Matey, J. M. et al., 2020. Ultraviolet-visible and high-resolution mass spectrometry for the identification of cyclopropyl-fentanyl in the first fatal case in Spain. Journal of Analytical Toxicology, 44(8), pp. 927–935.

Available at https://doi.org/10.1093/jat/bkaa081

Ultraviolet-Visible and High-Resolution Mass Spectrometry for the Identification of Cyclopropyl-Fentanyl in the First Fatal Case in Spain

Jose Manuel Matey1,2,*, Carmen García Ruíz2, Gemma Montalvo García2, Juan Carlos Gomez Soro3, Daniel Gutiérrez Delicado1, Jovita Rodriguez Gallardo1, and María Martínez1

1National Institute of Toxicology and Forensic Sciences, Chemistry and Drugs, Las Rozas de Madrid, Madrid, Spain, 2University of Alcala, Department of Analytical Chemistry, Physical Chemistry and Chemical Engineering and University Institute of Research in Police Sciences (IUICP), Alcala de Henares, Madrid, Spain and 3Anatomical Forensic Institute, Madrid, Spain

*Author to whom correspondence should be addressed.

INTRODUCTION

Fentanyl was first synthesized in 1959. It was followed by a series of related substances that constitutes new opioid narcotic analgesic drugs, a highly potent family of compounds commonly known as the fentanyls, fentanyl analogs or simply fentalogs [1]. N-phenyl-N-[1-(2-phenylethyl)piperidin-4-yl] cyclopropanecarboxamide, commonly known as cyclopropyl-fentanyl, is a fentanyl analog that was synthesized by Dr. Paul Jenssen [2], among other fentanyls.

The fentanyl family is based on the fentanyl chemical structure and shows important psychoactive effects, which include intense sedation, loss of consciousness or fatal human intoxication, and death. Fentanyl is a powerful synthetic opioid analgesic, similar to morphine, but 50-100 times more potent, and others fentanyls, such as, carfentanyl even 5,000-10,000 times higher than morphine, [3, 4].

Fentanyl and their analogs have been sold as legal substitutes of illicit opioids such as heroin, often leading to self-delivery and purchased in illegal markets [5]. In fact, illicit fentanyl and fentanyl analogs have been found in mixtures with heroin and other illicit opioids since 2013 and 2014 in United States [5, 6] and in Europe (2017, 2018) [7, 8]. Among fentanyl analogs, cyclopropyl-fentanyl is a strong μ-receptor agonist, which was first discovered on the illicit market in Europe in June 2017 [9, 10]. It has been involved in at least 78 deaths in Europe [1] and over a hundred in the United States [6, 8-10]. In Europe, during the second half of 2017, it was detected in postmortem evidences in a few countries, such as Sweden (74 cases), United Kingdom (3 cases) and Norway (1 case) [1].

The cyclopropyl-fentanyl is classified as a New Psychoactive Substance (NPS). In 2018 it was outlawed and subsequently, it was included as a scheduled substance under the *1961 Single Convention on Narcotic Drugs* [11]. The number of new psychoactive substances (NPS) that are synthetic opioids, mostly fentanyl analogues or fentanyls, reported on the market has been rising at an unprecedented rate. It rose worldwide from just 1 reported case substance in 2009 to 15 in 2015, and 46 in 2017, while the overall number of NPS present on the market stabilized at around 500 substances per year over the period 2015–2017. Synthetic opioids have become the second most important substance group, after stimulants, in terms of NPS reported for the first time. The group accounted for 29 per cent of the newly identified NPS in 2017 [12].

Fentanyl and others opioids have had a high prevalence, in some countries, especially in the USA, being minor in southern Europe countries. In spite of this, in these countries (including Spain) it is strongly necessary to be vigilant for an early detection and damage reduction in population, especially, in order to take preventive actions to avoid fatal cases, due to the high toxic risk of these substances. In this sense, the detection of the first fatality cases alerted on the real consumption of these substances in a country and allowed us to inform and to develop preventive actions.

In this work, we report the different analytical approaches followed in the National Institute of Toxicology and Forensic Sciences (INTCF) for cyclopropyl-fentanyl identification in a fatal case occurred in a poly-drug consumer in Spain in December 2017.

1.2. CASE HISTORY

A 24-years-old white male, was found dead at home in Madrid, Spain. The medical examiner indicated a dead person sat forward on the bed. The suspected cause of death was poly-drug intoxication, since drug paraphernalia was located at home and absence of injuries in arms, forearms and hands was observed. The autopsy was performed 48h later.

The histological examinations performed on stained samples showed no traumatic signs in both the cranial and brain cavity. Both lungs presented intense congestive parenchyma, with fluid and dark blood. Pericardial sac (240 grams heart) without findings of interest whereas spirits presented fluid and dark blood. In the abdominal cavity, the liver presented slight congestion. The stomach, with brown-dark pasty content, had the appearance of poorly digested blood, with hyperemia in the mucosa. Kidneys were very congested and there was a small volume of cloudy urine in the bladder.

