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CORRESPONDENCE CASE REPORT

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An ocfentanil-related death case: UHPLC–MS/MS analysis of the drug

1 | INTRODUCTION

Over the last decade, the consumption of new psychoactive substances (NPS) has rapidly increased in the recreational drug scene. Although cannabinoids and designer cathinones are the most popular among NPS, new synthetic opioids have recently emerged on the recreational drug market.[1-3] Ocfentanil (N-(2-fluorophenyl)-2-methoxy-N-[1-(2-phenylethyl) piperidin-4-yl]acetamide) is a new synthetic opioid described in a patent in 1986[4] and tested on humans in 1989 (code A-3217) in order to obtain an analgesic molecule with a better therapeutic index and fewer cardiovascular and respiratory effects than fentanyl, but never marketed. Indeed, its assumption causes analgesia and respiratory depression in a dose-dependent manner, reaching the maximum peak after six minutes of the injection; analgesia disappears largely after one hour, while respiratory depression tends to last longer than fentanyl.[5] The analgesic activity was determined to be 2.5 times as potent as fentanyl and around 200 times as potent as morphine.[6] This ability to act at very small concentrations, if used illicitly for recreational purposes, determines a high risk of overdose. Unfortunately, some of the attributes that make these drugs so precious are also responsible for their considerable potential for abuse, addiction, and overdose. Reports on the commercialization of non-controlled fentanyl derivatives through the web, such as butyrfentanyl and 4-fluoro-butyrfentanyl,[7] and of acetylfentanyl,[8] are particularly alarming because of the high risk associated with their consumption.[9] Another worrying trend is the use of fentanyl derivatives to adulterate heroin, which began three decades ago[10] and continues to this day.[9] A recent review has reported the presence of ocfentanil (OcF) in several samples sold as heroin in the hidden web.[8] Taken together, these data tell us how these designer opioids, such as OcF, represent a serious concern for public health, since they are both toxic at low dose and often sold as heroin to unsuspecting users. To date, a death involving OcF has been published in Belgium[11] and in Switzerland,[12] both in 2016. To our knowledge, this paper describes the first reported death involving OcF which occurred in Italy (in 2017). An ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) based method was developed and validated for OcF identification and quantitation.

2 | CASE REPORT

A 39-year-old white man with a history of heroin abuse was found dead in the cellar of his house. A syringe, a lighter, and a resealable plastic zipper bag (minigrip) containing several hundred milligrams of a brown powder were found close to the dead body by the police officers. First, a heroin overdose was suspected. After six years of detoxification and a severe relapse, he had started to attend a drug rehabilitation program and before his death he was being treated with buprenorphine and sedatives, in case of need. During the autopsy, a body examination revealed some injection sites at his elbow grooves, a pulmonary congestion, and an edema (left lung 950 g and right lung 1045 g), while his heart (410 g) and brain (1350 g) did not show any pathological findings. Samples of cardiac and femoral blood, bile, urine, brain, liver, gastric content, kidney, lung, hair, and nasal swab were collected and maintained at −20° C until toxicological analysis. In addition, samples were taken for histological analysis but not submitted to analysis, since they were not requested by the Public Prosecutor.

3 | MATERIAL AND METHODS

3.1 | Toxicological analysis of post-mortem specimens

A comprehensive systematic toxicological analysis was performed on the post-mortem tissue specimens to investigate alcohol, volatile substances, and illegal and medical drugs. First, peripheral post-mortem blood was screened for ethanol and volatile compounds by headspace gas chromatography with flame ionization detector (GC–FID). Immunological drug abuse screening (amphetamines, benzodiazepines, buprenorphine, cannabinoids, cocaine, methadone, and opiates) was performed on urine by Enzyme Multiplied Immunoassay Technique (EMIT) on VIVA-Twin™ Dade-Behring Analyzer (Cupertino, CA, USA). In addition, general unknown screening in blood, urine, and in the brown powder was conducted by gas chromatography mass spectrometry (GC–MS) in order to identify additional drugs or metabolites after acidic and basic extraction and derivatization according to Maurer et al..[13] This procedure was also used to identify OcF in the biological samples and in the powder as described in Section 3.2. Drugs of abuse, fentanyl, and new fentanyl derivatives were also investigated in hair specimens. After decontamination with 3 mL dichloromethane, proximal (0–1.5 cm) and distal (1.5–3 cm) hair sections were reduced in short cuts (2–4 mm) and dried. Hair extraction and analysis by GC–MS was performed in accordance to an internal validated procedure[14,15] in particular for the determination of fentanyl

