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## Title

Multiplex detection of 14 fentanyl analogues and U-47700 in biological samples: application to a panel of French hospitalized patients.

## Authors

Jean Jung<sup>1†</sup>, Allan Kolodziej<sup>1†</sup>, Elise Pape<sup>1,2</sup>, Michael Bisch<sup>3</sup>, Lucie Javot<sup>1</sup>, Valérie Gibaja<sup>1</sup>, Jean-Yves Jouzeau<sup>1,2</sup>, Julien Scala-Bertola<sup>1,2</sup>, Nicolas Gambier<sup>1,2,\*</sup>

1 Université de Lorraine, CHRU-Nancy, Department of Clinical Pharmacology and Toxicology, F-54000, Nancy, France.

2 Université de Lorraine, CNRS, IMoPA, F-54000 Nancy, France

3 Pôle Hospitalo-Universitaire du Grand Nancy, Centre Psychothérapique de Nancy, Département d'Addictologie, F-54520 Laxou, France

\* Author to whom correspondence should be addressed. *Email*: [n.gambier@chru-nancy.fr](mailto:n.gambier@chru-nancy.fr)

† Authors contributed equally to this work

1 **Title**

2 Multiplex detection of 14 fentanyl analogues and U-47700 in biological samples: application  
3 to a panel of French hospitalized patients.

4

5

6 **Abstract**

7  
8 Synthetic opioids (SO) associated with the recent alarming increase of deaths and  
9 intoxications in United States of America and Europe are not detected by the usual first-line  
10 opiates drug screening assays. We developed a liquid chromatography tandem mass  
11 spectrometry analytical method for the multiplex detection of 14 fentanyl analogues (2-  
12 furanylfentanyl, 4-ANPP, 4-methoxybutyrylfentanyl, acrylfentanyl, alfentanil, carfentanil,  
13 despropionyl-2-fluorofentanyl, fentanyl, methoxyacetylfentanyl, norfentanyl, ocfentanil,  
14 remifentanil, sufentanil and valerylfentanyl) and U-47700 in whole blood and urine samples.  
15 The method was validated according to the requirements of ISO 15189. A simple and fast  
16 liquid-liquid extraction (LLE) with De-Tox Tube-A was performed leading to better recovery  
17 of molecules in urine than in blood samples. Depending on the compound, the limits of  
18 detection (LODs) ranged from 0.01 to 0.10 ng/mL and from 0.02 to 0.05 ng/mL in whole  
19 blood and urine, respectively. Calibration curves were linear in the range 0.5-50.0 ng/mL and  
20 the limit of quantification (LOQ) ranged from 0.10 to 0.40 ng/mL in blood. Internal quality  
21 controls at 1 and 40 ng/mL showed intra-day and between-day precision and accuracy bias  
22 below 10% in urine and 15% in blood. The method was applied to the screening of 211 urine  
23 samples from patients admitted in emergency or addiction departments. The presence of legal  
24 fentanyl analogues in 5 urine samples was justified by their therapeutic use as analgesics.  
25 Only one patient was concerned by fentanyl misuse and addiction whereas no illegal SO was  
26 detected. This study is not in favor of a huge misuse of SO in the Lorraine region.

27

28

29 **Keywords:** fentanyl analogues; U-47700; synthetic opioids; misuse; HPLC-MS/MS

30

## 31 **1) Introduction**

32  
33 Fentanyl and its analogues are synthetic opioids (SO) (1)(2) classified as NPS “New  
34 psychoactive substances” (3)(4). Nowadays, more than 30 fentanyl analogues are globally  
35 identified as narcotics in the world (5), some of them, namely fentanyl, sufentanil, alfentanil  
36 and remifentanil being marketed for severe pain management (6).

37 However, the use of fentanyl analogues emerges as an international public health threat.  
38 Indeed, more than 72,000 people died from opioid overdose in 2017 in North America (7)(8)  
39 and fentanyl has become the first cause of overdose in the USA since 2018, exceeding those  
40 caused by heroin. The nonmedical use of fentanyl and its derivatives is also reported in  
41 Europe although at a lower frequency than in North America. Since the first misuse-related  
42 cases of fentanyl-related deaths reported in Sweden in 1997 (9), hundreds of people died from  
43 fentanyl overdose in Germany, Finland, United Kingdom, Greece (10)(11) and more  
44 generally across entire Europe (12). In France, some fentanyl-related deaths and intoxications  
45 were reported between 2010 and 2015 by the French Addictovigilance network (13) whereas  
46 illicitly manufactured fentanyl (IMF) were offending in few intoxications since 2016 (14)(15).  
47 One problem arising from the increased use of marketed or IMF and analogues is their  
48 inability to be detected by the first-line analytical methods used for the screening of opiates.  
49 As a consequence, their contribution to intoxications is likely underestimated due to both the  
50 lack of general knowledge regarding their possible use (especially if other drugs such as  
51 heroin, cocaine, amphetamines or benzodiazepines are already detected) and the technical  
52 challenge to detect them. To circumvent this limitation, several analytical methods have been  
53 developed to detect up to twenty-four IMF and analogues in biological fluids (16)(17). Whole  
54 blood is frequently used as a matrix, due to the search for IMF as the possible cause of deaths  
55 (18)(19)(20). However, urine is also a matrix of choice for documenting drug consumption in  
56 clinical or forensic toxicology due to the significant elimination of fentanyl analogues and  
57 metabolites in urine (16). Unconventional samples like vitreous humor or bile (21), hair (19)  
58 (22) or dried blood spot (23) have been proposed as alternate matrices. Mass spectrometry  
59 (MS) is the reference method to detect IMF and analogues with the coupling to liquid  
60 chromatography (LC) separation (18)(19)(20)(21)(23)(24)(25) being more frequently used  
61 than to gas chromatography (GC) (26). One plausible explanation could be the limitation of  
62 GC/MS to detect compounds such as carfentanil (27) although analytical performance can be  
63 comparable to LC-MS/MS for furanylfentanyl, ofentanil, acetylfentanyl and butyrylfentanyl  
64 (26).

