Activity-Based Detection and Bioanalytical Confirmation of a Fatal Carfentanil Intoxication

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Carfentanil, one of the most potent opioids known, has recently been reported as a contaminant in street heroin in the United States and Europe, and is associated with an increased number of life-threatening emergency department admissions and deaths. Here, we report on the application of a novel in vitro opioid activity reporter assay and a sensitive bioanalytical assay in the context of a fatal carfentanil intoxication, revealing the highest carfentanil concentrations reported until now. A 21-year-old male was found dead at home with a note stating that he had taken carfentanil with suicidal intentions. A foil bag and plastic bag labeled “C.50” were found at the scene. These bags were similar to a sample obtained by the Belgian Early Warning System on Drugs from a German darknet shop and to those found in the context of a fatality in Norway. Blood, urine and vitreous, obtained during autopsy, were screened with a newly developed in vitro opioid activity reporter assay able to detect compounds based on their µ-opioid receptor activity rather than their chemical structure. All extracts showed strong opioid activity. Results were confirmed by a bioanalytical assay, which revealed extremely high concentrations for carfentanil and norcarfentanil. It should be noted that carfentanil concentrations are typically in pg/mL, but here they were 92 ng/mL in blood, 2.8 ng/mL in urine, and 23 ng/mL in vitreous. The blood and vitreous contained 0.532 and 0.300 ng/mL norcarfentanil, respectively. No norcarfentanil was detected in urine. This is the first report where a novel activity-based opioid screening assay was successfully deployed in a forensic case. Confirmation and quantification using a validated bioanalytical procedure revealed the, to our knowledge, highest carfentanil concentrations reported in humans so far.

Keywords: synthetic opioids, untargeted screening, activity-based, bioassay, carfentanil, LC–MS/MS

INTRODUCTION

Carfentanil, a very potent derivative of the pharmaceutical opioid fentanyl, was developed in 1974 by Janssen Pharmaceutica (Van Bever et al., 1976). It is one of the most potent opioids known at ~10,000 times the potency of morphine and ~30–100 times the potency of fentanyl in the tail withdrawal test in rats (Van Bever et al., 1976). Commercially, it is always sold in combination with the µ-opioid antagonist naloxone due to its extreme toxicity in humans. Carfentanil is used to immobilize large exotic wildlife and has been implicated in the 2002 Moscow theater hostage...
crisis (Wax et al., 2003; Riches et al., 2012). Recently, carfentanil and other synthetic opioids have been reported as a contaminant in street heroin in the United States and Europe, and have been associated with an increased number of life-threatening emergency department admissions and deaths (EMCDDA and Europol, 2017; Papsun et al., 2017; Shanks and Behonick, 2017; Shulman et al., 2017). Here, we report on the application of a novel cell-based bioassay and a sensitive bioanalytical assay in the context of a fatal carfentanil intoxication, in which we found the highest carfentanil concentrations reported until now.

**CASE PRESENTATION**

A 21-year-old male was found dead at home along with a note stating that he had taken carfentanil with suicidal intentions, in addition notifying first responders that care should be taken, given the potency of the compound. A foil bag and plastic bag in addition notifying first responders that care should be taken, stating that he had taken carfentanil with suicidal intentions, A 21-year-old male was found dead at home along with a note (

Figure 1A labeled “C.50” were found at the scene (given the potency of the compound. A foil bag and plastic bag in addition notifying first responders that care should be taken, stating that he had taken carfentanil with suicidal intentions, A 21-year-old male was found dead at home along with a note (

