Development and Validation of a Method to Analyze Fentanyl and Its Analogues in Postmortem Blood Samples by LC-MS/MS

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The increase in the number of users, hospitalizations and deaths due to overdose of synthetic opioids made the scientific community to treat this problem as “the opioid crisis”, justifying the need for the development of analytical strategies to identify and quantify these drugs. Even with the global discussion, there is no Brazilian data regarding opioids consumption, addiction or fatal intoxication cases. The aim of this work was to develop, validate and applied an analytical method based on liquid chromatography-tandem mass spectrometry for determination of fentanyl and seven analogues (acetyl norfentanyl, acetyl fentanyl, thiofentanyl, acrylfentanyl, furanylfentanyl, carfentanil and valerylfentanyl) in postmortem blood samples, to the routine analysis of the Forensic Toxicology Laboratory of São Paulo State Police. Linearity was evaluated from 1-500 ng mL$^{-1}$ (correlation coefficient ($r$) value ($r \geq 0.99$), $1/x$ weight linear regression), with limit of quantification of 1 ng mL$^{-1}$. The method imprecision, bias and matrix effect were lower than 19.7%. No interference or carryover were observed, and extraction yield was greater than 57.7%. Analysis of postmortem blood samples ($n = 1,359$) showed that fentanyl was the most frequently detected opioid. The developed method proved to be useful for routine analysis of the Forensic Toxicology Laboratory.

Keywords: forensic toxicology, fentanyl, opioid crisis, new psychoactive substances, LC-MS/MS

Introduction

New psychoactive substances (NPS) are drugs that mimic the effects of traditional drugs of abuse and can pose a public health threat. These substances or mixtures of psychoactive compounds are produced in clandestine laboratories by chemical synthesis from precursor substances found in nature or not, or through minor modifications in the structure of the molecule that have known biological activity. The growing worldwide abuse of an important group of NPS, the synthetic opioids, has led to serious public health problems in several countries, mainly in the United States of America (USA). The significant increase in number of users, and consequently, individuals who became addicted, were hospitalized or died from overdose made the scientific community call this problem “the opioid crisis”, justifying the need for the development of analytical strategies that allow the identification and quantification of this class of drugs in the human system.

Synthetic opioids include (among other substances) fentanyl and its analogues, which are synthetic agonists of the $\mu$-opioid receptor of the phenylpiperidine class and they are high-potency drugs, approximately 80 to
100 times more potent than morphine. Toxic effects of these substances include respiratory depression and neurological complications that could lead to seizures; in addition to hypersensitivity reactions probably by immunoglobulin E (IgE)-mediated reactions leading to degranulation of mast cells and basophils, resulting in the release of inflammatory mediators. The severity of respiratory depression depends on patient response, pharmacokinetics, ingestion of other sedative medications, and other comorbidities that affect lung function.

Fentanyl is mixed with heroin to adulterate this drug of abuse, increasing its potency, but users do not know that fentanyl is present. The mix of these two drugs has serious consequences, and could be fatal even for users with a certain level of opioid tolerance, given fentanyl’s potency. Several cases of intoxications and deaths caused by these NPS have been described in the literature. In addition to new molecules, fentanyl derivatives that were used in other areas began to be used for recreational purposes. An example is carfentanil, a substance that has a potency 10,000 times higher than morphine, authorized only for veterinary applications (sedating large animals, such as elephants), has been used as a drug of abuse and has caused numerous deaths.

According to data from Drug Enforcement Administration (DEA), until 2018, more than 31,000 Americans died from overdose involving synthetic opioids other than methadone, accounting for 67% of opioid-involved deaths. Although several drugs are present in this category, fentanyl is the major responsible for these deaths. Moreover, the National Forensic Laboratory Information System (NFLIS) crime laboratory (NFLIS-Drug) accounted 100,378 fentanyl reports in 2019, an increase of 12% compared to 2018. Besides the global discussion about the “opioid crisis”, Brazil has no epidemiological data about consumption, addiction or fatal intoxications by these NPS.

The aim of this work was to develop and validate a method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the determination of fentanyl and 7 analogues (acyetyl norfentanyl, acetyl fentanyl, thiofentanyl, acrylfentanyl, furanyl fentanyl, carfentanil and valerylfentanyl) in postmortem blood samples. Then, the method was applied to the routine analysis of the Forensic Toxicology Laboratory, Institute of Legal Medicine, São Paulo State Police.