65 In the present study, we developed a new sensitive and specific LC-MS/MS method to detect  
66 and quantify 14 fentanyl analogues and U-47700 in whole blood and urines. We aimed to  
67 provide a fast, simple and sensitive analytical tool for addictologists and emergency  
68 physicians to estimate and/or follow the consumption of these emerging narcotics in our  
69 eastern French region sharing frontiers with 3 European countries. We validated its suitability  
70 by performing a retrospective screening of urine or blood samples of patients admitted either  
71 in one emergency or the addiction departments of our university hospital between March and  
72 September 2019.

73  
74

## 75 **1. Materials and method**

76  
77  
78

### 77 **a. Chemicals and standards**

79 Fentanyl and its analogues were obtained as powder or solution from different suppliers: 4-  
80 methoxybutyrylfentanyl and valerylfentanyl from Alsachim (Illkirch, France), acrylfentanyl,  
81 despropionyl-2-fluorofentanyl and methoxyacetylfentanyl from Cayman chemical (Ann  
82 Arbor, USA), alfentanil, carfentanil, 2-furanylfentanyl, ocfentanil, remifentanil, sufentanil  
83 and U-47700 from Cerilliant (Round Rock, USA), 4-ANPP from Chiron (Trondheim,  
84 Norway), fentanyl and norfentanyl from LGC (Luckenwalde, Germany). Five deuterated  
85 internal standards were also supplied: carfentanil-D5 (Cerilliant, Round Rock, USA),  
86 fentanyl-D5 and norfentanyl-D5 (LGC, Luckenwalde, Germany), alfentanil-D3 and  
87 remifentanil-C6 (TRC, North York, Canada). Methanol and ammonium formate were  
88 purchased from Honeywell (Charlotte, USA) and Sigma-Aldrich (Saint-Louis, USA),  
89 respectively. Acetonitrile, formic acid and DE-TOX TUBE A™ were purchased from VWR  
90 (Fontenay-sous-bois, France) and Dyna-Tek Industries (Lenexa, Kansas, USA), respectively.  
91 Standard solutions (0.1 mg/mL and 1 mg/mL) were prepared by diluting the drugs in  
92 methanol, according to the manufacturer's recommendations.

93 Blank human blood and urine were obtained from Ingen Biosciences (Chilly-Mazarin,  
94 France).

95  
96

### 97 **b. Instrumentation**

98  
99 The study was performed with an ultra-high-performance liquid chromatography Nexera XR®  
100 (Shimadzu, Japan) linked to a 3200 QTRAP triple quadrupole mass spectrometer (Life

101 Sciences Holdings France SAS SCIEX, Villebon sur Yvette, France). Analyst<sup>®</sup> software  
102 (version 1.6) was used for quantification.

103 Chromatography was performed using a Restek Pinnacle<sup>®</sup> DB PFP Propyl column (2.1 $\mu$ m  
104 particle size, 2.1 mm x 50 mm) maintained at 50°C in a thermostatically controlled oven. The  
105 following gradient of elution was applied using 5 mmol/L ammonium formate + 0.1% formic  
106 acid (mobile phase A) and acetonitrile (mobile phase B): 0.00-2.00 min: 5 - 20% B; 2.00-6.00  
107 min: 20 - 90% B; 6.00-8.00 min: 90 - 100% B; 8.00 - 8.20 min: 100 - 90% B; 8.20-8.40 min:  
108 90 - 5% B; 8.40-13.0 min: 5% B. The flow rate was 0.5 ml/min. Retention times of the  
109 molecules are shown in Table 1.

110 The column effluent was analyzed using a Turbo V ionization source equipped with  
111 electrospray ionization (ESI+) probe. Sources conditions were the following: spray voltage at  
112 5.5 kV; gas 1: nitrogen, 40 psi; gas 2: nitrogen, 60 psi; ion source temperature 500°C; curtain  
113 gas: nitrogen, 30 psi. MS parameter settings were optimized by infusing neat standards  
114 individually in methanol. Cone voltage (CV), collision energy (CE) and cell exit parameter  
115 (CXP) were adjusted to maximize the signal for the 2 most abundant product ion of each drug  
116 (Table 1). The instrument was operated in dynamic SRM (Selective Reaction Monitoring)  
117 mode.

118

119

### 120 **c. Method validation**

121

122 The method was validated according to the ISO15189 guidelines (28)(29). For that goal, the  
123 following parameters were checked: recovery, selectivity, carryover, matrix effect (ME), limit  
124 of detection (LOD), precision (repeatability and intermediate precision) and stability for  
125 identification in both whole blood and urine matrices. Analysis of accuracy, calibration model  
126 and limit of quantification (LOQ) were performed additionally to allow quantitative analysis  
127 in whole blood samples.