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norcarfentanil. To 250 µL sample (blood, urine, or vitreous), 10 µL of internal standard solution containing fentanyl-D₅ and norcarfentanil-D₅ (0.25 and 12.5 ng/mL, respectively) in methanol were added. Sample processing was as described above, except that reconstitution was with 55 µL acetonitrile, of which 50 µL were then mixed with 50 µL of mobile phase A (H₂O + 0.1% HCOOH) in an autosampler vial with 100 µL insert. For the analysis of carfentanil, the injection volume was 20 µL, whereas for the determination of norcarfentanil, 10 µL were injected. Chromatographic separation was achieved on a Kinetex Biphenyl column (50 mm × 2.1 mm, 2.6 µm) (Phenomenex, Utrecht, Netherlands) in a 3.7 min gradient using H₂O + 0.1% HCOOH and methanol + 0.1% HCOOH as mobile phases, at a flow rate of 0.6 mL/min. The method was validated in whole blood. Eight-point calibration curves were set up for carfentanil (range: 0.0025–2.5 ng/mL, linear regression with 1/x² weighting) and norcarfentanil (range: 0.025–25 ng/mL, linear regression with 1/x² weighting). Quality control samples at 0.015/0.25 ng/mL for carfentanil and at 0.15/2.5 ng/mL for norcarfentanil were run in sixPLICATE on 4 days, yielding acceptable intra- and inter-run imprecision (intra-run: <8.8%, inter-run: <14%) and bias (<±8.7%, n = 24 at two different concentrations). Matrix effects were assessed at the two above-mentioned concentrations by comparing the signal ratios of analyte to internal standard of post-extraction-spiked samples with those of standards spiked in neat injection solvent (n = 6). Matrix effects were 78% for carfentanil and 118% for norcarfentanil. Extraction efficiency, assessed by comparing the signal ratios of analyte to internal standard of pre- versus post-extraction-spiked samples, was 66% for carfentanil and 24% for norcarfentanil (n = 6, at the two
above-mentioned concentrations). Also, autosampler stability (change in concentration <9% for at least 3 days, n = 6, two different concentrations), specificity and carry-over (none within calibration range) were successfully evaluated. Dilution integrity was checked by spiking blood and aqueous samples with 100 ng/mL carfentanil and norcarfentanil, then diluting 1:1000 with blank matrix (n = 6) and comparing relative peak areas to control samples with 0.1 ng/mL (n = 6). Differences were ≤ ±13.5%.

The vitreous sample was quantified using a calibration curve in ultra-pure water. The urine sample was quantified by standard addition. To quantify carfentanil concentrations, blood and vitreous samples had to be diluted 1:1000 with blank blood and water, respectively, while the urine sample was diluted 1:100 with blank urine. For norcarfentanil, undiluted samples were analyzed. Carfentanil concentrations were 92 ng/mL in blood, 2.8 ng/mL in urine, and 23 ng/mL in vitreous. The blood and vitreous contained 0.532 and 0.300 ng/mL norcarfentanil, respectively. No norcarfentanil was detected in urine. It should be noted that carfentanil concentrations are typically in the sub-ng/mL range (Papsun et al., 2017: 0.1–14 ng/mL, median: 0.38 ng/mL; Shanks and Behonick, 2017: 0.0102–2 ng/mL, median: 0.0984 ng/mL; Hikin et al., 2018: 0.09–4 ng/mL, median: 0.234 ng/mL).

DISCUSSION

Given the continued emergence of novel synthetic opioids, the major disadvantage for their detection via immunoassays, GC–MS and LC–MS/MS analysis is that the methods are often targeted in nature or, for the latter two, limited by the availability of pre-established mass spectral libraries. Here in this case, the immunoassay for fentanyl did not pick up carfentanil, a fentanyl analog, due to the lack of cross-reactivity. Therefore, an alternative untargeted approach for the detection of (synthetic) opioids, not directly based on the structure of the opioids, but on their opioid activity, was applied. Such an approach may serve as a first-line screening tool, complementing the conventional analytical methods which are currently used.

The high ratio of carfentanil/norcarfentanil in blood and vitreous and the absence of norcarfentanil in urine can be explained by the presumably sudden death of the victim caused by the massive overdose. The detected concentrations of carfentanil are, to the best of our knowledge, the highest ever reported in a human being. Other intoxications always state sub-ng to low ng/mL levels of carfentanil (Müller et al., 2017; Papsun et al., 2017; Shanks and Behonick, 2017; Swanson et al., 2017; Elliott and Hernandez Lopez, 2018; Hikin et al., 2018). In conclusion, this is the first report in which a novel activity-based opioid screening assay was successfully deployed in a forensic case, where confirmation and quantification using a validated bioanalytical procedure revealed very high carfentanil concentrations.

ETHICS STATEMENT

We received permission from the Belgian Department of Justice to use the samples for this study.

AUTHOR CONTRIBUTIONS

AC was involved in the development and application of the bioassay and wrote the manuscript. LA worked on the development and validation of the LC–MS/MS method and wrote the manuscript. PB provided the carfentanil standard, gave additional information concerning the carfentanil package found at the scene, and checked the final version of the manuscript. CS was the forensic toxicologist in charge of the case and wrote the manuscript.