**Experimental**

**Standards and chemicals**

Solvents employed in the sample extraction and chromatographic analyses were formic acid (≥ 95%, from Sigma-Aldrich, St Louis, USA), ammonium formate, high performance liquid chromatography (HPLC) grade acetonitrile and methanol from Merck (Darmstadt, Germany), and ultra-pure deionized water was supplied by a Milli-Q RG unit from Millipore (Milford, USA). Reference standards of fentanyl, acetyl fentanyl, valeryl fentanyl, thiofentanyl, acrylfentanyl, furanyl fentanyl, carfentanil and isotopically labeled cocaine (cocaine-$d_3$ used as internal standard) were purchased from Cerilliant (Round Rock, USA) and Cayman Chemical (Ann Arbor, USA).

**Calibrators, quality controls and internal standards**

Working solutions were prepared by dilution of fentanyl and analog reference materials in methanol, in other to prepare calibrators at 10, 50, 100, 500 and 1000 ng mL$^{-1}$ and quality control (QC) solutions at three concentrations: low (30 ng mL$^{-1}$), medium (250 ng mL$^{-1}$) and high (750 ng mL$^{-1}$). To prepare the internal standard solution (IS), a cocaine-$d_3$ reference material was diluted in acetonitrile to reach a final concentration of 200 ng mL$^{-1}$. All solutions were stored at −20 °C in amber glass vials.

Blank postmortem human blood (250 µL) was fortified with 25 µL of calibrators or QC working solutions to achieve 1, 5, 10, 30, 50 and 100 ng mL$^{-1}$ (calibrators) and 3, 25 and 75 ng mL$^{-1}$ (QCs).

**Instrumentation and LC-MS/MS conditions**

The analysis was performed on a Nexera UHPLC chromatographic system coupled to a LCMS-8050 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). The analytes were separated using a Waters Atlantis-T3 column (150 mm x 4.6 mm, 3 µm) maintained at 40 °C, with mobile phase composed by (A) ultra-pure water and (B) methanolic solutions containing 0.1% formic acid and 2 mmol L$^{-1}$ ammonium formate. The flow rate was set to 0.35 mL min$^{-1}$ and gradient elution consisted of a linear change in percentage of mobile phase B, from 5 to 95%, holding at 95% B for 2.5 min and returning to 5% over 0.1 min, for a 15.5 min total run time. The mass spectrometer was equipped with an electrospray ionization source, operated in positive mode and source parameters optimized were: heat block temperature 400 °C; ion spray voltage 4.0 kV; nebulizer gas (N$_2$) flow 3 L min$^{-1}$; desolvation line temperature 526 °C; drying gas (N$_2$) flow 10 L min$^{-1}$; heating gas (synthetic air) flow 10 L min$^{-1}$; and collision induced dissociation gas pressure (Ar) 270 kPa. The analyses were performed in multiple reaction monitoring (MRM) mode. MRM conditions used are presented in Table 1, being the
entrance potential (EP), collision energy (CE) and collision cell exit potential (CCEP) optimized through the software LabSolutions (version 5.96, Shimadzu, Kyoto, Japan). Data were acquired and analyzed using the same software.

**Sample preparation**

This study was realized in accordance with the ethical standards of the University of Campinas committee (Comitê de Ética em Pesquisa da UNICAMP-CEP; CAAE 87316318.0.0000.5404) and Superintendence of the Technical-Scientific of Sao Paulo State Police (No. 766/2015/ATS/SPTC-SSP).

For sample preparation, 250 µL of postmortem blood were transferred to a polypropylene tube (2 mL), followed by 25 µL of internal standard (cocaine-\(d_3\) at 200 ng mL\(^{-1}\)) and 1000 µL of iced-cold acetonitrile. The tube was capped, vortexed for 5 min and centrifuged at 14,000 rpm for 10 min. Then, 600 µL of the organic phase were transferred to a vial and 2 µL were injected into the LC-MS/MS.