All biological samples (blood, vitreous humor and urine) were collected at autopsy by the medical examiner and sent to INTCF. Later, non-biological samples (paraphernalia) were also submitted from the police unit to INTCF, ordered by the judge for a comprehensive toxicological screening.

2. EXPERIMENTAL

2.1. Chemical, reagents and samples

Ethyl acetate, methanol, dichloromethane, isopropanol, (HPLC-grade) and formic acid (purity 99%, LC-MS grade), were purchased from (Scharlab, Barcelona, Spain). Millipore water (mili-Q grade) and methanol and acetonitrile

(LC–MS grade) were obtained from (Panreac, Barcelona, Spain), ammonium formate (LC–MS Ultra) was supplied by Sigma-Aldrich (Saint Louis, MO, USA). Chem Elut CE 1003 columns (3 mL of column reservoir volume) were all supplied by (Agilent Technologies, Palo Alto, CA, USA).

Cyclopropyl-fentanyl, and crotonyl-fentanyl certified reference materials (CRMs) were purchased from Cayman (Ann Arbor, Michigan, USA). The fentanyl and d5-fentanyl internal standards were acquired from Cerilliant Corporation (Round Rock, TX, USA). Figure 1 shows the chemical structure of these fentanyls compounds.

Non-biological samples (paraphernalia as capsules and a thermo sealed dust bag) and biological samples (whole-blood, vitreous humor and urine) were submitted to the INTCF laboratory. All biological samples were stored at frozen until their analysis.

2.2. Samples treatment

Non-biological samples were prepared by weighting a certain amount of powder and dissolved in methanol (2 mg in 2 mL for GC-MS and 10 mg in 25 mL for HPLC-DAD).

Whole-blood, vitreous humor and urine were subjected to basic (pH=9) liquid/liquid extraction (LLE) with solid-support (Chem-elut, Agilent) and elution with dichloromethane–isopropanol (85:15) based in our routine methods [13].

3. APPARATUS AND METHODOLOGY

Regarding the systematic toxicological analysis (STA) for this type of cases, briefly, it is described as follows: Drug paraphernalia analyses were performed

using GC-MS and HPLC-DAD. The analyses of biological samples were initially performed in the whole-blood and the urine samples for ethanol and other volatiles using HS-GC-FID. In urine were screened eight different drug families (cocaine metabolite, methadone, opiates, amphetamines, cannabis metabolite, benzodiazepines, barbiturates and tricyclic antidepressants) by enzyme-immunoassay (CEDIA). Confirmative and quantitative analysis were performed by GC-MS, HPLC-DAD, LC-MS/MS and LC-HR-MS/MS (Orbitrap).

4. METHODS

GC-MS analysis, involved an Agilent gas chromatograph 7890A model equipped with a capillary column (30 m, 0.25 mm i.d., 0.25 µm thick film of 5 % phenylmethylsiloxane) and coupled with an Agilent MS/EI detector 5975C. 1 µL biological samples extracts were injected (1 µL) in splitless and selected-ion monitoring (SIM) and screening full scan (SCAN) using simultaneous detection modes also called (SCAN/SIM) sincrono [14]. Non-biological samples were screening injected in split mode into the Agilent system but only using SCAN detection mode. The GC conditions consisted of helium gas at constant pressure as carrier, and the injector and quadrupole temperatures at 230 ºC and 150 ºC, respectively. The column temperature was set to 100 ºC for 1 min, then 10 ºC / min up to 320 ºC, and finally 5 min in isothermally hold.

The standards were studied in a LC-HR-MS/MS (orbitrap) system. These standards (1uL) were injected into a ultra-high performance liquid chromatograph Vanquish Flex Binary UHPLC system, equipped with a Thermo Scientific Accucore phenyl-hexyl column (100 mm x 2.1 mm x 2.6 µm). The temperature was set at 40 ºC and coupled with an Orbitrap Q Exactive Focus

system (ThermoFisher, Hemel Hempstead, UK) for full scan (FS) ion monitoring, with data dependent acquisition (DDA) for fragmentation in MS/MS spectrometry. The mobile phases consisted of 2 mM aqueous ammonium formate plus 0.1 % formic acid at pH 3 (eluent A) with acetonitrile:methanol (50:50, v/v; 1 % water) plus 0.1 % formic acid at pH 3 (eluent B). An initial isocratic elution, followed by a gradient elution, added major percentage in eluent organic B, made at a flow rate set to 0.5 mL/ min. An electrospray ionization (H-ESI II) set in the positive ionization mode was performed. The FS ion monitoring settings were those reported by Hans H. Maurer [15], modifying the list of fragmentation acquisition (inclusion list) for different NPS and their metabolites.