and new fentanyl derivatives, such as norfentanyl, acetyl fentanyl, fentanyl, sufentanyl, noralfentanyl, alfentanyl, mefentanyl, carfentanyl, lofentanyl, furanyl fentanyl, n-benzyl phenyl norfentanyl, remifentanyl, thiofentanyl, valerylfentanyl, mirefentanyl, benzylcarfentanil, acrylfentanyl and buprenorphine.

3.2 | Ocfentanil confirmation analysis

Since no commercial OcF standard was available in the beginning for analysis, a prior evaluation of OcF concentration was performed on a fentanyl-based calibration curve, set up in blank plasma using remifentanil as internal standard (IS). Once an OcF reference compound was available, a UHPLC–MS/MS was developed and validated for OcF identification and quantitation, according to Food and Drug Administration (FDA) guidelines.[16]

3.2.1 | Chemicals

Ultrapure water, acetonitrile, dichloromethane, isopropanol, methanol, and sodium hydroxide were of analytical grade and purchased from Carlo Erba (Milan, Italy). The certified reference ocfentanil (N-(2-fluorophenyl)-2-methoxy-N-[1-(2-phenylethyl)-4-piperidyl]acetamide) was obtained from Alsachim (Illkirch Graffenstaden, Strasbourg, France). Remifentanil (100 µg/mL solution in methanol) was purchased from Cerilliant (Milan, Italy). Formic acid (98%–100%), tris (hydroxymethyl) aminomethane (TRIS) ≥99% and protease from Bacillus licheniformis type VIII were from Sigma–Aldrich (Milan, Italy). The 96-well plate SPEC MP1 was obtained from Agilent Technologies (Palo Alto, CA, USA).

3.2.2 | Instrumental conditions

UHPLC–MS/MS analyses were performed on a 1290 Infinity ultra-high-performance liquid chromatography system (Agilent Technologies, Palo Alto, CA, USA) coupled to a Q Trap 5500 linear ion trap triple quadrupole mass spectrometer (Sciex, Darmstadt, Germany) and equipped with an electrospray ionization (ESI) source. Chromatographic separation was carried out on a Kinetex HPLC XB-C18 column (100 mm length x 2.1 mm i.d, 2.6 particle size) at 30°C using a linear gradient elution with two solvents: 0.1% formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Solvent A and B were 90% and 10% at 0.00 minutes, respectively. Solvent B was increased to 25% from 0.00 to 2.00 minutes, then increased to 90% from 2.00 to 3.50 minutes and to 98% from 3.50 to 4.00 minutes, held at 98% from 4.00 to 5.40 minutes, and then decreased back to 10% from 5.40 to 5.50 minutes and held at 10% from 5.50 to 7.50 minutes for re-equilibration. The flow rate was kept constant at 0.5 mL/min during the analysis. The separated analytes were detected with a triple quadrupole mass spectrometer operated in multiple reaction monitoring (MRM) mode via positive ESI using the precursor ion and product ions transition shown in Table 1. The instrumental conditions were optimized by direct infusion (flow rate 7 µL/min) of OcF solution (100 ng/mL) and were as follows: Entrance potential 10 eV, curtain gas 25 psi, ion spray voltage 5500 eV, ion source temperature 500°C, ion source gas 1 45 psi, and ion source gas 2 40 psi. Data acquisition and processing was performed using Analyst®1.6.2 and MultiQuant®2.1.1 software (Sciex, Darmstadt, Germany), respectively.

