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Determination of nine new fentanyl analogues and metabolites in consumers' urine by ultra-high-performance liquid chromatography-tandem mass spectrometry

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Abstract. – OBJECTIVE: New fentanyl analogues have been constantly emerging into the illegal drug market as cheap substitutes of heroin posing a serious health threat for consumers because of their high toxicity. Analytical methods to disclose the presence of these compounds in biological fluids of intoxicated individuals need to be updated to keep up with the new trends. In this study, we updated an ultra-high-performance liquid chromatography-tandem mass spectrometry method previously developed, for detecting some new fentanyl analogues and metabolites (sufentanil and norsufentanil, cis-3-methylnorfentanyl, trans-3-methylnorfentanyl, metabolites of cis and transmethylfentanyl, beta-phenylfentanyl, phenylfentanyl, para-fluoro furanyl fentanyl, isobutyryl fentanyl and ocfentanil) in urine sample.

MATERIALS AND METHODS: Urine samples were simply diluted before injection in the chromatograph equipped with a reversed phase microcolumn. Detection was achieved with a triple quadrupole mass spectrometer with an electrospray ionization source in positive ion mode and operated in multiple reaction monitoring.

RESULTS: The chromatographic separation was short (5 min) and the method was fully validated with a high sensitivity being limits of quantifications from 0.003 to 0.066 μg/L urine for the analytes under investigation.

CONCLUSIONS: The suitability of the method was tested with urine specimens from former heroin addicts, which resulted positive by immunological screening to the class of fentanyl analogues. This method represents a valid tool to document recent exposure to the above-reported compounds for clinical and forensic purposes.

Key Words:
Fentanyl analogues, UHPLC-MS/MS, Urine, New psychoactive substances.

Introduction

Fentanyl is a μ-opioid receptor agonist synthesized more than 50 years ago as narcotic-analgesic medication. The drug presents 50- to 100- fold higher potency than that of morphine. It is mainly used for intraoperative analgesia and chronic severe pain due to cancer and non-cancerous illnesses[1]. Since 2009 illicit fentanyl analogues have been introduced to illegal drug market as cheaper substitute of heroin or mixed with it as cutting agents. Fentanyl derivatives have similar or higher potency compared to primary drug, and for this reason represent a very high risk of poisoning to consumer[2]. Indeed, overdose cases and fatalities by respiratory depression and anaphylactic reactions caused by fentanyl analogues misused in place of heroin have been reported[3,4].

From 2009 to 2020, 57 new synthetic opioids were described in the European drug market, of which 36 were fentanyl analogues. In 2018, about 1000 seizures of new opioids were registered in EU area, with the majority being fentanyl analogues[5-8]. This trend even increased during COVID-19 pandemic[9].

Intoxications, overdoses, and fatalities by fentanyl derivatives request constantly updated ana-

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lytical methods to objectively assess the presence of parent drug and metabolites in biological matrices of related cases[10].

To the best of our knowledge, international literature reports only three analytical methods for detection of fentanyl analogues in human urine from real cases[11-13].

The one concerning the detection 22 fentanyl and metabolites in different biological matrices has been set up and validated by our investigation group in 2019[8]. However, in the meanwhile, new analogues entered the illicit European market, creating the exigence to update the existing method and analyzed unsolved intoxication cases.

In this study, we updated the above-reported ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method to identify and quantify: sufentanil and norsufentanil, cis-3-methylnorfentanyl, trans-3-methylnorfentanyl, metabolites of cis and transmethylfentanyl, beta-phenylfentanyl, phenylfentanyl, para-fluoro furanyl fentanyl, isobutyryl fentanyl and ocfentanil. We confirmed the suitability of the updated method by testing 11 urine samples from former heroin addicts, which resulted positive by immunological screening to the class of fentanyl analogs.

Materials and Methods

Chemicals and Reagents

Working standards (beta-phenyl fentanyl, phenyl fentanyl, para-fluoro furanyl fentanyl, isobutyryl fentanyl, ocfentanil, sufentanil, norsufentanil, cis-3-methylnorfentanyl, trans-3-methylnorfentanyl) and deuterated internal standards (IS; acetyl norfentanyl-D5 and fentanyl-D5) were purchased from Cayman Chemical (Ann Arbor, MI, USA) and stored at -20°C until use. LC-MS grade water, acetonitrile, methanol were obtained from Sigma-Aldrich® (Milano, Italy) and ULC grade formic acid was obtained from Biosolve Chimie SARL® (Dieuze, France). Ammonium acetate buffer was prepared with ≥ 97% purity ammonium acetate salt (Carlo Erba®, Cornaredo, Milan, Italy) dissolved in LC-MS water.