Validation and quantification was performed by a LC-MS/MS (triple quadrupole mass spectrometer), with the aim of including cyclopropyl-fentanyl in a routine method, which currently includes more than 200 compounds (amphetamine and related compounds, cocaine, opioids, hallucinogens, cathinones, synthetic cannabinoids and other NPS, and therapeutic drugs). Briefly, 1µL aliquots of extracts were injected into a ultra-high performance liquid chromatograph Agilent Infinity 1290 II, flex quaternary UHPLC system, equipped with an Agilent InfinityLab Poroshell 120 EC-C18, column (100 mm x 2.1 mm x 1.9 µm), whose temperature was set at 55 ºC and coupled with a tandem spectrometry mass system (triple quadrupole mass spectrometer Agilent, 6420) for dynamic multiple reaction monitoring (dMRM). The mobile phases consisted of 5 mM aqueous ammonium formate plus 0.01 % formic acid at pH 3 (eluent A) with methanol and 0.01 % formic acid (eluent B) at a constant flow rate of 0.5 mL/min. The starting gradient conditions were 10 % B, and the increased ramp

were 15 % B over 0.5 min, 50 % B over 3 min, 95 % B by 6.20 min and holding it for 2.3 min. The system was then equilibrated back to initial conditions for 1.5 min, with a total run time of 10 min. The MS analyzed the compound using electrospray ionization under positive mode. The source parameters included a gas temperature 350 ºC, gas flow at 12 L/ min, nebulizer at 50 psi and capillary voltage at 2 kV. All data were processed using Agilent MassHunter Quantitative Analysis for QQQ (B.10.0.707.0). The three most abundant product ions of multiple reaction monitoring (MRM) transition were chosen for cyclopropyl-fentanyl and two other product ions for ISTD d5-fentanyl; the first for quantitation and the two others for ion ratio comparison as confirmation, see Table 1.

LC-MS/MS method validation

Method validation was performed for this case report. Different literature references were consulted [16-17] for the quantification of cyclopropyl-fentanyl by the LC-MS/MS method using d5-fentanyl as internal standard (IS) for this case report. Calibrators and quality controls (QCs) were prepared by fortifying blank whole-blood samples. The analytical parameters studied in this case were: calibration model, limit of detection (LOD), limit of quantification (LOQ), intra and inter day precision and accuracy, matrix effect with the drug-free only for whole-blood, selectivity and carryover. The selectivity of the method was demonstrated by analyzing ten different blank blood matrices from different real cases which had not detected this substance before. Blanks of the whole blood (central and peripheral) samples were extracted as described above.

The matrix effect was studied by fortifying working standards solutions with cyclopropyl-fentanyl and IS into ten sources of whole-blood, the same samples studied for selectivity of negative whole-bloods. This set (set-1) contained neat samples at low (1 ng/ mL) and high concentration (200 ng/ mL) compared with another set (set-2) of standards at same concentration (1 ng/ mL and 200 ng/ mL), matrix effect values should be within 20 % [17].

Calibration curves were plotted in the range of 1-200 ng/ mL in whole-blood, with six levels (1, 5, 10, 50, 100 and 200 ng / mL). Working standard solutions were gradually diluted with methanol to prepare 0.1 and 1 mg/ L of standard mixture solutions. The internal standard consisted in a solution of 0.1 mg/ L by dilution of 100 mg/ L of d5-fentanyl with methanol.

The calibration curves were constructed by plotting the concentration against the ratio of the peak area for each compound against the peak area of the IS (Area Compound / Area ISTD), with a weighing of $1/x^2$. LOD and LOQ were estimated from the calibrators, which equivalent to 3 and 10 times of signal to noise, respectively. The intra-day and inter-day accuracy and precision were determined by analyzing three different levels of QC, fortifying 1, 10, 200 ng in one milliliter of blank whole-blood, respectively. Triplicate analysis was performed for each QC everyday (n=3), in three different days (n=9). The criteria of acceptance for both the accuracy (%) and precision RSD (%), were within ≤ 20% [17].

Carryover studies determined by injecting a blank after the injection of the highest level of calibration (200 ng/ mL) and the next sample analyzed (blank) was not detected carryover, or the analytes of interest were < LOD.

Results and Discussion

Identification of cyclopropyl-fentanyl in the first fatality case reported in Spain.

When the reported case was received and initially studied in 2017, cyclopropyl-fentanyl was not a controlled substance in Spain. It was under risk assessment study and the European Monitoring Centre of Drugs and Drug Addiction (EMCDDA) was monitoring it as NPS due to its toxicity, even lethal in many cases, and because it had been detected in some countries [1]. At that time, the INTCF did not include it in the routine screening analysis and did not have the certified reference material (CRM) for its determination. Under these unfavorable circumstances, a first approach was performed to identify the presence of cyclopropyl-fentanyl in the studied case.

Initially, non-biological and biological samples were studied by GC-MS. Heroin metabolites, cocaine and metabolites, amphetamines and related substances, benzodiazepines and a fentanyl derivative were detected in all biological samples. From the different non-biological samples, a lethal mixture, consisting of heroin and a fentanyl analog was detected in one of the analyzed samples. The fentanyl analog was identified using GC-MS due to a high correlation (> 70 % in the matching library) that contain two fentanyls compounds in different libraries (SWGDRUG 3.4 and Cayman Library of electronic impact of GC-MS). Interestingly enough, they were two isobaric candidates (cyclopropyl-fentanyl and crotonyl-fentanyl), which had the same empirical formula (C_3H_5) in the radical alkyl chains of the amide group (structural isomers). Oddly, they both were presented a similar fragmentation pattern and could not be distinguished by MS detection due to their similar ions and relative abundance, compared

with library of spectrometry mass [even similar intensity, with the carbocation of the amide group C_4H_5O+ (m/z value 69) and their corresponding alkyl chains C_3H_5+ (m/z value 41)].