128

#### 129 **i. Recovery**

130 Samples were submitted to liquid-liquid extraction (LLE) procedure. Briefly, 25  $\mu$ L of IS  
131 (100 ng/mL) mixture of carfentanil-D5, fentanyl-D5, norfentanyl-D5, alfentanil-D3 and  
132 remifentanil-C6 were added to 500  $\mu$ L of blank whole blood or urine aliquots. After addition  
133 of 2.7 mL deionized water, the mixture was transferred into DE-TOX TUBE A<sup>™</sup> (DTI),

134 which allows extraction of basic and neutral drugs at pH 9.0 using a patented mixture of  
135 heptane/isopropyl alcohol/dichloromethane. After agitation on a rotating mechanism for 5  
136 min and centrifugation at 2.500 g for 5 min, the organic layer was gently evaporated to  
137 dryness under a nitrogen stream then reconstituted with 200  $\mu$ L of mobile phase A. A volume  
138 of 20  $\mu$ L was subsequently injected into the LC-MS/MS system. The recovery was calculated  
139 as the ratio of the peak areas measured post extraction over those obtained without extraction  
140 on blood and urine samples spiked at 1 ng/mL (low level) or 40 ng/mL (high level). Recovery  
141 was expressed as mean percentage of 5 values for both levels.

142

143 ii. Matrix effect (ME)

144 ME was determined using the experimental design proposed by Matuszewski *et al.* (30). ME  
145 was determined by comparing the ratio of the mean peak area obtained for five different blank  
146 urine and whole blood samples fortified with all analytes at 1 ng/mL (low level) or 40 ng/mL  
147 (high level) after extraction to the mean peak area obtained with pure solutions of the same  
148 analytes. Matrix effect was expressed as mean percentage for both levels.

149

150 iii. Selectivity and carryover

151 Selectivity was assessed by analyzing six blank urine samples and six blank blood samples to  
152 check for interfering compounds. To evaluate carryover, an extracted blank sample was  
153 analyzed immediately after running three replicates of the highest calibration standard (50  
154 ng/mL).

155

156 iv. Linearity

157 Six calibration standards including a blank sample and five spiked concentrations over a  
158 range from 0.5 to 50 ng/mL were analyzed over a six-day period. The peak area ratio of  
159 analyte over corresponding internal standard (IS) versus analyte concentration was plotted for  
160 each molecule. Linearity was determined by analyzing and processing calibrators with 1/x  
161 weighting.

162

163 v. Limits of detection (LOD) and of quantification (LOQ)

164 The LODs and LOQs were determined by spiking blank urine and whole blood samples  
165 which were then serially diluted. A signal-to-noise (S/N) ratio greater than 3 standard  
166 deviations defined the LOD as the lowest concentration. The LOQ was defined as the lowest



167 concentration of the linearity for which accuracy between 80 and 120% (bias < 20%) and  
168 precision (coefficient of variation, CV) of  $\pm 20\%$  or less were obtained over six  
169 measurements.

170

171 vi. Precision and accuracy

172 Precision and accuracy were measured on spiked blood samples and urine samples, used as  
173 internal quality control (QC), at two concentration levels: low (1 ng/mL) and high (40  
174 ng/mL). Both concentration levels were analyzed in 6 replicates.

175

176

#### 177 **d. Sample preparation and storage**

178

179 Blood and urine samples were submitted to the LLE procedure described above. Aliquots of  
180 500 $\mu$ L of whole blood or urine were used. Urine and blood samples were frozen within 24h  
181 after collection and stored at  $-20^{\circ}\text{C}$  until synthetic opioids (SO) screening by mass  
182 spectrometry.

183 Influence of storage conditions on the detection of fentanyl analogues and U-47700 was  
184 studied by measuring repeatedly analytes levels over a 30 days period. Compounds were  
185 analyzed at two concentrations (1 ng/mL and 40 ng/mL) following two different storage  
186 conditions ( $+4^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$ ).

187

188

#### 189 **e. Patients population**

190

191 Using the presently described method, we performed a retrospective study on samples of  
192 patients admitted in the Addiction (AD) or Emergency Departments (ED) of our University  
193 Hospital from March to September 2019. ED included the intensive care unit and the  
194 emergency medical services. All patients underwent urine narcotics screening (UNS) by an  
195 enzyme immunoassay (EIA) which included the detection of cocaine (COC), opiates (OPI),  
196 cannabis (THC), amphetamine/methamphetamine (AMPH) and related compounds by cross-  
197 reactivity. The immunoassay screens were performed on a Beckman AU5800 chemistry  
198 analyzer using Syva Emit II Plus reagents (Siemens Healthcare Diagnostics; Newark, DE).  
199 EIA results were reported as negative or positive based on whether the detected level  
200 exceeded a specific threshold for a given substance. The compounds used for calibration in

201 the UNS were benzoylecgonine for COC (cut-off level 150 ng/mL), morphine for OPI (cut-  
202 off level 300 ng/mL), 11-nor-delta<sup>9</sup>-THC-9-COOH for THC (cut-off level 20 ng/mL) and d-  
203 Metamphetamine for AMPH (cut-off level 300 ng/mL).