Calibrators and Quality Control Solutions

Stock solutions of each standard at 10 mg/L were prepared in methanol. Standard stock solution containing all 9 non-deuterated standards was prepared in methanol at 1 mg/L. IS standard stock solution with acetyl norfentanyl-D5 and fentanyl-D5 was prepared in methanol at 1 mg/L. Stock solutions were stored in glass vials at – 20°C.

Calibrator working solutions were daily prepared from the standard stock solution in methanol (5 calibrators along the working range). Low, medium, and QC working solutions were daily prepared from the standard stock solution in methanol. IS working solution was daily prepared from the IS stock solution in methanol to reach a concentration of 5 µg/L in urine.

Human Samples

Blank urine samples were obtained from the laboratory storehouse of blank biological samples. Urine specimens from authentic cases of consumption were provided as discarded material from different collaborative European projects by the Department of Excellence of Biomedical Science and Public Health, University "Politecnica delle Marche" of Ancona (Ancona, Italy).

Sample Preparation

Aliquots of 100 µL of urine were fortified with 5 µL IS working solution in glass tubes, vortexed and added with 3 mL mobile phase A:B 95:5 (v/v). Tubes were then capped, vortexed for 10 s and centrifuged at 15,000 g for 5 min. About 200 µL of supernatant was transferred into autosampler glass vials, prior to injection (10 µL) onto the chromatographic system.

Instrumentation

UHPLC-MS/MS analysis was performed on a Waters® Xevo® TQ-S micro mass spectrometer (triple quadrupole) equipped with an electrospray ionization source in positive ion mode (ESI+) and interfaced with an ACQUITY UPLC® I-Class (Waters®; Milan, Italy). Data were acquired with MassLynx® software version 4.1 (Waters®).

Separation was performed on an ACQUITY UPLC® BEH C_{18} column from Waters® (length: 50 mm, internal diameter: 2.1 mm, particle size: 1.7 µm). Run time was 8 min with a gradient mobile phase composed of 0.1% formic acid in 5 mM ammonium acetate buffer (A) and 0.05% formic acid in acetonitrile (B) at a flow rate of 0.35 mL/min. Initial conditions were 5% B, held for 1 min, increased to 30% B within 3.5 min, increased to 95% B within 0.5 min, held for 0.5 min, returned 5% B within 0.1 min, and then held for 2.4 min. LC flow was directed to waste the first 1.5 min of the separation and after 6 min. Autosampler and column oven temperatures were 10°C and 50°C, respectively.

The mass spectrometer operated in scheduled multiple reaction monitoring (MRM) mode, with two transitions for each analyte and one transition for each IS (Table I). MS parameter settings were optimized by infusing neat standards individually in methanol and ramping cone voltage and collision energy (Table I). Scan speed (dwell time) was adjusted in the chromatographic conditions of the analysis to produce 15 to 20 scans per chromatographic peak. ESI+ conditions were optimized as follows: capillary voltage = 0.5 kV, source temperature = 150°C, desolvation temperature = 650°C, cone gas flow rate = 20 L/h, desolvation gas flow rate = 1,200 L/h.

Method Validation

The method was validated over five subsequent days in urine following the most recent criteria for method development and validation in analytical toxicology[14,15].

Working ranges were LOQ-100 µg/L, for all analytes. Selectivity, linearity, sensitivity (limits of detection and quantification), accuracy, precision, carryover, analytical recovery, and matrix effects were calculated using five different daily replicates of calibration points (five points for each calibration curve, including the limit of quantification as the lowest point) and five replicates of QC samples (low QC = 0.015 µg/L, medium QC = 10 µg/L, and high QC = 80 µg/L) along three subsequent working days, as previously described. Dilution integrity was tested for over-the-curve samples with a concentration 10 and 50 times higher than the highest calibrators, with a dilution in mobile phase A:B 95:5 (v/v) before sample treatment. Calibration points and QC samples were prepared by two different staff members.