Due to these two fentanyl analogs presented different characteristic chromophore groups (see Figure 1), and based on literature [18], we confirmed two possible candidates (cyclopropyl-fentanyl and crotonyl-fentanyl) by GC-MS (SCAN mode) in non-biological samples (capsules and a heat-sealed powder bag). Then, when comparing the UV-Vis spectra of the fentanyl standard, with that of the non-biological samples, the cyclopropyl-fentanyl was identified (see Figure 2).

This first analytical approach based on the identification by MS of two fentanyl analogs, which were differentiated by their UV-Vis spectra, resulted in a positive identification for cyclopropyl-fentanyl. In the non-biological sample, the cyclopropyl-fenanyl was mixed together with heroin. The case was notified within the framework of the Spanish Early Warning System (SEAT) of NPS in 13th of April of 2018, which in turn is the Spanish focal point and member of the European Early Warning System (EWS). It was notified to EMCDDA on 30th of May of 2018, and the same day, the National Plan on Drugs (PNSD, Spain, https://pnsd.sanidad.gob.es) published a special “alert about derivatives of fentanyl in heroin users” [19]. Later, the valuable information provided by the UV-Vis spectra of cyclopropyl-fentanyl and crotonyl-fentanyl was published by Maher S. et al. [20]. As a result, the UV-Vis spectra information has a major significance for laboratories that do not have CRMs, due to their great utility and simplicity in the differentiation of those structural isomeric compounds.

Identification of fentanyl derivatives/metabolites by high-resolution mass spectrometry (LC-HR-MS/MS).

Due to the high potential of LC-HR-MS/MS, several objectives were pursued in this case: (i) to inspect if differentiation of the two-fentanyls isobaric compounds was possible; and (ii) to study the metabolites of the cyclopropyl-fentanyl. After about half a year of a complex process due to the illegalization of cyclopropyl-fentanyl, CRM standards of cyclopropyl-fentanyl and crotonyl-fentanyl were acquired by the INTCF. In order to test if the differentiation of the two-fentanyls isobaric compounds was possible, the study of both, CRM standards and a biological sample (urine) was performed by the LC-HR-MS/MS method (see the experimental section). The coincidence of the retention time (5.6 minutes) and the characteristic HR-MS/MS spectrum (same precursor [M-H$^+$] and ion fragments MS/MS, given in Table 2) of cyclopropyl-fentanyl and crotonyl-fentanyl did not allow their unequivocal identification by HR-MS/MS (see Figure 3).

As seen in Figure 3, it is not possible to distinguish those compounds neither by the precursor nor by the main generated fragments, with a similar abundance (see Table 2). This conclusion is also supported by literature [20],

High-resolution mass spectrometry technique (LC-HR-MS/MS) also allowed the study of the cyclopropyl-fentanyl metabolism in the urine sample with high sensitivity. In this case, the main metabolite of cyclopropyl-fentanyl (cyclopropyl-norfentanyl) [10] and its posterior methylation to (N-methyl cyclopropyl-

norfentanyl) were identified showing the characteristic HR-MS/MS spectra of Figure 4. Whereas the cyclopropyl-norfentanyl ($C_{16}H_{22}N_2O$) appeared at 4.22 minutes and at m/z = 259.1798 (error of -2.31 ppm from theoretical precursor), the N-methyl cyclopropyl-norfentanyl ($C_{15}H_{20}N_2O$) appeared at a retention time of 4.25 minutes and at m/z= 245.1640 (error of -2.04 ppm from theoretical precursor).

In this case, only two of the main fragments of cyclopropyl-fentanyl (3*, 4*, see figure 3) were found in these metabolites ($C_4H_5O^+$/69.0334) and ($C_{15}H_{18}NO$+/228.1383) for cyclopropyl-norfentanyl and N-methyl cyclopropyl-norfentanyl (see figure 4). However, if we compare the structures of other main ion fragments of parent drug (cyclopropyl-fentanyl), we can see more coincidences in both metabolites. The principal ion fragment structure (1*, see figure 3) of cyclopropyl-fentanyl ($C_{13}H_{18}N^+$/188.1430), is also found as the most abundant in the metabolites, but with a different formula composition due to its phenyl-ethyl loss during metabolization in a piperidine group ($C_5H_{10}N^+$/84.0813) for cyclopropyl-norfentanyl and its later N-methylation for N-methyl cyclopropyl-norfentanyl ($C_6H_{12}N^+$/98.0668) . The second more abundant ion fragment (2*) of cyclopropyl-fentanyl ($C_8H_9^+$/105.0704) is not present because of the above mentioned loss during the process of metabolization (phenyl-ethyl group). For adquisition and study of all common ion fragments, it was only applied the information routine method (normal energy NE, 35ev ± 50%). In addition, these two metabolites of cyclopropyl-fentanyl were in a proportion of 55 % (cyclopropyl-norfentanyl) and 16 % (N-methyl cyclopropyl-norfentanyl), when their corresponding abundances were expressed in absolute area of the

precursor compared to that obtained from the parent drug (cyclopropyl-fentanyl).