204 This study was approved by the local research ethics committee (request N°354).

205

206

## 207 **2. Results and Discussion**

208

### 209 **a. Method validation**

210

211 The analytical method was validated to detect (urine) and quantify (whole blood) 14 fentanyl  
212 analogues and U-47700. Additional compounds failed unfortunately to be provided by the  
213 suppliers. The fifteen compounds are separated in a single run of 13 min. A representative  
214 chromatogram of a urine sample spiked with 5 ng/mL of each analyte is shown in Figure 1.  
215 Almost all analytical methods described in the scientific and medical literature use mass  
216 spectrometry, especially in tandem with coupling to LC, for specificity and sensitivity reasons  
217 (5)(16)(17)(26)(31). The validation data for the determination of SO in whole blood and urine  
218 samples are summarized in Table 1 and 2, respectively.

219 For the different compounds, the matrix effect ranged from 71% to 118% and from 74% to  
220 121% for whole blood and urines, respectively (Tables 2 and 3). The matrix effect was in the  
221 same range for most molecules except for furanylfentanyl and valerylfentanyl which were  
222 more easily detected from urines than from whole blood. These values are in the range of the  
223 data reported previously for multiplex detection methods (18)(20)(23)(26) and confirms the  
224 higher impact of the matrix on low analytes concentration. The lowest value observed for  
225 valerylfentanyl in blood samples remains unexplained but this molecule is one of the less  
226 frequently searched amongst fentanyl analogues (20)(22)(23)(24) and is rarely found in case  
227 of intoxications and/or during post-mortem analysis (18)(19)(32). The recovery was  
228 satisfactory for all compounds in urines (over 58%) but remained below 39% in blood  
229 samples for valerylfentanyl and sufentanil. Such result may be due to the LLE methodology  
230 used which is less efficient than cleaning-up methods such solid-phase extraction (18)(20)(32)  
231 or microextraction with packed sorbent (MEPS) (33) to concentrate analytes (27). However,  
232 as simple precipitation methods (24)(34) our LLE protocol allows a fast, simple and cheap  
233 extraction of fentanyl analogues (19)(35) from only 500µL of sample. The 13 min separation  
234 is consistent with previously published methods (18)(20)(34) and suitable for routine analysis,

235 even if shorter runs have been logically reported using ultra-high performance liquid  
236 chromatography (UPLC) systems (19)(22)(32).

237 Using five hundred microliters of biological samples, the LODs ranged from 0.02 to 0.05  
238 ng/mL and 0.01 to 0.10 ng/mL in urines and blood, respectively. These values are lower than  
239 previously published data in urines (33)(35) and in the range of those reported in blood  
240 (18)(32)(35) with the exception of the extremely low levels (0.7 to 2 ng/L) reported recently  
241 for a UPLC-MS/MS method (19). In our experimental conditions, the LOQs values ranged  
242 from 0.1 to 0.4 ng/mL for whole blood samples. Once again, our data are in the range of  
243 previously reported methods (18)(32)(35). Of particular interest is the ability of any method to  
244 detect and/or quantify compounds in the context of intoxication or autopsy (27). In published  
245 post-mortem blood analysis, 4-ANPP (18)(19)(32), furanylfentanyl (18)(19)(20)(32),  
246 methoxyacetylfentanyl (19)(20), fentanyl (18)(19)(24), norfentanyl (18)(19), acrylfentanyl  
247 (20)(32), carfentanil (18)(24)(32) and U-47700 (20)(32) were amongst the most frequently  
248 retrieved molecules. When provided, the mean or median blood concentrations were found  
249 above 2.5 ng/mL (18)(19)(20)(24)(32), excepted for carfentanil and acrylfentanyl which were  
250 in the range 0.24 to 0.86 ng/mL (24)(32) and at 1.86 ng/mL (32), respectively. For all these  
251 molecules, we determine LODs ranging from 0.01 to 0.1 ng/mL and LOQs ranging from 0.10  
252 to 0.40 ng/mL (0.20 for carfentanil). This means that our method is enough sensitive to detect  
253 them adequately in biological samples (36) and even to quantify them with an acceptable  
254 accuracy.

255 Except for remifentanil which was only stable for 5 days at +4°C, stability testing showed  
256 urine concentrations within the range of 80-120% for all the compounds after 30 days of  
257 storage at +4°C and -20°C, whatever analytes concentration (data not shown). In whole blood  
258 samples, remifentanil was stable at -20°C but very unstable during storage at +4°C. Indeed, a  
259 significant decrease of its concentration (> 50%) was observed after 1 day, which is consistent  
260 with a former study having shown a 20% decrease after 5 hours at +4°C in samples without  
261 acidification (37). Concerning alfentanil, methoxyacetylfentanyl, norfentanyl, ofentanil and  
262 U-47700, whole blood concentrations remained stable after 30 days whatever storage  
263 conditions. At both low and high concentrations, other fentanyl analogues were stable for at  
264 least 2 days at +4°C and 8 days at -20°C, respectively. Taken together, these data support that  
265 urine samples can be analyzed for fentanyl analogues until 5 days after collection if stored  
266 refrigerated but that blood samples must be frozen at -20°C, as soon as possible after  
267 sampling, to avoid analytes degradation.

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269

270

## **b. Retrospective study**

271

272 Population characteristics are summarized in Table 4. Within the 6-month period of the study,  
273 1 whole blood sample and 211 urine samples were analyzed by LC-MS/MS for the presence  
274 of 15 SO. Among these samples, 66.5 % (n=141) and 33.5 % (n=71) came from Emergency  
275 (ED) and Addiction (AD) Departments, respectively. The number of males (49%) and  
276 females (51%) was roughly equal and mean age was  $31.5 \pm 16.6$  years. Fifteen infants (7.1%)  
277 were hospitalized in the neonatal intensive care unit for close monitoring of neonatal  
278 abstinence syndrome (NAS). All urine samples from the AD were from patients aged over 18  
279 years.