Results

A chromatogram of a urine sample fortified with all the analytes under investigation at 0.01 µg/L and their ISs is displayed in Figure 1 and validation parameters are reported in Table II. Limits of quantification (LOQ) ranged from 0.003 to 0.006 µg/L urine, and analytical recovery ranged from a minimum of 89.1% to 99.3%. No interfering peaks due to endogenous substances appeared at retention times of the investigated analytes and ISs and no significant ion suppression (less than 10% analytical signal suppression) due to matrix effect occurred during chromatographic runs. All QCs were quantified within ±15% accuracy and intra- and inter-assay precision. Sample contamination by carryover was not observed for any of the nine analytes. Diluted over-the-curve samples well fitted into the calibration curves with precision and accuracy (within ±15% of target concentration).

Over a group of 11 urine samples, previously screened by Randox Evidence MultiSTAT Immuno Analyser and MultiSTAT NPS I Urine Array cartridges/kit (Randox, Milan Italy), and resulted positive to the class of fentanyl analogs, five samples were confirmed by our developed method to contain: 13.7 µg/L phenyl fentanyl (sample n. 3),

Table I. Mass spectrometry parameters for analytes under investigation and internal standards. Scan speed (dwell time) and detection windows were adjusted accordingly.

Compound	IS	Cone voltage (V)	Q1 mass (m/z)	Quant. transition		Conf. transition		RT (min)
				Q3 mass (m/z)	CE (eV)	Q3 mass (m/z)	CE (eV)	
Acetyl norfentanyl-D5 (A-D5)	-	25	224.2	84.0	18	-	-	2.23
Fentanyl-D5 (F-D5)	-	25	342.2	105.2	38	-	-	4.76
Beta-phenyl fentanyl	A-D5	30	413.2	105.1	42	188.2	24	4.53
Phenyl fentanyl	F-D5	30	385.2	105.2	36	188.3	20	4.05
Para-fluoro furanyl fentanyl	F-D5	25	393.2	105.1	40	188.1	22	3.70
Isobutyryl fentanyl	F-D5	30	351.3	105.1	42	188.1	22	3.99
Ocfentanil	F-D5	30	371.2	105.2	40	188.2	24	2.92
Sufentanil	F-D5	16	387.2	111.0	38	238.1	18	5.15
Norsufentanil	A-D5	25	277.1	96	25	128.1	15	2.80
Cis-3-methylnorfentanyl	A-D5	25	247.1	69.1	30	98	16	2.34

Quant. transition, quantification transition; Conf. transition, confirmation transition; IS, internal standard; CE, collision energy; RT, retention time.

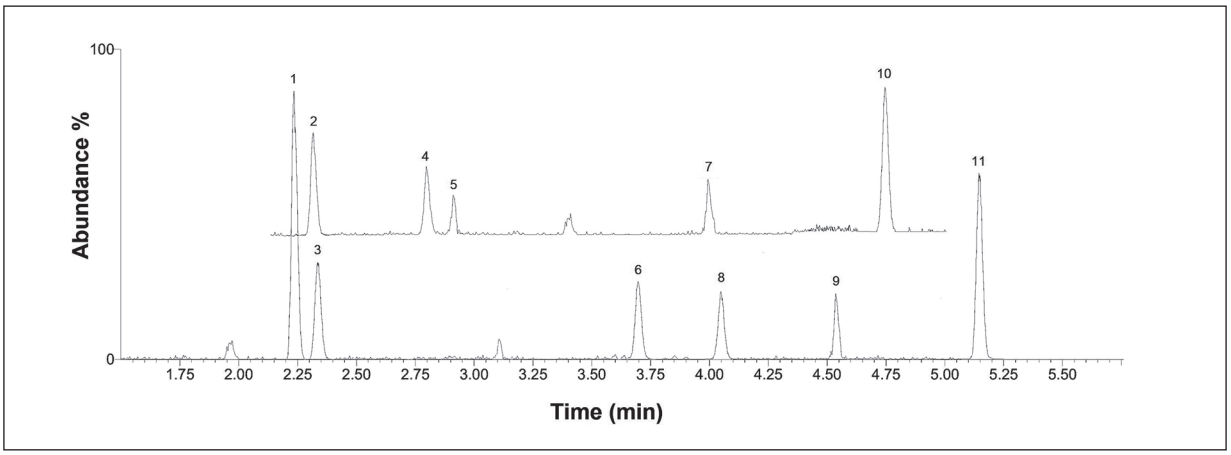

Figure 1. UHPLC-MS/MS chromatogram of urine sample fortified with the analytes at 0.01 µg/L and their ISs. 1, acetyl norfentanyl-D5 (A-D5); 2, cis-3-methylnorfentanyl; 3, trans-3-methylnorfentanyl; 4, norsufentanil; 5, ocfentanil, 6, para-fluoro furanyl fentanyl; 7, isobutyryl fentanyl; 8, phenyl fentanyl; 9, beta-phenyl fentanyl, 10, fentanyl-D5 (F-D5); 11, sufentanil.