These identifications were done together with the study of its ion fragments (figure 4) and comparison with parent drug (figure 3), where therefore the main metabolite (cyclopropyl-norfentanyl) detected showed the same ion fragments, supported by literature [10]. But this identification is only as a mode of tentative in both cases, as the standards were not available.

Quantification of cyclopropyl-fentanyl by LC-MS/MS

For the determination of fentanyl derivatives, the CRM standards of cyclopropyl-fentanyl and crotonyl-fentanyl were incorporated to the systematic routine method (LC-MS/MS), which was validated for the quantification of fentanyl derivatives in whole-blood samples (see the experimental section). This method could be complemented by a method based on retention times, with CRM standards and the same column published by Lee et al. [21], which allow the separation of the two fentanyls.

The linearity obtained was in the range of 1-200 ng/ mL (n=6), with a linear calibration model (R^2>0.99), weighted $1/x^2$. LOD concentration was determined injecting decreasing analyte concentration in blood samples. In this case only the half of the first point of quantification 0.5 ng/ mL whereas the LOQ corresponded to the first point of calibration 1.0 ng/ mL.

The precision of the method was evaluated with spiked samples of negative blood samples at three concentration levels QC (1, 10, 200 ng/ mL). The results showed good interday RSD values, comprised among 10.7 % and 12.3 %. Accuracy of the method was also good, ranging from -5.9 % to 6.3 %, as shown

in Table 2.

The quantitative analysis of cyclopropyl-fentanyl in whole-blood revealed a concentration of 20.4 ng/ mL. This value is in the range of the values (median value) of this fentanyl derivative reported in other fatal cases in different countries. In Sweden, the median was 8.2 ng/ mL (n=73) [1]; in the United States, it was 11.8 ng/ mL (n=416) [22]; in the United Kingdom was 25.4 ng/ mL (n=3) [1] and in Italy 30.0 ng/ mL (n=42) [23]. As a consequence, the high concentration of cyclopropyl-fentanyl in this studied case led to the conclusion that the fentanyl analogue had a primary role as cause of death.

Conclusions

This case involves a violent death of presumably accidental etiology. The cause of death was due to poly-drug toxicity, and match with a preponderant role of a fentanyl analogue, specifically cyclopropyl-fentanyl.
Also other drugs of abuse that were detected: heroin, cocaine, amphetamine and others stimulants, as well as other therapeutics drugs. The necropsy findings and background allow us to establish a date of death, approximately in the afternoon/evening of the previous day.

The experience in the analysis of drugs by the INTCF personnel, and the handling and transverse knowledge of different instrumental techniques, together with the non-biological samples finally sent to the same laboratory, allowed a positive elucidation of cyclopropyl-fentanyl by two different analytical approaches. A first one, using GC-MS allowed us to focus in two isobarics fentanyls and to limit the possible substances presents and a posterior UV-Vis

spectra differentiation of the two structural isomers of fentanyl (cyclopropyl and crotonyl) due to the presence of an additional chromophore group in the case of crotonyl. The detection of cyclopropyl-fentanyl allowed communicating an alert to Spanish Early Warning System (in Spanish SEAT) and contributing to the surveillance and detection of this dangerous substance, mixed in doses of clandestine sale heroin.

Moreover the use of high-resolution instrumentation (LC-HR-MS/MS) assisted the identification of cyclopropyl-fentanyl and its metabolites with a high sensitivity. Sometimes these instruments can be helped to solve and identify certain structural isomers by UV-Vis; as happen in this case, especially when standards are not available. Two tentative metabolites were identified by the high-resolution technique: cyclopropyl-norfentanyl and N-methyl cyclopropyl-norfentanyl. Then, the incorporation of the CRM standards of cyclopropyl-fentanyl and crotonyl-fentanyl to the systematic routine method (LC-MS/MS) allowed the validation and quantification of cyclopropyl-fentanyl in whole-blood sample (20.4 ng/ mL). Finally, it should be noted that the availability of urine containing a high concentration of parent drugs and their metabolites, as well as the delivery of paraphernalia, were crucial to clarify this fatality involving common drug abuse, including cyclopropyl-fentanyl. As far as we know, this is the first fatal case involving cyclopropyl-fentanyl reported in Spain.

ACKNOWLEDGEMENTS

We would like to acknowledge Rocío González Lira for the English revision, Adrián López Fernández for the graphics assistance, and María Andreu Tena for Figure 2. C. García-Ruiz also thanks the project IUICP-2019/07 for financial support.

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figure captions

Figure 1. Chemical structure of the fentanyls studied in this work. The functional groups of interest in this study are lighter gray. Cyclopropyl and crotonyl aliphatic groups had the same empirical formula (C_3H_5) in the radical alkyl chains of the amide group.