**Validation of the method**

Blank postmortem blood for preparation of fortified matrix samples used during the validation was collected in blood collection tubes containing sodium fluoride and ethylenediaminetetraacetic tripotassium salt. The validation of the method was carried out in accordance with American National Standards Institute (ANSI), American Academy of Forensic Sciences (AAFS) Standards Board (ASB) Standard 036, Standard Practices for Method Validation in Forensic Toxicology. Identification criteria were symmetrical peak eluting within ±2% of average calibrator retention time, signal-to-noise ratio of 3 and ion ratio of the quantifying and qualifier MRM between ±20 to ±50% according to ANSI/ASB. The lower limit of quantitation (LLOQ) was determined as the lowest concentration fulfilling the identification criteria, a signal-to-noise ratio of at least 10 and quantifying within 20% of each target concentration. Linearity was assessed with six-point calibration curves (1, 5, 10, 30, 50 and 100 ng mL\(^{-1}\)) over 5 days and calibrators must quantify within ±20% relative standard deviation (RSD) of each target concentration. For the construction of the calibration curve, six weighting factors were analyzed (1/x, 1/x\(^2\), 1/x\(^{0.5}\), 1/y, 1/y\(^2\), 1/y\(^{0.5}\)), being chosen the one with the lowest sum of residual regression errors (\(\Sigma RE\%\)). A high correlation coefficient (r) value (r ≥ 0.99) and inaccuracy better than ±15% for all calibrators were used as criterion of adequate linearity. Blank matrix samples were analyzed immediately after a fortified blood extract at the highest calibrator concentration to evaluate carryover. Negative postmortem blood samples fortified with forty-six drugs of abuse and Table 1. Mass spectrometer parameters and retention times of fentanyl, its analogues and internal standard for the analysis in postmortem blood by LC-MS/MS

<table>
<thead>
<tr>
<th>Analyte</th>
<th>MRM transitions (m/z)</th>
<th>EP / V</th>
<th>CE / V</th>
<th>CCEP / V</th>
<th>Retention time / min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl norfentanyl</td>
<td>219.1 &gt; 84.1(^a)</td>
<td>−16</td>
<td>−19</td>
<td>−15</td>
<td>6.17</td>
</tr>
<tr>
<td></td>
<td>219.1 &gt; 55.1</td>
<td>−16</td>
<td>−34</td>
<td>−21</td>
<td></td>
</tr>
<tr>
<td>Cocaine-(d_3) (IS)</td>
<td>307.0 &gt; 185.0(^b)</td>
<td>−15</td>
<td>−20</td>
<td>−19</td>
<td>7.19</td>
</tr>
<tr>
<td></td>
<td>307.0 &gt; 85.0</td>
<td>−15</td>
<td>−33</td>
<td>−16</td>
<td></td>
</tr>
<tr>
<td>Acetylfentanyl</td>
<td>323.3 &gt; 105.2(^c)</td>
<td>−17</td>
<td>−40</td>
<td>−19</td>
<td>7.51</td>
</tr>
<tr>
<td></td>
<td>323.3 &gt; 188.2</td>
<td>−10</td>
<td>−24</td>
<td>−12</td>
<td></td>
</tr>
<tr>
<td>Thiofentanyl</td>
<td>343.1 &gt; 111.0(^b)</td>
<td>−13</td>
<td>−35</td>
<td>−19</td>
<td>7.82</td>
</tr>
<tr>
<td></td>
<td>343.1 &gt; 194.1</td>
<td>−25</td>
<td>−22</td>
<td>−19</td>
<td></td>
</tr>
<tr>
<td>Acrylfentanyl</td>
<td>335.3 &gt; 188.2(^a)</td>
<td>−17</td>
<td>−39</td>
<td>−18</td>
<td>7.87</td>
</tr>
<tr>
<td></td>
<td>335.3 &gt; 105.1</td>
<td>−17</td>
<td>−23</td>
<td>−20</td>
<td></td>
</tr>
<tr>
<td>Furanyl fentanyl</td>
<td>375.3 &gt; 105.1(^c)</td>
<td>−19</td>
<td>−40</td>
<td>−20</td>
<td>7.92</td>
</tr>
<tr>
<td></td>
<td>375.3 &gt; 188.2</td>
<td>−11</td>
<td>−24</td>
<td>−12</td>
<td></td>
</tr>
<tr>
<td>Fentanyl</td>
<td>337.3 &gt; 188.1(^c)</td>
<td>−10</td>
<td>−25</td>
<td>−19</td>
<td>8.01</td>
</tr>
<tr>
<td></td>
<td>337.3 &gt; 105.1</td>
<td>−10</td>
<td>−42</td>
<td>−10</td>
<td></td>
</tr>
<tr>
<td>Carfentanil</td>
<td>395.3 &gt; 335.1(^c)</td>
<td>−20</td>
<td>−19</td>
<td>−22</td>
<td>8.19</td>
</tr>
<tr>
<td></td>
<td>395.3 &gt; 113.2</td>
<td>−11</td>
<td>−30</td>
<td>−11</td>
<td></td>
</tr>
<tr>
<td>Valeryl fentanyl</td>
<td>365.2 &gt; 188.2(^a)</td>
<td>−14</td>
<td>−43</td>
<td>−20</td>
<td>8.85</td>
</tr>
<tr>
<td></td>
<td>365.2 &gt; 105.1</td>
<td>−18</td>
<td>−25</td>
<td>−18</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Quantifier transitions. MRM: multiple reaction monitoring; EP: entrance potential; CE: collision energy; CCEP: collision cell exit potential; m/z: mass-to-charge ratio; V: volt; IS: internal standard.
pharmaceuticals at 200 ng mL\(^{-1}\) were analyzed to evaluate possible exogenous interferences. Analyzes of 10 different postmortem blood samples were performed for possible endogenous interferences.

