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Comparison of two automated solid phase extractions for the detection of ten fentanyl analogs and metabolites in human urine using liquid chromatography tandem mass spectrometry☆

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ABSTRACT

Two types of automated solid phase extraction (SPE) were assessed for the determination of human exposure to fentanyls in urine. High sensitivity is required to detect these compounds following exposure because of the low dose required for therapeutic effect and the rapid clearance from the body for these compounds. To achieve this sensitivity, two acceptable methods for the detection of human exposure to seven fentanyl analogs and three metabolites were developed using either off-line 96-well plate SPE or on-line SPE. Each system offers different advantages: off-line 96-well plate SPE allows for high throughput analysis of many samples, which is needed for large sample numbers, while on-line SPE removes almost all analyst manipulation of the samples, minimizing the analyst time needed for sample preparation. Both sample preparations were coupled with reversed phase liquid chromatography and isotope dilution tandem mass spectrometry (LC–MS/MS) for analyte detection. For both methods, the resulting precision was within 15%, the accuracy within 25%, and the sensitivity was comparable with the limits of detection ranging from 0.002 ng/mL to 0.041 ng/mL. Additionally, matrix effects were substantially decreased from previous reports for both extraction protocols. The results of this comparison showed that both methods were acceptable for the detection of exposures to fentanyl analogs and metabolites in urine.

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1. Introduction

Fentanyls, potent opioid analgesics, have been used for chronic pain treatment, for palliative care, and for use as an anesthetic. Since the initial synthesis of fentanyl in 1960 by Janssen Pharmaceuticals, multiple analogs have been developed with varying potencies for use in the medical and veterinary fields. Additionally, analogs with no approved medical use have been synthesized and sold illegally under several names including “China White” [1]. Overdose cases have been reported in California, Illinois, and Pennsylvania; resulting in hospitalization and, in some cases, death [2–4]. Fentanyls have also been reported to have applications as incapacitating agents [5,6].

Clinical manifestations from a significant exposure to fentanyl, which include euphoria, sedation, and respiratory depression, are the same as exposure to other opioids such as morphine and heroin. Thus, symptomology alone cannot be used to differentiate among exposure to different opioids; therefore, a selective analytical method is needed to distinguish fentanyl exposure from other opioids. The high potency of fentanyls, 50–100 times more potent than morphine, along with the low renal clearance of fentanyl analogs, results in low concentrations (0.8–4 ng/mL) of the intact fentanyl excreted via urine following therapeutic doses [7]. The biological half-life of fentanyl is 1–3.5 h [8]; however, the nor-metabolite, the oxidative *n*-dealkylation at the piperdine nitrogen of the parent compound, has been detected at concentrations of 0.3–0.7 ng/mL up to 96 h following therapeutic doses [9]. It is important to note that the common nor-metabolites are not unique to each fentanyl analog; therefore, to correctly identify the exposure agent, the native compound of all suspected fentanyls must

☆ *Disclaimer*: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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be also monitored (e.g. sufentanil and alfentanil both metabolize to the metabolite norsufentanil) [10–12].

Detection of fentanyls and their corresponding nor-metabolites has been used previously to confirm exposures. Analysis of fentanyl in biological matrices has been achieved using immunoassays; but these tests are prone to cross-reactivity issues, or are not able to detect multiple analogs [11,13,14]. Analytical techniques such as liquid chromatography with ultraviolet detection (LC–UV), gas chromatography with nitrogen phosphorous detection, gas chromatography–mass spectrometry (GC–MS) and liquid chromatography tandem mass spectrometry (LC–MS/MS) [15] have also been used to successfully quantitate fentanyls. LC analysis has been preferred over GC because GC analysis requires a derivitization step [16]. Tandem mass spectrometry (MS/MS) has proven to be a valuable tool for fentanyl detection because it achieves high selectivity between fentanyl, its analogs, and the metabolites while maintaining low detection limits (estimated range from 0.003 ng/mL to 0.027 ng/mL) [17].

To obtain high sensitivity, sample preparation and clean-up is often required before LC–MS/MS analysis. Although fentanyl compounds have been successfully extracted from biological matrices using liquid–liquid extraction (LLE) [15,18], these extractions were time consuming and required large volumes of solvents. Solid phase extraction (SPE) [16,17] has been used for the isolation of fentanyls from biological matrices with success, and has several benefits over other sample preparation approaches, including less solvent use, smaller sample volume requirements, and it is easily automated.

Automation of solid phase extraction allows a large number of samples to be prepared with minimum variability while maintaining high levels of productivity and sample throughput. Applications using automated 96-well plate off-line SPE have been documented in many publications [19–22], including fentanyl analysis in plasma [23]. The use of on-line SPE automation has further minimized the steps required by the analyst for sample preparation. Multiple methods using commercially available on-line SPE systems have demonstrated great success [24–26]. Described in this paper is the comparison of off-line SPE with on-line SPE for the automated sample preparation of human urine before the analysis and quantitation of seven fentanyls and three nor-metabolites using LC–MS/MS.