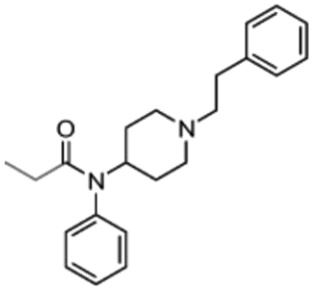
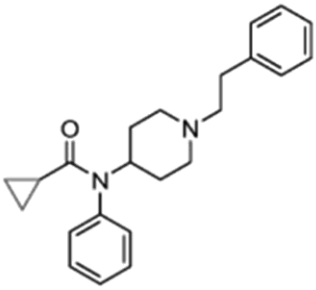
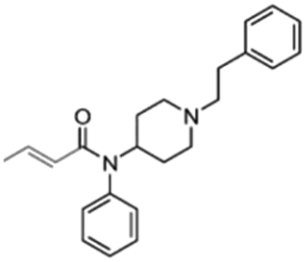
Fentanyl	Cyclopropyl-fentanyl	Crotonyl-fentanyl
		

Figure 2. UV-Vis spectra of fentanyl and cyclopropyl-fentanyl, which are similar, and crotonyl-fentanyl standards.

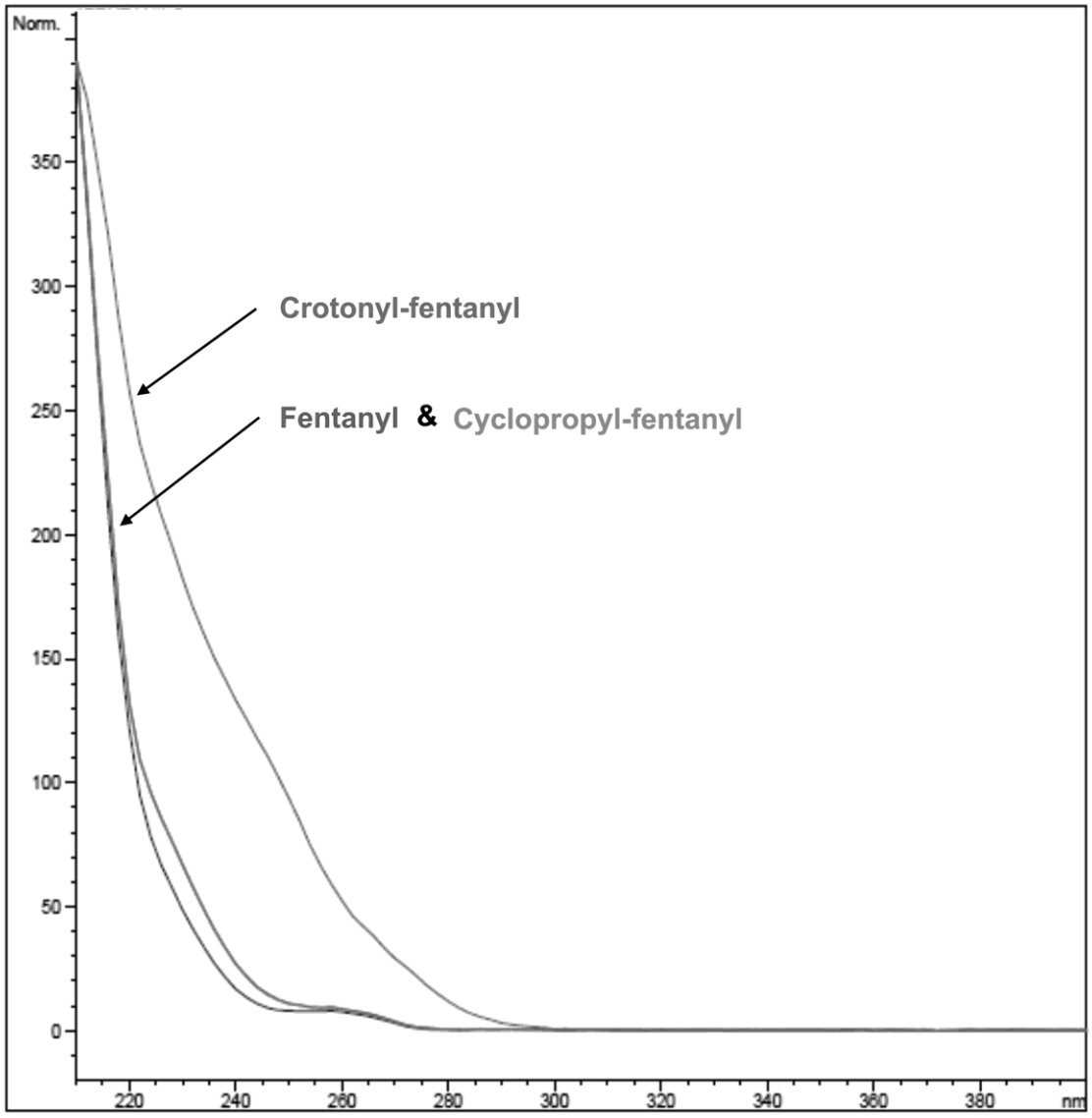


Figure 3. HRMS-MS spectra of a measured precursor ([M-H$^+$]/$C_{23}H_{28}N_2O^+$) for cyclopropyl-fentanyl and crotonyl-fentanyl CRMs and the biological sample (urine) from the decedent.