280 211 individual screening immunoassays for narcotics were performed with an overall screen  
281 positive rate of 50.2% (n=106). There was a high percent of positive screens for OPI (31.3%),  
282 THC (17.1%) and COC (19.9%) but a very low one for AMPH (1.9%). Most of the 66  
283 positive urine samples screened for opiates were found on heroin addicts, a population of  
284 interest for the detection of possible SO use. SO are in fact consumed voluntarily either as  
285 opioid substitutes or unwittingly as heroin, cocaine or even drugs like oxycodone adulterants  
286 at very low doses, in order to reduce production costs of these substances and to potentiate  
287 their effects (24)(26)(38)(39)(40)(41)(42). However, SO usually fail to be detected by  
288 immunoassays which are based on the cross-reactivity towards the morphinane backbone of  
289 the natural opiates (43).

290 In our retrospective study, there is a quite low percentage (2.8%) of patients positive for SO  
291 identification (Table 5). As expected from an analytical point of view, all cases that were  
292 positive for NSO were negative for opiates immunoassay screening. The total number of SO  
293 exposures is thus likely underreported and underrated, because routine toxicology screens fail  
294 to systematically detect these compounds (44). As proposed by Seither and Reid (45), this  
295 result demonstrates the need to complete routine toxicology analysis with a sensitive  
296 methodology that can detect synthetic opioids in situations where opioids use may be  
297 implicated.

298 In the present study, all SO detected were related to pharmaceutical fentanyls. In 4 cases  
299 sufentanil was the detected drug, while fentanyl and its metabolite norfentanil were identified  
300 in 3 other cases. Among the 211 patients screened in our study, no consumption of illicit  
301 fentanyl or fentanyl analogues has been detected. This suggests that our eastern French  
302 region, despite its close vicinity with transit lanes like Belgium, Luxembourg or Germany,

303 might be preserved to date from the opioid crisis observed in North America. This result is in  
304 accordance with the recent observations of the French Addictovigilance Network which  
305 recorded only 16 intoxications related to the consumption of non-medicated fentanyl (n=8),  
306 namely ocfentanil (5 overdoses including 2 deaths), carfentanil (n=2) and butyrylfentanyl  
307 (n=1) in our country between 2012 and 2017 (46).

308 In our study, five of the six positive cases were admitted into intensive care and were  
309 administered sedation, explaining the presence of pharmaceutical fentanyl or analogues in  
310 their biological samples, whereas no consumption of illicit fentanyl was detected. These data  
311 are encouraging about the apparent low-rate consumption of SO even if our study has been  
312 performed on a limited number of addicted patients. However, one case of medical  
313 prescription-induced addiction to fentanyl was observed (case 6 in Table 5). The medical  
314 story of this patient began in 2012 when he was hospitalized for chronic pain of unknown  
315 origin and was given ketamine and morphine to relieve it. However, progressive failure of  
316 pain relief led prescriber to start a fentanyl-based treatment in early 2018 using intra-nasal and  
317 transdermal routes which appeared efficient. Since then, the patient was suspected to develop  
318 addiction to opiates due to withdrawal syndrome features (nausea, vomiting, abdominal  
319 chronic pain) recorded during a consultation 6 months later. A substitution therapy with  
320 methadone was initiated and the patient was supported by hospital addiction department  
321 which carried out toxicological screening. The patient claimed that he stopped taking fentanyl  
322 three weeks ago but his urines were still positive for fentanyl and norfentanyl, which is not  
323 consistent with the pharmacokinetics of this molecule. He refused any further urinary tests  
324 and ask for a blood screening, including SO which returned negative results two weeks later.  
325 One may underline that one limitation of this retrospective study is that it did not address  
326 patients suffering from rheumatic or oncologic diseases and consulting for pain management.  
327 These patients can also be exposed to fentanyl or sufentanil and are not routinely controlled  
328 by toxicology screening. This matter has to be put in regards to the opioid crisis in North  
329 America which found its origin in an evolution from the consumption of opiates to those of  
330 licit then illicit fentanyl analogues (8)(47)(48)(49). Fentanyl analogues are, in fact, potent,  
331 short-acting synthetic opioids commonly prescribed for pain relief (1), usually by transdermal  
332 delivery system (patch), but also by transmucosal (sublingual, buccal, nasal) and intravenous  
333 route of administration (2)(3) in rheumatology, cancerology or neurology. The intra-nasal  
334 route is fast-acting but may also be more addictive, as has been observed in our retrospective  
335 NSA screening.

336

337

### 338 **3. Conclusion**

339

340 We developed a simple and routine LC-MS/MS method for the analysis of 14 fentanyl  
341 analogues and U-47700 in urines and whole blood. The method was validated according to  
342 the requirements of ISO 15189 and successfully applied to the screening of clinical samples.  
343 Amongst 211 urine samples tested, 6 were found positive to pharmaceutical fentanyl  
344 analogues. Neither illicitly manufactured fentanyl analogues, nor heroin or cocaine  
345 adulteration was observed. The presence of fentanyl analogues in urine samples was the result  
346 of a medical prescription with one case of misuse. Although the emerging use of these SO  
347 seems more limited in Europe than in North America, a routine toxicological analysis with a  
348 sensitive methodology able to detect them in biological samples should be used  
349 systematically. Indeed, the consumption of fentanyl analogues is likely underestimated due to  
350 the analytical limitations of first-line opiates screening methods, all the more that patients  
351 suffering from rheumatic or oncologic diseases are also exposed. The method is sensitive  
352 enough to be usable on biological samples in postmortem toxicological casework.

