Clinical Biochemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Short Communication

# Rapid identification of cyclopropyl fentanyl/crotonyl fentanyl in clinical urine specimens: A case study of clinical laboratory collaboration in Canada

Jan Palaty[a,*], Danijela Konforte[b], Theano Karakosta[b], Ernest Wong[c], Cristiana Stefan[c]

[a] LifeLabs Medical Laboratories, Burnaby, British Columbia, Canada
[b] LifeLabs Medical Laboratories, Toronto, Ontario, Canada
[c] Centre for Addiction and Mental Health, Toronto, Ontario, Canada

## 1. Introduction

The current worldwide opioid crisis continues to challenge both law enforcement laboratories evaluating seized drugs and clinical laboratories monitoring use by patients. In particular, both facilities face the problem of identifying new street drugs as they are introduced to the illicit market such as the fentanyl (FEN) analogues that have been increasingly detected in overdoses and fatalities [1–6]. The controlled substance regulation processes drive the rapid changes of drugs on the street market, while analytical constraints related to technology and/or availability of certified standards delay the process of street drug identification. In this communication, we describe a collaborative strategy combining powerful and innovative mass spectrometry algorithms that led to the identification of an unexpected pair of fentanyl analogue isomers, cyclopropyl fentanyl (Cp-FEN)/crotonyl fentanyl and their metabolites, in a large number of urine samples from substance use disorder patients in two Canadian provinces.

### 1.1. Materials

For LC/MS/MS, all solvents were from EMD Millipore (Billerica, MA, USA), Formic acid from Sigma-Aldrich (Oakville, ON, Canada) and stock solutions of fentanyl, fentanyl-$D_5$, norfentanyl and norfentanyl-$D_5$ were from Cerilliant (Round Rock, TX, USA). Abalone glucuronidase was from KURA Biotec (Los Angeles, CA, USA): the BG100 product was used in Ontario (ON) and the initial British Columbia (BC) work, while KURA Turbo was used in the subsequent BC studies. Fentanyl urine screening was by EMIT (Enzyme Multiplied Immunoassay Technique) immunoassay from Thermo Scientific (Waltham, MA, USA) at a cut-off of 1 ng/mL. For high-resolution LC/MS/MS, methanol was from VWR International (Mississauga, ON, Canada), ammonium formate from Sigma-Aldrich and the internal standards morphine-$D_3$ and methadone-$D_9$ from Cerilliant.

### 1.2. Patient samples

Patient samples were selected from those submitted to LifeLabs Medical Laboratories in BC or ON for routine urine fentanyl testing. Most samples originated in community-based substance use disorder clinics.

### 1.3. LC/MS/MS: sample preparation and instrument parameters

The initial precursor ion scan studies were done by the BC lab. Sample preparation consisted of mixing 125 µL urine with 125 µL KURA BG100 glucuronidase (pH 4.8 0.1 M acetate buffer), hydrolysis (60 °C/15 min), salt-assisted liquid-liquid extraction (700 µL acetonitrile plus 100 µL brine), evaporation and reconstitution (200 µL 0.1% aqueous formic acid). Sample (25 µL) was loaded on a 2.1 × 50 mm Kinetex Biphenyl column (Phenomenex, Torrance, CA, USA) interfaced to an Agilent model 6410 tandem mass spectrometer (Santa Clara, CA, USA). Mobile phases consisted of 0.2% formic acid in water (solvent A) and 0.2% formic acid in methanol (solvent B). MS detection of Cp-FEN initially used a precursor ion scan of m/z 200–400 for fragments m/z 188 and m/z 84 using same source and collision cell conditions as fentanyl and norfentanyl. For multiple reaction monitoring (MRM), the instrument was set to transitions of m/z 337 > 188 (quantifier) and 337 > 105 (qualifier) for fentanyl, 342 > 188 for fentanyl-$D_5$, 233 > 84 (quantifier) and 233 > 55 (qualifier) for norfentanyl, and 238 > 84 for norfentanyl-$D_5$. In the subsequent BC studies with Cp-FEN, the procedure was modified as follows in order to enhance throughput *via* automation. Sample (200 µL) was heated with 400 µL pH 6.8 0.15 M phosphate buffer containing internal standards and KURA Turbo glucuronides for 30 min at 50 °C, following which 200 µL was applied to a 1 mL CEREX HP SCX mixed-mode SPE column (Tecan SP, Baldwin Park, CA, USA), which was then washed with water (700 µL), 0.1 M hydrochloric acid (300 µL) and water (500 µL) before drying and elution with dichloromethane/isopropanol/ammonium hydroxide (70:28:2, v/v). Following reconstitution with water (200 µL), 20 µL was injected to the same column type as above with a gradient profile as follows: 5% B for 0.5 min, 5% to 25% B for 1.5 min, 25% B to 80% B for 3 min, 95% B for 0.5 min, 95% B to 5% B for 0.5 min. The same Agilent 6410 mass spectrometer was used with MRM settings as before, but with the addition of transitions of 349 > 188 (quantifier)