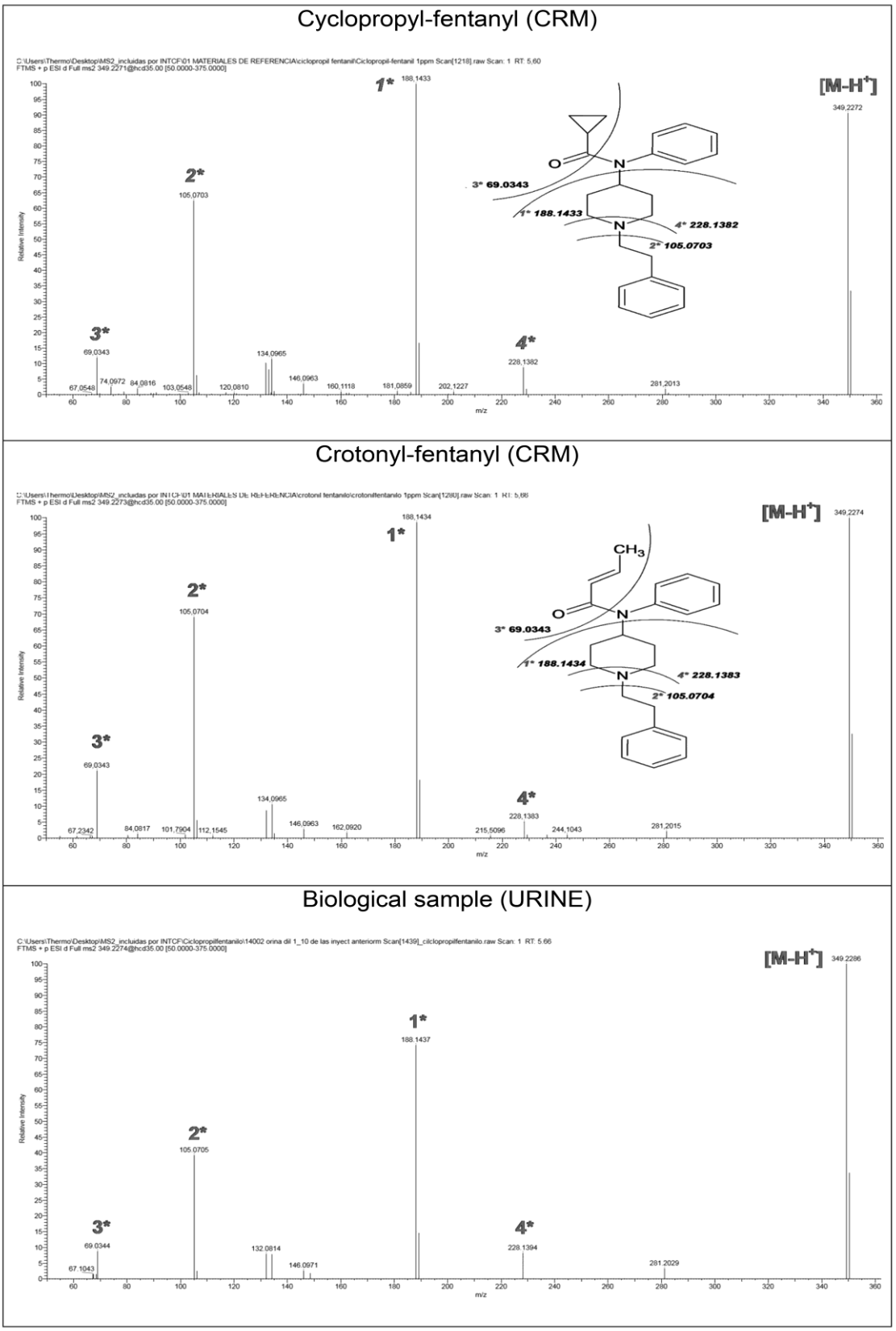


Figure 4. HRMS-MS spectra of main metabolites searched in a biological sample (urine).