353

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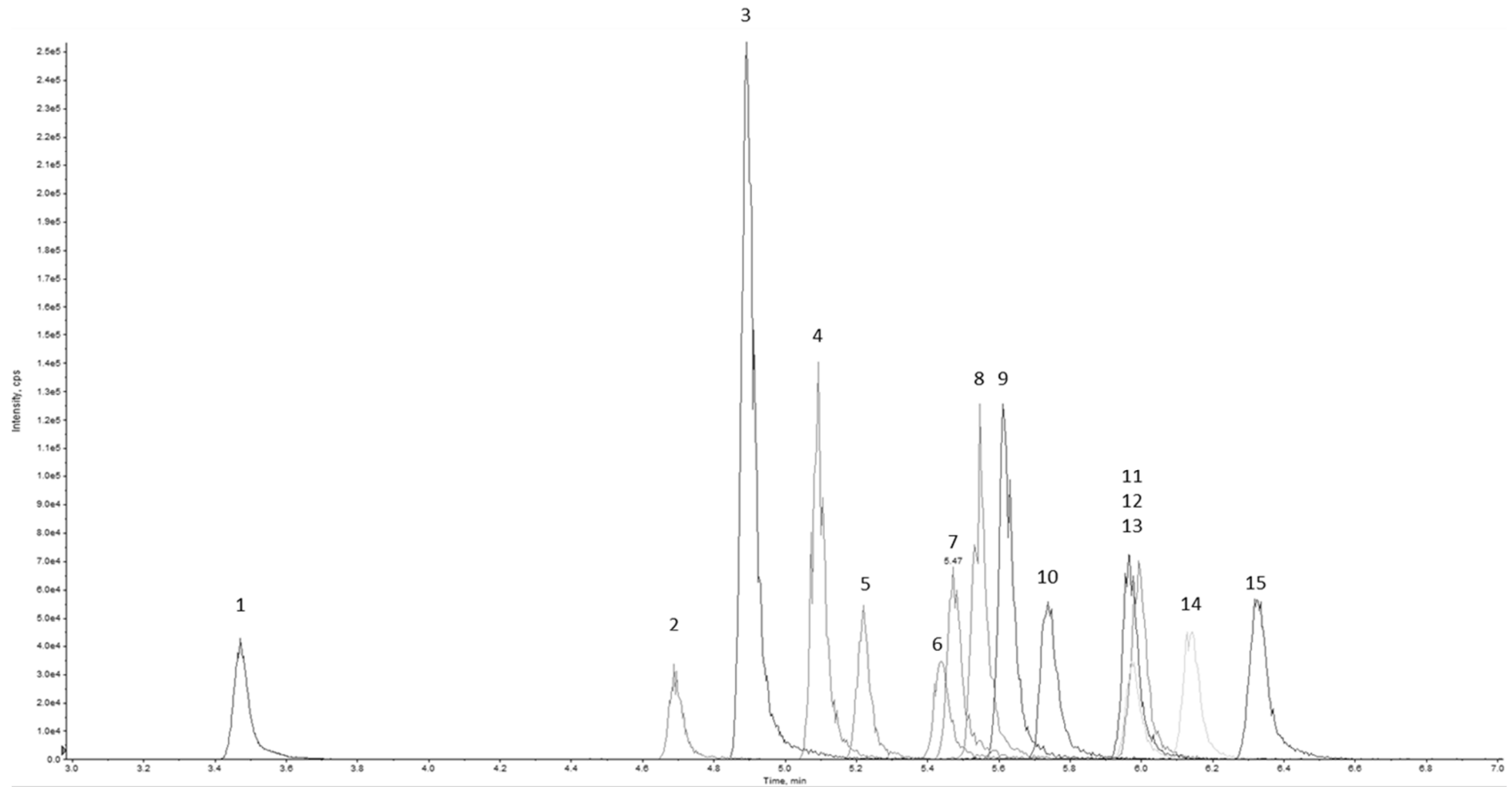
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**Figure 1.** LC-MS/MS chromatogram of a whole blood sample spiked with fentanyl and its analogs at 5ng/ml. **1:** Norfentanyl (RT=3.49). **2:** Remifentanyl (RT=4.70). **3:** Methoxyacetylfentanyl (RT=4.92). **4:** Ocfentanil (RT=5.11). **5:** Alfentanil (RT=5.25). **6:** U-47700 (RT=4.70). **7:** Acrylfentanyl (RT=5.47). **8:** Fentanyl (RT=5.52). **9:** Furanylfentanyl (RT=5.65). **10:** 4-ANPP (RT=5.77). **11:** 4-méthoxybutyrfentanyl (RT=6.01). **12:** Carfentanil (RT=6.01). **13:** Despropionyl-2-fluorofentanyl (RT=6.03). **14:** Sufentanil (RT=6.18). **15:** Valeryfentanyl (RT=6.38)

**Table 1.** Selective reactions monitoring (SRM) transitions and retention times for fentanyl analogues and U-47700 and corresponding internal standards (IS).