2. Materials and methods

2.1. Chemicals, standards, and reagents

Fentanyl, norfentanyl, fentanyl-d_5, and norfentanyl-d_5 were purchased from Cerilliant (Round Rock, TX). Carfentanil, sufentanil, norsufentanil, norcarfentanil, and their corresponding N-phenyl-d_5 labeled forms were custom synthesized by Battelle Laboratories (Columbus, OH). The remaining analytical standards, lofentanil, alfentanil, 3-methylfentanil, and α-methylfentanil, were generous gifts from a variety of sources listed in the acknowledgements. High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were purchased from Tedia Company Inc. (Fairfield, OH). Formic acid (99%) and ammonium hydroxide (28.58%) were purchased from Sigma Aldrich (Pittsburgh, PA). Deionized water (>18 mΩ) was prepared on-site using an installed water purification system (Aqua Solutions Inc., Jasper, GA).

2.2. Calibrator, internal standard, and quality control (QC) materials preparation

A working solution containing fentanyl, norfentanyl, sufentanil, norsufentanil, carfentanil, norcarfentanil, lofentanil, alfentanil, 3-methylfentanil, and α-methylfentanil each at a concentration of 500 ng/mL was prepared in methanol. Calibrators were prepared from this solution in pooled human urine from healthy volunteers, purchased from Tennessee Blood Services (Memphis, TN) at the following concentrations: 0.010, 0.025, 0.050, 0.10, 0.25, 0.50, 1.0, 5.0, and 10 ng/mL. Quality control samples (QCs) were prepared in the same manner at concentrations of 0.075, 0.75, and 7.5 ng/mL. An internal standard solution was prepared as a mixture of the six isotopically labeled versions of fentanyl, norfentanyl, sufentanil, norsufentanil, carfentanil, and norcarfentanil each at a concentration of 25 ng/mL in methanol.

2.3. Instrumentation

On-line SPE was automated using a Spark Holland Symbiosis (Emmen, The Netherlands) system and off-line SPE was automated using a Tomtec Quadra 4 (Hamdem, CT). The Symbiosis system was comprised of a refrigerated autosampler, an automated cartridge exchanger (ACE), two high pressure dispensing pumps (HPD) for SPE solvent delivery, two high performance liquid chromatography (HPLC) pumps, and a column oven. Liquid chromatography for both methods was performed using the Symbiosis system. Analytes were detected using an Applied Biosystems API 5500 Triple Quadrupole MS (Foster City, CA).

2.4. Sample preparation

For on-line SPE analysis, 10 μL of the prepared internal standard solution was spiked into 100 μL of sample, calibrator, or QC in a 300-μL autosampler plate (Eppendorf, Hauppauge, NY) and mixed via pipet aspiration and by shaking for 5 min using a Thermo Labsystem Wellmix plate mixer (Rochester, NY). Samples were then heat sealed with foil and loaded into the autosampler that was cooled to 4 °C. An Oasis HLB 30-μm particle, 10.6-mg bed size cartridge (Waters, Milford, MA) was loaded into the ACE solid phase extraction unit. Automated on-line SPE was controlled with Analyst (Applied Biosystems, Foster City, CA) and Symbiosis Pro (Spark Holland, Emmen, The Netherlands) companion software. SPE cartridges were conditioned with 1 mL of acetonitrile and equilibrated with 1 mL of aqueous 1% ammonium hydroxide. Fifty microliters of sample, calibrator, or QC was loaded onto the cartridge for extraction. The cartridge was then washed with 1 mL of a 90:10 solution of aqueous 1% ammonium hydroxide: acetonitrile and eluted with the LC gradient directly onto the HPLC column for the entirety of the run. Each cartridge was only used once.

Off-line SPE samples were prepared by adding 25 μL of the internal standard solution to 500 μL of sample, calibrator, or QC in a 2-mL 96-well Nunc plate (Thermo Scientific, Rochester, NY). This solution was then diluted with 500 μL of aqueous 1% ammonium hydroxide. Samples were extracted using a 96-well Oasis HLB 30-μm particle, 30-mg plate (Waters, Milford, MA) on the Tomtec Quadra 4 system. Each well was conditioned with 1 mL of acetonitrile and equilibrated with 1 mL of aqueous 1% ammonium hydroxide. The entire sample mixture was then loaded onto the plate and washed with 1 mL of a 84:15:1 solution of water:acetonitrile:ammonium hydroxide. The sample was then eluted with 1 mL of acetonitrile containing 1% formic acid. The extracts were evaporated to dryness using a 96-well Turbovap evaporator (Caliper, Hopkinton, MA) set at 50 °C under a continuous flow of nitrogen to aid in evaporation. Dried extracts were reconstituted with 50 μL of water and briefly mixed via a Thermo Labsystems Wellmix plate mixer (Rochester, NY) and by pipet aspiration. The reconstituted samples were transferred to a 300-μL autosampler plate (Eppendorf, Hauppauge, NY), heat sealed with foil, and loaded into the autosampler that was cooled to 4 °C in preparation for LC–MS/MS analysis.

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