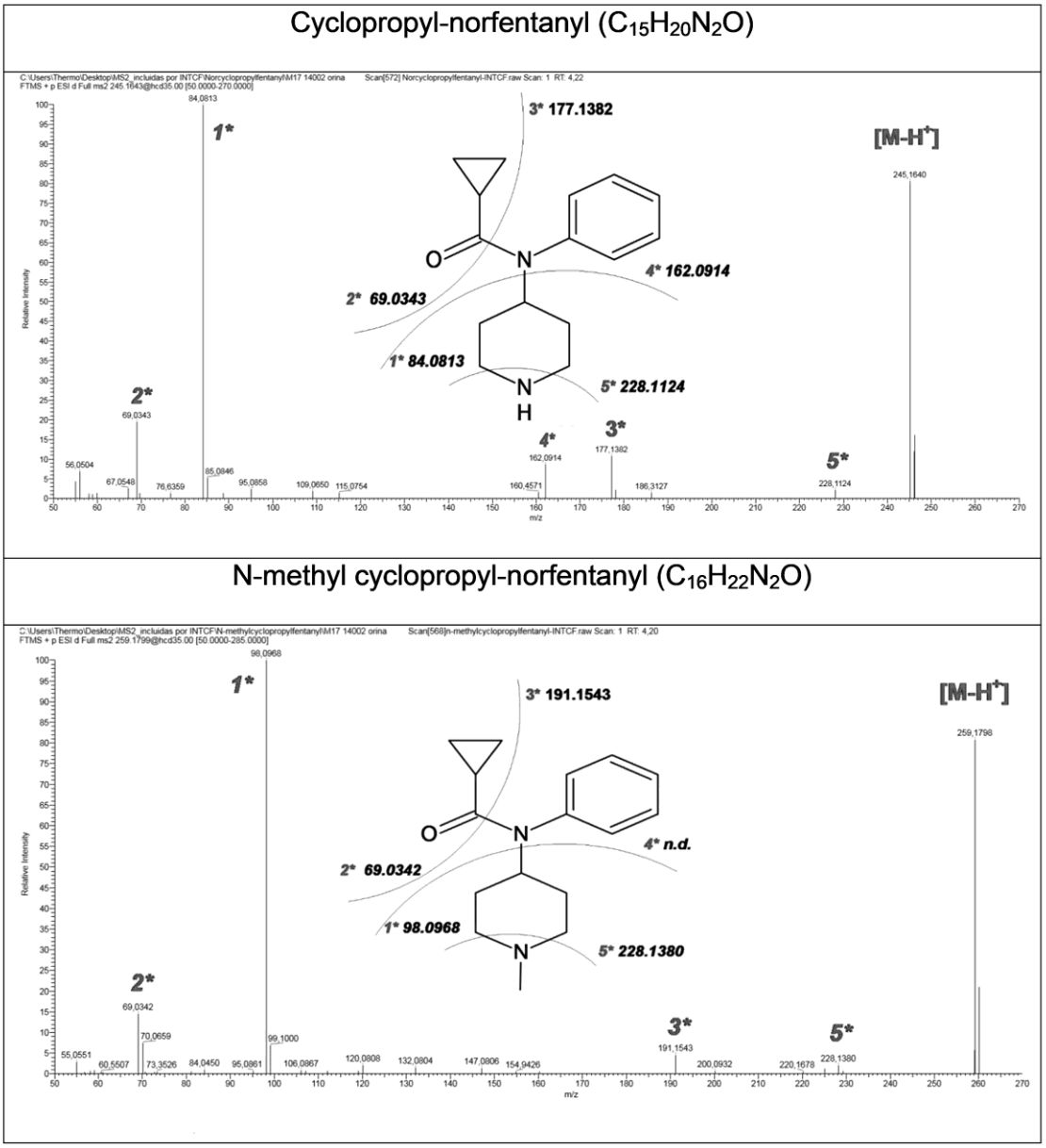


Table I. MRM Parameters for Cyclopropyl-Fentanyl and ISTD (Fentanyl-d_5)

Compound name	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (V)
Cyclopropyl-fentanyl	4.26	349.2	188.1	144	37
			105.1		25
			69.1		45
Fentanyl-d_5 (ISTD)	4.03	342.3	105.1	150	20
			188.1		40

Table II. Main Fragments of HRMS-MS for Cyclopropyl-Fentanyl, Crotonyl-Fentanyl and the Biological Sample (Urine) by LC–HRMS-MS

Code Figure-3	**Formula [M+H]+ Products**	**Theoretical [M+H]+ Products**	**Cyclopropyl-fentanyl [M+H]+ Products (ppm)**	**Crotonyl-fentanyl [M+H]+ Products (ppm)**	**Urine sample [M+H] + Products (ppm)**
1*	**$C_{13}H_{18}N^+$**	188.1430	188.1436 (3.19)	188.1434 (2.1)	188.1437 (3.72)
2*	**$C_8H_9^+$**	105.0704	105.0704 (0.0)	105.0704 (0.0)	105.0705 (0.95)
3*	**$C_4H_5O^+$**	69.0334	69.0344 (14.5)	69.0343 (13.0)	69.0342 (11.6)
4*	**$C_{15}H_{18}NO^+$**	228.1383	228.1384 (0.44)	228.1383 (0.0)	228.1384 (0.44)
[M-H$^+$]	**$C_{23}H_{28}N_2O^+$**	349.2274	349.2274 (0.0)	349.2274 (0.0)	349.2271 (-0.86)

Table III. Method Validation for Cyclopropyl-Fentanyl in Whole Blood by LC–MS-MS*

Analyte	Intraday (n=3)						Interday (n=9)						n=10		LOD	LOQ	R2	Weighting	Linearity
	Accuracy (bias%)			Precision (RSD %)			Accuracy (bias%)			Precision (RSD %)			Matrix effect CV(%)						
	1†	10†	200†	1†	10†	200†	1†	10†	200†	1†	10†	200†	1†	200†					
Cyclopropyl-fentanyl	1.1	−0.7	4.5	4.5	3.6	4.2	4.2	−5.9	6.3	11.8	12.3	10.7	−2%	3.8%	0.5*	1	>0.99	$1/x^2$	1–200*

* The internal standard was fentanyl-d_5.

† Each concentration for the method validation is expressed in ng/mL.