Compound	IS	Cone Voltage (V)	Q1 mass (m/z)	Quantification transition		Confirmation transition		RT (min)
				Q3 mass (m/z)	CE (eV)	Q3 mass (m/z)	CE (eV)	
4-methoxybutyrylfentanyl	Fentanyl-D5	56	381.2	188.1	33	105.1	53	6.01
Acrylfentanyl	Fentanyl-D5	51	335.1	188.1	29	105.1	47	5.47
Alfentanil	Alfentanil-D3	46	417.2	268.1	27	165.2	45	5.25
Carfentanil	Carfentanil-D5	46	395.2	335.1	27	113.1	41	6.01
Despropionyl-2-fluorofentanyl	Fentanyl-D5	41	299.1	188.2	25	105.1	39	6.03
4-ANPP	Fentanyl-D5	41	281.1	188.2	23	105.0	51	5.77
Fentanyl	Fentanyl-D5	56	337.1	188.1	31	105.2	47	5.52
Furanylfentanyl	Fentanyl-D5	66	375.1	188.2	29	105.1	51	5.65
Methoxyacetylfentanyl	Fentanyl-D5	51	353.2	188.1	29	105.2	49	4.92
Norfentanyl	Norfentanyl-D5	51	233.2	84.1	25	56.1	39	3.49
Ocfentanil	Fentanyl-D5	56	371.2	188.1	31	105.2	49	5.11
Remifentanil	Remifentanil-C6	41	377.1	116.1	37	113.1	41	4.70
Sufentanil	Fentanyl-D5	46	387.1	111.1	47	238.1	27	6.18
U-47700	Fentanyl-D5	41	329.0	284.1	23	173.0	41	4.70
Valerylfentanyl	Fentanyl-D5	56	365.2	188.1	31	105.1	53	6.38
Alfentanil-D3	/	41	420.2	165.1	45	/	/	5.25
Carfentanil-D5	/	46	400.2	105.2	59	/	/	6.01
Fentanyl-D5	/	51	342.2	105.0	49	/	/	5.52
Norfentanyl-D5	/	41	238.1	84.2	25	/	/	3.49
Remifentanil-C6	/	41	383.1	113.1	41	/	/	4.70

V: cone voltage in Volts ; CE: collision energy; RT: retention time; D: deuterium; C: carbon

**Table 2.** Validation parameters for fentanyl analogues and U-47700 in whole blood.

Substance	Accuracy (% bias)				Precision (% bias)				LOQ (ng/mL)	LOD (ng/mL)	Matrix effect		Recovery	
	Within-day		Between-day		Within-day		Between-day				(%)	(%)	(%)	(%)
	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High		
4-methoxybutyrylfentanyl	10.5	0.7	1.0	3.2	6.1	3.80	3.2	14.6	0.30	0.05	101	118	56	53
Acrylfentanyl	1.4	1.2	5.3	1.7	6.7	4.2	8.9	8.6	0.40	0.01	99	105	63	52
Alfentanil	0.7	2.5	4.3	1.6	4.3	4.2	6.6	4.5	0.10	0.05	90	103	75	65
Carfentanil	5.2	1.5	7.3	2.2	5.9	3.1	8.0	4.6	0.20	0.10	89	95	61	57
Despropionyl-2-fluorofentanyl	4.0	3.0	1.0	2.8	8.7	4.5	3.8	11.4	0.30	0.05	87	118	61	58
4-ANPP	12.3	1.9	0.5	0.1	5.7	4.8	10.3	9.8	0.20	0.10	87	99	55	50
Fentanyl	7.5	6.4	1.7	6.8	4.9	3.2	7.4	3.0	0.10	0.01	91	107	61	62
Furanylfentanyl	3.3	3.0	1.3	4.0	5.0	4.3	3.5	5.9	0.20	0.05	71	94	71	60
Methoxyacetylfentanyl	17.5	3.9	0.8	7.1	8.8	3.9	6.1	13.3	0.10	0.03	109	114	78	77
Norfentanyl	6.6	3.3	4.7	3.0	1.7	1.9	6.1	2.1	0.10	0.01	92	91	71	67
Ocfentanil	5.7	5.6	1.3	7.6	5.8	3.6	4.1	9.1	0.10	0.01	108	114	73	55
Remifentanil	2.1	2.7	2.3	7.2	5.3	3.4	6.0	8.3	0.10	0.05	92	95	65	66
Sufentanil	11.7	5.5	1.5	5.6	6.7	4.0	3.0	11.1	0.40	0.10	97	106	47	33
U-47700	8.3	4.7	3.2	9.5	7.3	4.3	8.0	9.1	0.40	0.05	97	113	67	56
Valerylfentanyl	9.8	1.4	3.0	5.4	4.8	4.4	3.7	5.4	0.20	0.03	69	83	49	39

**LOQ:** limit of quantification; **LOD:** limit of detection; **Low:** internal quality control 1 ng/mL; **High:** internal quality control 40 ng/mL

