with

Table 1

Determination of % Recovery of Fentanyl Spiked at Three Different Levels (10, 100 and 250 ng/mL) with $n = 5$ Replicates

Spike level (n)	Mean % recovery	RSD, %	Determined concentration (ng/mL) range
10 ng/mL (5)	102.7	12.8	8.36 − 11.9
100 ng/mL (5)	93.6	7.3	86.8 − 103.4
250 ng/mL (5)	88.9	4.3	211.9 − 237.1

$n = 5$ replicates at each level (Table 1). Samples were prepared for GC−MS analysis via the optimized DLLME method with the IS (100 ng/mL) added just before analysis. The mean recoveries ranged from 88.9 to 102.7% with RSD values from 4.3 to 12.8%.

Analysis of human urine samples

In the final study, the optimized DLLME method was utilized to determine concentrations of fentanyl in urine samples in forensic cases where fentanyl had previously been identified as a significant finding. Urine samples from these cases were retrieved from archived sample sets and re-analyzed by both the DLLME and the traditional LLE. The comparison of these data is included in Figure 1 (correlation plot) and Figure 2 (Bland−Altman difference plot).

Briefly, sample concentrations by LLE ranged from 19.8 to 656.8 ng/mL for which concentrations were determined by a historical calibration curve prepared from the whole blood matrix (where standards ranged from 2.5 to 100 ng/mL). By DLLME, concentrations in the same samples ranged from 25.5 to 920.8 ng/mL. By a regression analysis, these methods compare well (Figure 1) for the 11 forensic cases and 1 positive control (PM 100 reference solution at 10 ng/mL) included in the study where the correlation coefficient (R^2) was 0.9877. There were two cases for which the determined fentanyl concentrations were very high (in excess of 300 ng/mL by LLE) and exceeded the highest standard concentration in the LLE historical calibration curve. When these two cases were removed (Figure 1, inset), the correlation between the samples was still strong ($R^2 = 0.6431$) and the relationship statistically significant ($P = 0.0053$). As well, the slope of the correlation line was nearly one ($m = 1.0939$) and the intercept was 0.72 ng/mL, a level that would be found in urine of living patients receiving anesthesia [13].

The agreement between the LLE protocol and the DLLME protocol for determination of fentanyl concentrations in urine was assessed through the Bland−Altman difference plot (Figure 2) [27]. From this analysis, it was determined that the mean difference between these two protocols was 4.9 ng/mL, which is at a high concentration level determined in urine for patients receiving anesthesia (0.8–4.0 ng/mL) [13] and below the experimentally determined instrument LOD (10 ng/mL) and near the DLLME method LOD (1.0 ng/mL). The limits of agreement (mean ± 2SD, the range into which all values fell) ranged from −32.2 ng/mL (lower limit) to 42.0 ng/mL (upper limit).

While peripheral blood concentrations of fentanyl ≥25 ng/mL were indicative of fentanyl as probable cause of death and levels ≥10 ng/mL were strongly correlated with fentanyl as a possible cause of death [6], the relationship of urinary fentanyl concentrations with cause of death is less clear. Urine is a waste
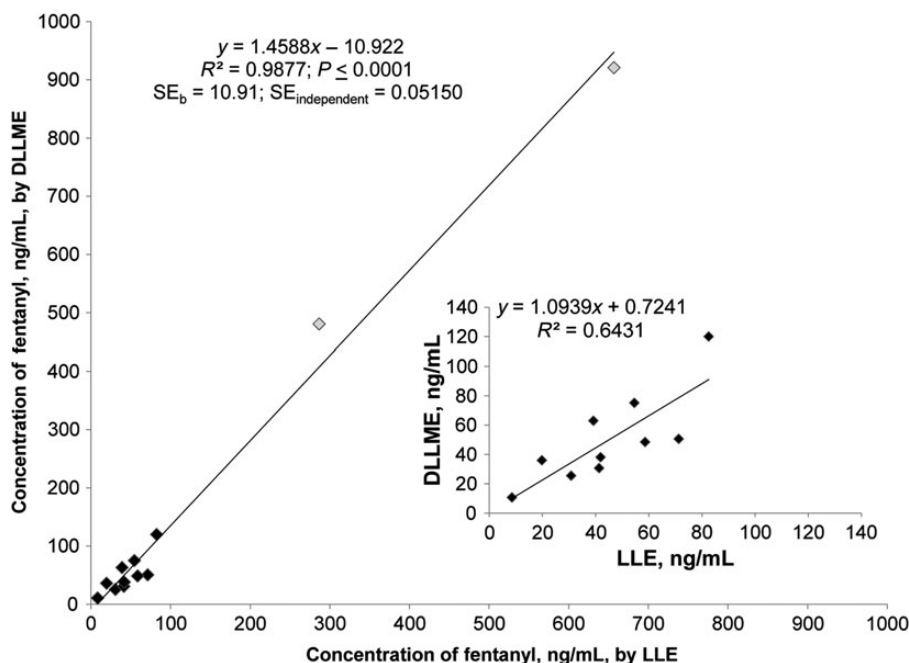
Figure 1. Correlation plots comparing the extraction of fentanyl (ng/mL) from urine samples from forensic toxicology cases by LLE (*x*) versus the extraction of fentanyl by DLLME (*y*). Standard error of the intercept and estimate (independent) are included with the correlation coefficient and *P*-values indicating the strength of the correlation. The insert plot is for the comparison of fentanyl concentrations below 150 ng/mL.
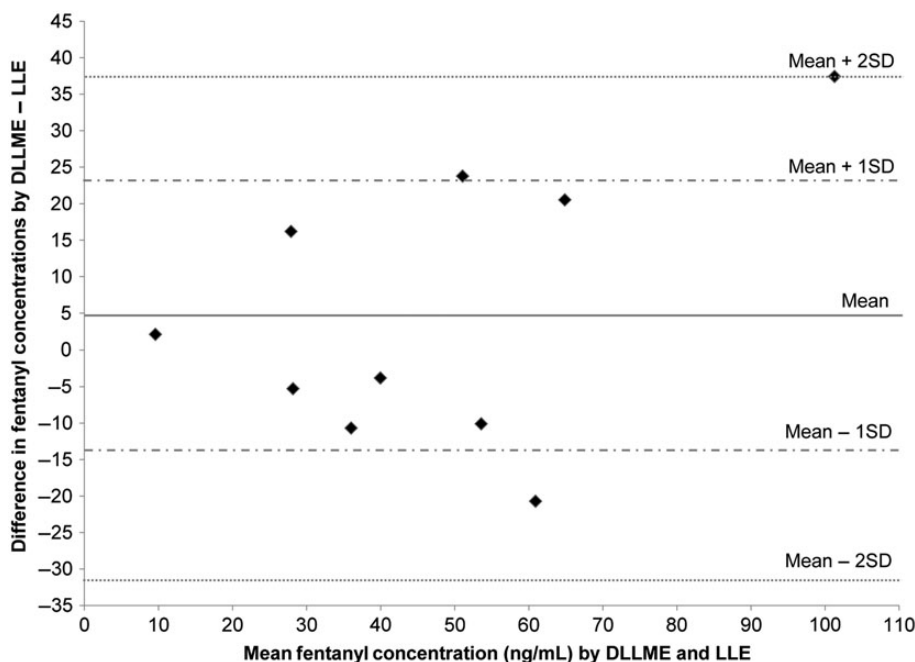