* Corresponding author.
*E-mail address:* jan.palaty@lifelabs.com (J. Palaty).

https://doi.org/10.1016/j.clinbiochem.2018.01.013
Received 5 December 2017; Received in revised form 5 January 2018; Accepted 20 January 2018

and 349 > 105 (qualifier) for Cp-FEN, 245 > 84 (quantifier) and 245 > 55 (qualifier) for cyclopropyl norfentanyl (Cp-NFEN).

The Ontario studies were performed on a Sciex 4500 tandem quadrupole mass spectrometer (Concord, ON, Canada) using a similar SPE procedure described elsewhere [7].

Samples were considered positive when Cp-FEN and Cp-NFEN were both present at a 20:1 signal-to-noise ratio with retention times and ion ratios within 2SD of those observed for the high resolution MS-verified reference sample.

### *1.4. High-resolution LC/MS/MS: sample preparation and instrument parameters*

Urine samples were diluted ten-fold with water and 15 μL injected for LC separation on a 100 × 2.1 mm Kinetex F5 column (Phenomenex) at a 0.5 mL/min flow rate in a 15 min run using the following step gradient program: 1) 0–10 min with 98% solvent A (10 mM ammonium format in water) and 2% solvent B (10 mM format in methanol); 2) 10–14 min with 2% solvent A and 98% B; 3) 14–15 min with 98% solvent A and 2% solvent B. The LC effluent was scanned from m/z 100 to 800 at 70,000 resolution (at m/z 200) using a Q-Exactive Orbitrap mass spectrometer with a HESI source (Thermo Scientific). MS/MS fragments were generated in the high collision dissociation chamber with 3-step normalized collision energy (NCE, %) of 30, 70 and 100 and analyzed by the Orbitrap detector with 17,500 resolution.

## 2. Results and discussion

Given that fentanyl and its metabolite norfentanyl exhibit MS fragments common to many of their analogues, we reasoned that re-analysis of extracted samples with a precursor ion scan for their respective product ions should be able to detect fentanyl analogues with substituents on the acyl and/or amide phenyl moieties. The predominant pattern of these LC/MS results in 50 consecutive urine samples consisted of large peaks exclusively due to fentanyl and norfentanyl, but in three samples we observed a recurring pattern of an m/z 188 precursor at m/z 349 and an m/z 84 precursor at m/z 245. In both cases, the respective retention times lagged slightly behind those of fentanyl and norfentanyl, consistent with a more lipophilic compound (and its metabolite) that we tentatively identified as Cp-FEN and its dealkylated (nor) metabolite Cp-NFEN (Fig. 1).

However, there was no mention of this drug in PubMed at the time, nor had it been reported in seizures by local law enforcement [8]. As no commercial standard was available without lengthy delays due to controlled substance import restrictions, no further action was taken until two months later when we became aware of Cp-FEN in a small Ontario town through a report published in its local newspaper [9], at which point the putative MRM parameters were added to the fentanyls confirmation panel at the LifeLabs BC lab. The first samples processed by the updated program had been sent from the LifeLabs ON laboratory to verify the presence of carfentanil, and it was from this group of 20 samples that two were tentatively identified as containing Cp-NFEN with and without parent Cp-FEN, respectively. These two samples were sent to the Centre for Addiction and Mental Health (CAMH) reference laboratory in Toronto, Ontario where compound identifiers were verified by high-resolution MS. Two peaks corresponding to the calculated exact masses of the protonated molecular ions (M + H) of Cp-FEN (m/z 349.2274) and Cp-NFEN (m/z 245.1648) were extracted from the total ion chromatogram (TIC) with a 5 ppm mass accuracy window at retention times of 11.20 min and 8.48 min respectively (Fig. 2A; 2B upper; 2C upper). Note the fragments characteristic of Cp-FEN (Fig. 2B, lower panel: m/z 228, 166, 69) and Cp-NFEN (Fig. 2C, lower panel: m/z 162, 69) in addition to the signature fragments of fentanyl (m/z 188, 105) and norfentanyl (m/z 177, 84, 55). The structure of the fragments is also shown.