**Table 3.** Validation parameters for fentanyl analogues and U-47700 in urine.

Substance	Accuracy (% bias)				Precision (% bias)				LOD (ng/mL)	Matrix effect		Recovery	
	Within-day		Between-day		Within-day		Between-day			(%)	(%)	(%)	(%)
	Low	High	Low	High	Low	High	Low	High		Low	High	Low	High
4-methoxybutyrylfentanyl	0.1	4.3	0.7	5.5	3.6	6.2	8.2	6.4	0.02	95	110	68	65
Acrylfentanyl	2.7	1.7	0.1	2.1	3.6	8.6	9.5	6.8	0.02	109	104	58	65
Alfentanil	1.0	4.5	4.5	3.8	5.7	3.8	5.0	8.3	0.02	94	91	72	73
Carfentanil	0.5	2.6	2.3	3.1	3.4	4.5	3.5	7.3	0.02	96	101	66	66
Despropionyl-2-fluorofentanyl	2.1	0.5	2.8	6.8	7.0	6.5	2.3	8.6	0.02	98	102	71	72
4-ANPP	5.0	0.3	5.6	2.5	2.6	6.4	9.8	9.7	0.03	87	98	80	68
Fentanyl	0.7	1.0	4.2	4.2	5.9	3.0	4.6	7.7	0.02	89	104	70	67
Furanylfentanyl	1.9	1.3	0.5	4.3	4.6	6.8	6.6	8.2	0.02	89	106	72	68
Methoxyacetylfentanyl	3.9	2.2	0.8	0.6	6.2	5.1	7.9	7.0	0.02	121	98	59	78
Norfentanyl	2.7	5.6	0.5	3.8	1.3	1.5	1.4	7.0	0.05	86	100	73	64
Ocfentanil	4.6	2.1	2.5	3.3	4.5	5.1	5.6	7.9	0.02	109	104	66	68
Remifentanil	3.1	2.3	0.8	1.6	4.7	3.2	5.5	6.5	0.02	87	97	72	72
Sufentanil	3.9	2.1	0.4	5.9	4.1	6.1	6.0	6.8	0.03	87	107	82	65
U-47700	5.5	2.5	2.7	2.2	4.8	6.5	9.1	8.9	0.03	74	86	66	74
Valerylfentanyl	1.4	0.5	1.3	3.7	2.3	4.9	3.9	9.5	0.02	86	109	72	62

**LOQ:** limit of quantification; **LOD:** limit of detection; **Low:** internal quality control 1 ng/mL; **High:** internal quality control 40 ng/mL

**Table 4.** Demographic characteristics of the population studied. Urine narcotics screening (UNS) and synthetic opioids (SO) identification in patients admitted in Nancy university hospital emergency departments (ED) and addiction departments (AD) from March to September 2019. Data are expressed as absolute number with corresponding percentage in brackets ().

<b>Characteristics</b>	<b>ED + AD</b>	<b>ED</b>	<b>AD</b>
<b>Number</b>	<b>211 (100)</b>	<b>141 (66.8)</b>	<b>70 (33.2)</b>
<b>Gender</b>			
• Male	104 (49.3)	80 (37.9)	25 (11.8)
• Female	107 (50.7)	61 (28.9)	45 (21.3)
<b>Age group in years</b>			
• <2	15 (7.1)	15 (7.1)	0
• 2-11	6 (2.8)	6 (2.8)	0
• 12-17	27 (12.8)	27 (12.8)	0
• 18-75	163 (77.3)	93 (44.1)	70 (33.2)
<b>UNS –</b>	<b>105 (49.8)</b>	<b>95 (45.1)</b>	<b>10 (4.7)</b>
<b>UNS +</b>	<b>106 (50.2)</b>	<b>46 (21.8)</b>	<b>60 (28.4)</b>
• OPI +	66 (31.3)	23 (10.9)	43 (20.4)
• THC +	36 (17.1)	13 (6.2)	23 (10.9)
• COC +	42 (19.9)	12 (5.7)	30 (14.2)
• AMPH +	4 (1.9)	2 (0.9)	2 (0.9)
• SO +	6 (2.8)	5 (2.3)	1 (0.5)

**OPI:** Opiates; **THC:** Tetrahydrocannabinol; **COC:** Cocaine; **AMPH:** Amphetamines; **SO:** Synthetic Opioids, **UNS:** Urine narcotics screening.

**Table 5.** Characteristics of patients detected positive to fentanyl analogues in urine (n = 6).

Case	Age (years)	Gender	Department of admission	Medical history	UNS	SO	Indication of fentanyl and its analogs
#1	15	F	ICU	Head trauma following a fall from a height of 3 meters.	THC +	Fentanyl Norfentanyl Sufentanil	Sedation
#2	2	M	ICU	CRA following inhalation of a foreign body.	Negative	Sufentanil	Sedation
#3	38	F	ICU	CRA following occlusion of the AIV artery.	THC +	Sufentanil	Sedation
#4	49	F	ICU	Coma following hepatic encephalopathy.	Negative	Sufentanil	Sedation
#5	27	F	ICU	Coma following deliberate drug overdose	THC +	Fentanyl Norfentanyl	Sedation
#6	53	M	AD	Chronic chest pain without etiology initially treated by morphine. Methadone prescription by his GP because of morphine withdrawal symptoms. Introduction of Fentanyl (PO) and Instanyl™ (IN) by his GP for pain management. Then development of prescribed fentanyl addiction.	Negative	Fentanyl Norfentanyl	Pain management

**UNS:** urine narcotics screening; **SO:** synthetic opioids; **ICU:** intensive care unit; **AD:** addiction department; **CRA:** cardiorespiratory arrest; **AIV:** anterior inter-ventricular; **PO:** per os; **IN:** intranasal; **GP:** general practitioner; **THC:** tetrahydrocannabinol