TABLE 1 MRM parameters: Dwell time, de-clustering potential (DP), collision energy (CE), precursor and product ion transitions for Ocfentanil and Remifentanil

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Dwell Time (msec)	DP (eV)	CE (eV)
Ocfentanil	371.1	188.1	70	250	30
		102.1	70	230	45
		77.1	70	180	104
Remifentanil	377.1	285.1	70	80	30
		228.0	70	80	30

3.2.3 | Validation procedure

UHPLC–MS/MS procedure for OcF determination was performed in accordance with international recommendations for the validation of new analytical methods endorsed by FDA guidelines.[16] Calibration standards and quality controls (low: 0.25 ng/mL; intermediate: 2.5 ng/mL; high: 25 ng/mL) were obtained by spiking 500 µL blank plasma aliquots with appropriate amounts of OcF working solutions (100 ng/mL) in the range 0–25 ng/mL. Eight points calibration curves (0–0.25 – 0.5 – 1 – 2.5 – 5 – 10 – 25 ng/mL) were generated based on the peak area ratios of the analytes to the IS against nominal analyte concentration using a weighted 1/x2 linear regression. The correlation was tested over the whole range of concentration to ensure linear regression. Linearity was considered satisfactory if $r^2 \geq 0.990$ and CV ≤ 15%. Sensitivity was expressed in terms of LOD (limit of detection) and LOQ (limit of quantification). The LOQ was determined as the lowest concentration with values for precision and accuracy within ±20% and a signal-to-noise (S/N) ratio of the peak areas ≥10. The LOD was determined as the lowest concentration with a signal-to-noise (S/N) ratio of the peak areas ≥3. Precision and accuracy of the method were determined through the analysis of six independent replicates of QC materials extracted from blank plasma samples. Precision and accuracy were determined by calculating the coefficient of variation (CV%) and the Bias (BIAS%). The analytes recovery was determined by comparing the mass spectrometric response of a first set of OcF-free plasma samples (n = 3) fortified with OcF prior to extraction at a final concentration of 0.25, 2.5 and 25 ng/mL respectively, and a second set of OcF-free plasma samples (n = 3) fortified with analytes at the same final concentration after extraction. Absolute recovery was determined by comparing the peak areas of the two sets of samples and expressed as percentage. The matrix effect (%) was determined by comparing the peak areas of a first set of extracted aqueous samples (n = 3) in the low, intermediate and high concentration range with the peak areas of a second set of OcF-free plasma samples (n = 3), both fortified with OcF after to extraction.

3.2.4 | Specimens preparation and extraction

Biological specimens as kidney, liver, lung, and brain were homogenized in TRIS (5 mL for 1 g) by addition of the protease from Bacillus licheniformis (9.3 units/mg solid) and ultrasonicated for two hours. An aliquot of diluted tissue (500 µL) and liquid specimens such as cardiac and femoral blood (500 µL), urine (50 µL), and bile (20 µL) was added by 5 µL IS (remifentanil 1 µg/mL) and 2 mL of acetate buffer solution at pH 4 under vortex-mixed agitation. After centrifugation, the upper layer was extracted following a previous validated

procedure.[14] The organic phase was evaporated to dryness with a gentle stream of nitrogen and the residue was dissolved with methanol (100 µL). 1 µL aliquot was then injected into the UHPLC-MS/MS system, and analyzed as described in Section 3.2.2.

4 | RESULTS

As a first step, GC–MS screening was applied to the brown powder and typical cutting agents of street heroin such as acetaminophen and caffeine were found, in addition to OcF (2.48%). The identification was obtained by means of a good fit of the obtained mass spectra with the SWGDRUG library version 2.3 installed on the Agilent Chemstation (as described in Figure 1 for OcF).

Same analysis was performed on the previously mentioned post-mortem specimens, showing a therapeutic concentration of acetaminophen in blood (0.13 µg/mL; acetominophen concentration in fatalities 248 mg/L[17]) and an irrelevant level of caffeine (1.61 µg/mL; caffeine concentration in fatalities 183 mg/L[17]), in addition to OcF.

As previously described, a hair sample was also analyzed in GC–MS, after splitting in proximal and distal section, with the following results: cocaine (proximal: 2.33 ng/mg; distal: 1.69 ng/mg) and benzoylecgonine (proximal: 0.31 ng/mg; distal: 0.15 ng/mg).

As soon as the OcF reference compound was available, both automated extraction procedure and UHPLC–MS/MS method were developed and validated for OcF identification and quantitation, as described in Section 3.