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REFERENCES

Cannaert, A., Franz, F., Auwärter, V., and Stove, C. P. (2017). Activity-based detection of consumption of synthetic cannabinoids in authentic urine samples using a stable cannabinoid reporter system. Anal. Chem. 89, 9527–9536. doi: 10.1021/acs.analchem.7b02552

Cannaert, A., Storme, J., Franz, F., Auwärter, V., and Stove, C. P. (2016). Detection and activity profiling of synthetic cannabinoids and their metabolites with a newly developed bioassay. Anal. Chem. 88, 11476–11485. doi: 10.1021/acs.analchem.6b02600

Cannaert, A., Vasudevan, L., Friscia, M., Mohr, A. L. A., Wille, S. M. R., and Stove, C. P. (2018). Activity-based concept to screen biological matrices for opiates and (synthetic) opioids. Clin. Chem. (in press).

Elliott, S. P., and Hernandez Lopez, E. (2018). A series of deaths involving carfentanil in the UK and associated post-mortem blood concentrations. J. Anal. Toxicol. doi:10.1093/jat/bkx109 [Epub ahead of print].

EMCCDA and Europol (2017). EMCCDA-Europol Joint Report on A New Psychoactive Substance: Methyl 1-(2-Phenylethyl)-4-[Phenyl (Propionyl)Amino]Piperidine-4-Carboxylate (Carfentanil). Lisbon: European Monitoring Centre for Drugs and Drug Addiction.

Feasel, M. G. (2017). The Use of In Vitro and In Silico Technologies for Predicting Human Pharmacology and Toxicology of Carfentanil. Doctor of Philosophy, University of Maryland, College Park, MD.

Hikin, L., Smith, P. R., Ringland, E., Hudson, S., and Morley, S. R. (2018). Multiple fatalities in the North of England associated with synthetic fentanyl analogue exposure: detection and quantitation a case series from early 2017. Forensic Sci. Int. 282, 179–183. doi: 10.1016/j.forsciint.2017.11.036

Marlin, M., and Hoyte, C. (2017). The characterization of carfentanil sales on a major darknet cryptomarket. Clin. Toxicol. 55:701. doi: 10.1080/15563650.2017.1348043

Müller, S., Nussbaumer, S., Plitzko, G., Ludwig, R., Weinmann, W., Krähenbühl, S., et al. (2017). Recreational use of carfentanil – a case report with laboratory confirmation. Clin. Toxicol. 56, 151–152. doi: 10.1080/15563650.2017.1355464

Papsun, D., Isenschmid, D., and Logan, B. K. (2017). Observed carfentanil concentrations in 355 blood specimens from forensic investigations. J. Anal. Toxicol. 41, 777–778. doi:10.1093/jat/bks068

Riches, J. R., Read, R. W., Black, R. M., Cooper, N. J., and Timperley, C. M. (2012). Analysis of clothing and urine from Moscow theatre siege casualties reveals carfentanil and remifentanil use. J. Anal. Toxicol. 36, 647–656. doi:10.1093/jat/bks078
Shanks, K. G., and Behonick, G. S. (2017). Detection of carfentanil by LC-MS-MS and reports of associated fatalities in the USA. J. Anal. Toxicol. 41, 466–472. doi: 10.1093/jat/bkx042
Shulman, J., Nunnally, B., Marino, R., and Lynch, M. (2017). Laboratory confirmed intravenous carfentanil exposure requiring naloxone infusion. Clin. Toxicol. 55, 787–788. doi: 10.1080/15563650.2017.1348043
Stove, C. P., De Letter, E. A., Piette, M. H., and Lambert, W. E. (2013). Fatality following a suicidal overdose with varenicline. Int. J. Legal Med. 127, 85–91. doi: 10.1007/s00414-012-0695-5
Swanson, D. M., Hair, L. S., Strauch Rivers, S. R., Smyth, B. C., Brogan, S. C., Ventoso, A. D., et al. (2017). Fatalities involving carfentanil and furanyl fentanyl: two case reports. J. Anal. Toxicol. 41, 498–502. doi: 10.1093/jat/bkx037
Van Bever, W. F., Niemegeers, C. J., Schellekens, K. H., and Janssen, P. A. (1976). N-4-Substituted 1-(2-arylethyl)-4-piperidinyl-N-phenylpropanamides, a novel series of extremely potent analgesics with unusually high safety margin. Arzneimittelforschung 26, 1548–1551.
Vevelstad, M., and Drange, E. (2017). En versting blant opioider. Tidsskr. Nor. Legeforen. 137. doi: 10.4045/tidsskr.17.0419
Wax, P. M., Becker, C. E., and Curry, S. C. (2003). Unexpected “gas” casualties in Moscow: a medical toxicology perspective. Ann. Emerg. Med. 41, 700–705. doi: 10.1067/mem.2003.148

Conflict of Interest Statement: 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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