Figure 2. Bland–Altman difference plot to demonstrate the agreement between urine samples from forensic toxicology cases prepared by DLLME versus LLE prior to GC–MS analysis. The outliers (in excess of 200 ng/mL fentanyl in urine) were removed from the analysis. The mean difference between the methods was 4.9 ng/mL fentanyl (which is below the instrument LOD) and all nine forensic toxicology samples and the QC standard were within 2 SDs of the mean difference.

product and may not reflect blood or plasma concentrations. Strano-Rossi *et al.* recently validated a method for the analysis of fentanyl and analogs in urine for cases of anesthesia and suicide. In their validated method, urinary fentanyl concentrations ranged from 0.8 to 4.0 ng/mL for patients who had received

fentanyl as part of an anesthesia protocol whereas the fentanyl concentration in the suicide case was 183 ng/mL in urine (13). Ebrahimzadeh *et al.* quantified fentanyl in urine collected from a patient after application of a single 50 µg h^{-1} Duragesic fentanyl patch and 10.1 ng/mL fentanyl was found in the urine of

this patient after sample work-up by LLLME (9). In another study, urine was collected from nine patients after receiving anesthesia during heart surgery. Seven of the nine patients received fentanyl as part of the anesthesia regimen (0.01 mg/kg dose). Concentrations found in urine (ng/mL) ranged from <LOD (1.4 ng/mL) to 160 ng/mL after HS-SPME pre-concentration (10). Thus, the levels determined by LLE and DLLME in this study are reasonable and within the range of reported urinary levels.

Finally, a urine sample (prepared by LLE) from a forensic case was re-analyzed after a full-scan analysis with sample preparation by DLLME with full-scan analysis (rather than the SIM method described above) to determine how many other compounds of forensic or toxicological interest could be identified by the DLLME method. By LLE, reportable compounds included: nicotine, cotinine (nicotine metabolite), doxylamine and dextromethorphan. In comparison, the DLLME approach yielded the following: nicotine (but no cotinine), doxylamine, dextromethorphan, dextrorphan (a metabolite of dextromethorphan), sertraline, nortriptyline, desmethyldoxepin (a metabolite of doxepin) and paroxetine. Thus, the DLLME approach may offer some advantages for identifying additional compounds beyond the LLE protocol for screening basic drugs and is of future research interest. The ability of DLLME to screen basic drugs was replicated in the analysis of the Liquichek Urine Toxicology Control (Supplementary data, Figure S7). Future work will include assessing the suitability of the DLLME approach for many other basic drugs (opioids, tricyclic antidepressants, selective serotonin reuptake inhibitors etc.) in many other forensically relevant matrices (blood, cerebrospinal fluid or brain tissue).

In conclusion, we have described a method, DLLME, for the recovery of fentanyl in synthetic and real urine samples with comparable results obtained by this methodology and the traditionally used LLE protocol. The method was validated, showing good within-day and between-day precision, excellent accuracy and reasonable limits of quantitation. As well, the stability of fentanyl in synthetic urine was also demonstrated over 12 weeks at three different storage temperatures with extraction by three different protocols. Comparison of method parameters between the three protocols is presented in Supplementary data, Table S2.

Acknowledgments

Supplementary data

Supplementary data are available at *Journal of Analytical Toxicology* online.

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