All calibration curves showed good linearity (r^2 > 0.9994) over the entire investigated range when using linear correlation. The % CV at the low concentration point was found to be 5.3%, whereas LOD and LOQ obtained were 0.03 and 0.10 ng/mL, respectively. An overview of the assessed validation data is shown in Table 2.

The validated method was then applied to obtain a distribution of OcF in the post-mortem fluids (urine, bile, cardiac, and femoral blood) and tissue specimens (kidney, liver, lung, and brain), following an internal extraction procedure[14] previously set up for hair samples, with some minor modification in the pre-extraction step, as described in Section 3.2.4. Figure 2 reports MRM chromatograms relative to OcF (A) and the certified reference OcF spiked in blank plasma (B). OcF was detected in all post-mortem specimens, at different level, as reported in Table 3.

TABLE 2 Validation parameters

Validation Parameter	OcF		
	Low	Intermediate	High
Amount (ng)	0.25	2.50	25.0
Measured (ng/mL)	0.26	2.42	25.2
Precision (%)	5.3	11.6	7.7
Accuracy (%)	7	11	12
Recovery (%)	88.6	84.0	86.0
Matrix Effect (%)	−13.8	−9.82	13.1

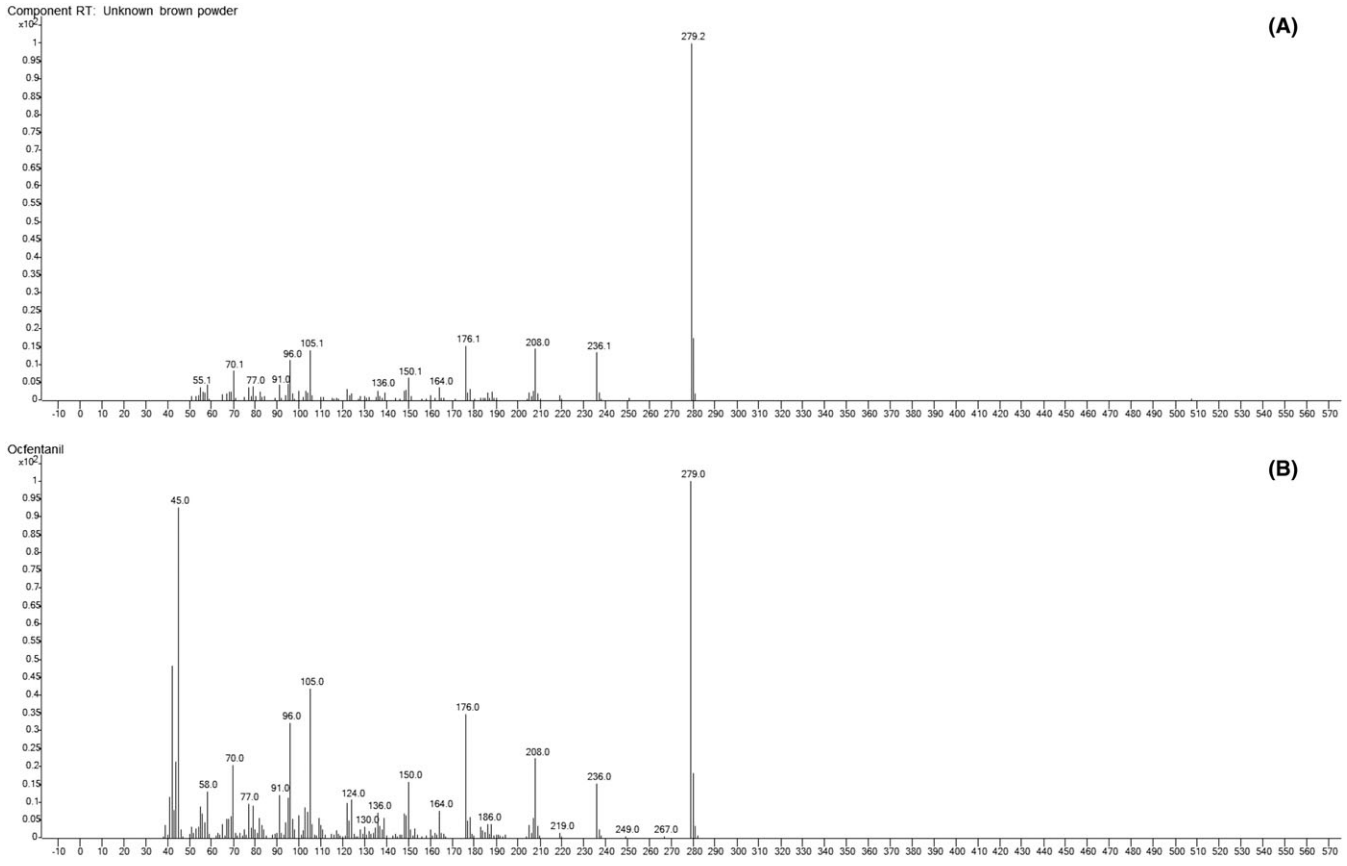

FIGURE 1 Mass spectra relative to OcF from A, brown powder and B, reference from SWGDRUG library

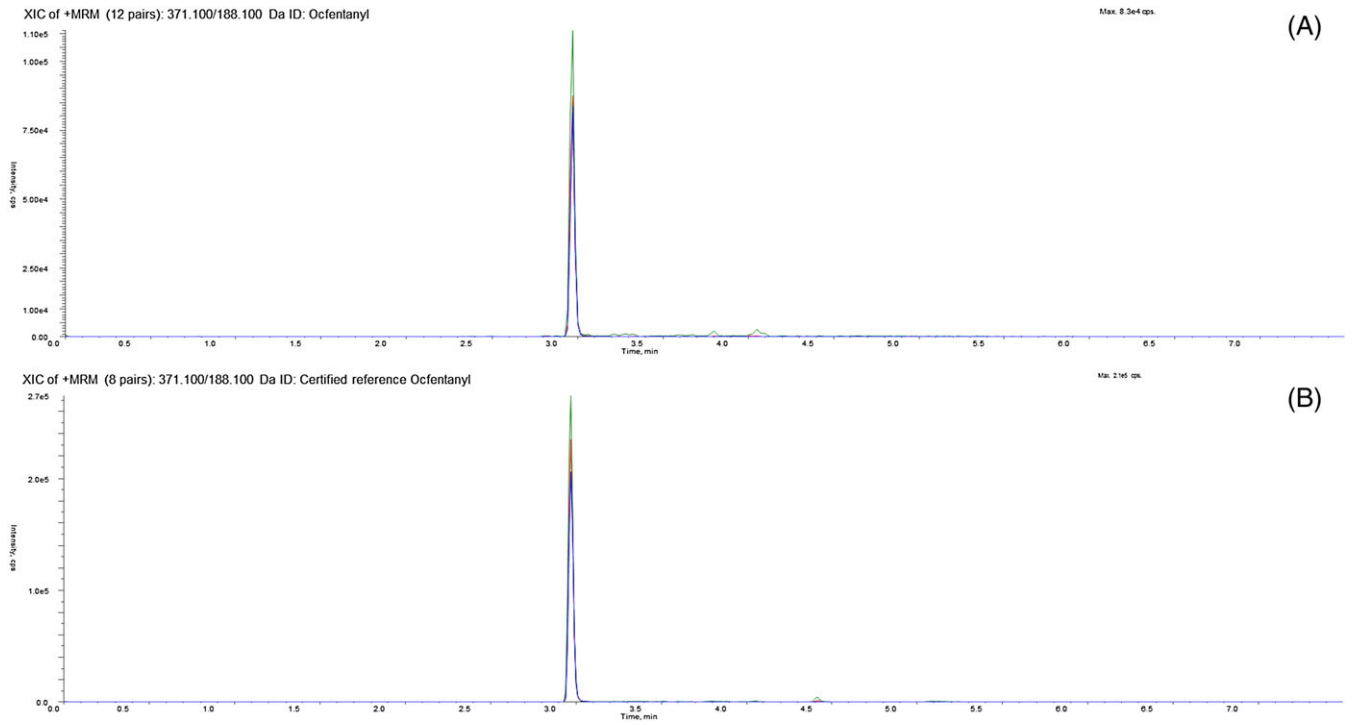

FIGURE 2 MRM chromatograms relative to A, OcF and B, the certified reference OcF [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Distribution of OcF in post-mortem biological samples in the present case and in Coopman et al[11] and average of values of lethal fentanyl-related intoxication[17]