11.3 and 54.3 µg/L isobutyryl fentanyl (samples 5 and 6), 2.5 µg/L ocfentanil (sample n.8) and 1.2 µg/L sufentanil and 25.6 µg/L norsufentanil (sample No. 1). In addition, two other samples contained norfentanyl and a latter one fentanyl itself. In the remaining four ones, we could not detect any fentanyl analogue included in our method nor in the previous developed one[13].

Discussion

The application on real samples demonstrated the robustness of our validated UHPLC-MS/MS assay. The latter, combined with the one validated by our investigation team in 2019[13], which included twenty-two fentanyl, provides us the most comprehensive targeted screening for quantifying fentanyl and analogues in urine up to date. Norsufentanil, cis-3-methylnorfentanil and trans-3-methylnorfentanil, metabolites of sufentanil, cis-3-methylfentanil and trans-3-methyl fentanyl, respectively, were included for the first time in an analytical method for urine. In this matrix it is essential to mainly analyze the metabolites when they are known and commercially available.

Conclusions

We have updated an ultra-high performance liquid chromatography tandem mass spectrometry method previously developed for detection of twenty-two different fentanyl analogues and metabolites, for clinical and forensic applications. The method is quick and easy and has been validated in urine with high sensitivity for all analytes. This is the first method reported to simultaneously quantify sufentanil and norsufentanil, cis-3-methylnorfentanyl, trans-3-methylnorfentanyl, metabolites of cis and transmethylfentanyl, beta-phenylfentanyl, phenylfentanyl, para-fluoro furanyl fentanyl, isobutyryl fentanyl and ocfentanil.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Table II. Validation parameters for fentanyl and analogues in urine. Low, medium, and high-quality control working solutions contained all standards at 0.01, 10, and 80 µg/L, respectively.

Compound	Determination coefficient (r^2)	LOD (µg/L)	LOQ (µg/L)	Accuracy (% error)			Intra-assay precision (% CV)			Inter-assay precision (% CV)			Matrix effect (%)	Recovery (%)
				Low QC	Mid QC	High QC	Low QC	Mid QC	High QC	Low QC	Mid QC	High QC		
Beta-phenyl fentanyl	0.998 ± 0.003	0.002	0.006	9.5	9.4	12.1	11.4	6.4	5.9	13.1	12.9	6.2	91.2	89.1
Phenyl fentanyl	0.999 ± 0.001	0.001	0.004	11.4	8.5	6.5	10.4	5.9	7.1	14.1	9.9	7.4	91.3	95.1
Para-fluoro furanyl fentanyl	0.996 ± 0.003	0.002	0.006	8.9	10.1	8.5	9.6	7.9	6.4	13.1	9.4	9.4	89.9	93.2
Isobutyryl fentanyl	0.996 ± 0.005	0.002	0.005	10.4	10.4	10.1	11.4	6.6	6.3	12.5	11.4	7.1	91.1	90.1
Ocfentanil	0.994 ± 0.004	0.002	0.005	10.5	7.9	9.9	12.4	6.7	8.1	10.1	11.4	10.7	92.3	97.5
Sufentanil	0.991 ± 0.002	0.001	0.003	9.0	8.5	7.9	9.1	10.8	9.2	12.1	11.2	13.5	81.3	89.1
Norsufentanil	0.994 ± 0.002	0.001	0.004	9.2	10.2	10.1	10.1	8.3	3.9	12.3	9.8	8.1	90.6	99.3
Cis-3-methylnorfentanyl	0.994 ± 0.004	0.001	0.004	9.8	7.6	11.2	8.9	9.2	3.7	13.4	10.5	8.5	89.1	95.1
Trans-3-methylnorfentanyl	0.997 ± 0.001	0.001	0.003	11.5	8.2	7.9	9.4	10.6	5.1	11.1	13.2	9.1	92.2	96.6

CV, coefficient of variation; LOD, limit of detection; LOQ, limit of quantification, QC, quality control samples.

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