We consequently added MRMs for Cp-FEN and Cp-NFEN to the BC fentanyl confirmatory testing menu (which also included carfentanil, norcarfentanil, furanyl fentanyl and U-47700 and metabolites) and over 8 days identified Cp-FEN/Cp-NFEN in 56 of 1048 (5.3%) samples which had screened positive by immunoassay. The mean ion ratios (qualifier/quantifier) of 0.92 (Cp-FEN) and 0.38 (Cp-NFEN) were very similar to those obtained for the corresponding fragmentation of fentanyl (1.01) and norfentanyl (0.43), further supporting the compounds' identity. Interestingly, levels observed for the two compounds were typically far from low; assuming the same detector response as their fentanyl/norfentanyl counterparts, estimated median concentrations for Cp-FEN and Cp-NFEN were 24 ng/mL (range: 1–3200 ng/mL) and 573 ng/mL (range: 43–11,860), respectively. During a similar period (November 2017) LifeLabs ON detected Cp-FEN and Cp-NFEN in 8 of 104 samples (7.7%) testing positive for fentanyl by LC/MS. Estimated median concentrations were lower than those detected in BC at 6 ng/mL (range: 3–27 ng/mL) and 44 ng/mL (range: 5–134 ng/mL) for Cp-FEN and Cp-NFEN, respectively. A retrospective analysis of fentanyl/carfentanil co-positive samples from February to October 2017 showed that Cp-FEN was present in 4 of 79 samples (5.1%) positive for fentanyl. Estimated median concentrations in those samples for Cp-FEN and Cp-NFEN were more consistent with the BC findings at 16 ng/mL (range: 6–119 ng/mL) and 228 ng/mL (150–372 ng/mL), respectively. Combined with the novelty of the cyclopropyl substituent and these levels, the presence of this drug in two Canadian provinces suggests deliberate addition rather than a synthetic by-product or accidental contamination.

In conclusion we have applied innovative algorithms using data obtained independently through conventional and high resolution mass spectrometry technologies to identify new fentanyl analogue(s) in clinical specimens. Compound identification in clinical laboratory settings traditionally requires the use of certified standards, but we have overcome this analytical constraint by combining the exact mass data with fragmentation, ion ratios, as well as metabolite data of structurally related compounds. Using similar algorithms we have recently also identified furanyl fentanyl and its metabolites in urine specimens as part of a clinical case investigation and revealed that furanyl fentanyl pills were sold illicitly as fentanyl in Toronto, ON [10]. A limitation of this current work is that the mass spectral properties of Cp-FEN are shared by its isomer, crotonyl fentanyl, which is predicted to have a similar retention time [11]. Unlike Cp-FEN, however, this compound has not been highlighted in any of the recent media reports from the United States and Canada describing seizures by law enforcement, making it less likely in our opinion that crotonyl fentanyl is being sold illicitly at present in significant amounts.

Our work also demonstrates the importance of collaboration among

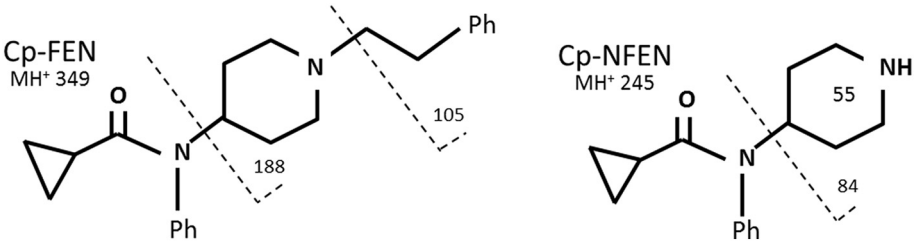


**Fig. 1.** Structure and fragmentation of Cyclopropyl fentanyl (Cp-FEN) and Cyclopropyl norfentanyl (Cp-NFEN). Ph = Phenyl.