	Present Case OcF	OcF[11]	Fentanyl[17]
Femoral blood	36.4 ng/mL	15.3 ng/mL	8.3 ng/mL (3.00–28.0)
Cardiac blood	49.8 ng/mL	23.3 ng/mL	/
Urine	67.9 ng/mL	6.00 ng/mL	28.0 ng/mL (5.00–93.0)
Bile	365 ng/mL	13.7 ng/mL	/
Brain	72.0 ng/g	37.9 ng/g	20.0 ng/mL (9.20–30.0)
Liver	106 ng/g	31.2 ng/g	37.0 ng/mL (5.90–78.0)
Kidney	75.5 ng/g	51.2 ng/g	18.0 ng/mL (6.10–42.0)
Lung	108 ng/g	/	/

5 | DISCUSSION AND CONCLUSION

This article reports a case of acute intoxication caused by OcF injection. To obtain a diagnosis of death, a forensic-based method involving anamnestic, clinical, and circumstantial, anatomo-pathological and toxicological criteria was used. It was known that the deceased person had a substantial anamnestic history of drug addiction when in life, which may direct the diagnosis toward a death correlated with a chemical cause. As far as the anatomo-pathological findings are concerned, limbs lesions were found to be significant and consisted with a narcotic substance injection. Moreover, these typical findings are frequently reported among intravenous drug users. Indeed, the toxicological investigations performed on the brown powder and subsequently on the biological specimens taken from the body of the deceased person, showed the presence of OcF in the femoral and cardiac blood, in urine, bile, brain, liver, lung, and kidney, together with paracetamol and caffeine. In particular, OcF determination in the blood as well as in the brain proves the administration of the substance just before the time of death and a state of acute intoxication. With regard to fentanyl compounds determination, it should be noted that the toxicological framework emerging from post-mortem samples may not reflect the actual situation at the time of death, since before analysis, OcF may undergo the same in vitro transformations demonstrated for fentanyl (due to oxidation, temperature variations, and acidic and basic environment).[18] The extent of these post-mortem phenomena could only be assessed throughout the determination of the concentration ratio between unchanged molecule and metabolites, here not performed, due to the lack of metabolites standards. However, the toxicological data obtained in the present study, taken together with the well-known potency and danger of the substance and with the already discussed congruence between anamnestic, circumstantial, and anatomopathological data with the hypothesis of acute narcosis, allow us to support the lethal power of OcF. The OcF levels we have determined in the different post-mortem specimens are summarized in Table 3. Our data were then

compared with those reported by Coopman et al[11] – to our knowledge the only documented case of lethal OcF-related intoxication reporting OcF levels in tissues – and with average fentanyl levels reported in lethal fentanyl-related intoxication.[17] As we can see, in our case the OcF levels were higher than the others, in all the specimens, confirming acute intoxication. This statement is supported in case of lack of tolerance to the synthetic opioids such as fentanyl and new fentanyl derivatives and/or to other opioids, as in this subject. Indeed, the 3 cm-hair analysis revealed the only presence of cocaine and no opioid compounds at all, to confirm regular abuse only for the first one. In particular, no traces of buprenorphine, fentanyl, and other new fentanyl derivatives were detected in the keratin matrix. Nothing can be said about the will of the subject to take OcF; it is not clear if the consumer was aware of the real composition of the powder he gave himself or whether he thought he was taking heroin. Indeed, there are documented cases in which the drug, purchased online as heroin, has showed no trace of it but a composition based on (in order of magnitude) paracetamol, caffeine, and OcF[19] as in this case. In conclusion, based on circumstantial evidence, autopsy findings, and toxicological analysis, the medical examiner certified the cause of death as most likely acute OcF intoxication. To our knowledge, this is the first published case, reported in Italy, with a fatal outcome related to OcF and with OcF concentration in tissues.

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