Intraday (n = 3) and interday (n = 15) imprecision and bias were evaluated over 5 days and in triplicate, for low, medium and high QC concentrations. Imprecision was calculated through one-way analysis of variance (ANOVA) for each QC to evaluate the occurrence of significant variability (p < 0.05), values less than 20% were acceptable. Bias were calculated as the mean of the difference between the concentration expected for each QC and the value estimated for each observation and expressed as a percentage. Values in the range of 80-120% were considered acceptable.

Matrix effect and extraction yield were evaluated fortifying matrix samples at low and high QC concentrations. For matrix effect, two groups of six replicates were compared, using the equation: (a/b) × 100, being “a” the absolute area of the peak obtained from postmortem blood samples fortified before the extraction and “b”, those obtained from fortified ultrapure water. For extraction yield, the same equation was used, being “a” the results obtained from postmortem blood samples fortified before the extraction and “b”, those obtained from samples fortified after the extraction. Samples were extracted according to the method previously described and results were expressed as percentages.

Processed sample (autosampler) stability was evaluated at low and high QC samples in triplicate. After extraction and analysis, samples were stored on the autosampler at 10 °C and re-inject after 24 h. Peak areas obtained were compared with those from the analysis of a freshly extracted calibration curve.

**Results and Discussion**

**Method validation**

According to the ANSI/ASB guideline,\(^{21}\) the LLOQ could be determined by using a decision point concentration, i.e., defining the LLOQ as the value of an administratively defined decision point, even though a lower LLOQ is analytically achievable. The therapeutic range for fentanyl is 0.3-1.2 ng mL\(^{-1}\).\(^{25,26}\) Previously studies\(^{26-35}\) have shown that in postmortem cases involving fentanyl and its analogues, most of these analytes were above 1 ng mL\(^{-1}\). This method was focused on detecting fentanyl and analogues acute intoxications, situations were the concentrations used to be far from the therapeutic levels. Thus, the LLOQ of 1 ng mL\(^{-1}\) fits for method purpose and the MRM chromatograms of all analytes at this concentration are shown in Figure 1.

The developed method was able to analyze fentanyl and 7 analogues with LLOQ of 1.0 ng mL\(^{-1}\). A linear curve from 1 to 100 ng mL\(^{-1}\) was evaluated and showed adequate performance using a 1/x weight linear regression, presenting a satisfactory linearity (r > 0.99). The errors of all calibrants were also evaluated, with inaccuracy always better than ±15%. No carryover or exogenous interference were observed, demonstrating the specificity of the method.

For evaluation of the imprecision, the ANOVA test was applied; the highest values found were 14.5% (intraday imprecision) and 19.7% (interday imprecision), both for the LLOQ of carfentanil (1 ng mL\(^{-1}\)), being all the values accepted according to ANSI/ASB guideline. The bias was greater than 11.3, 10.7 and 8.0% for the LLOQ of furanyl fentanyl, low QC of acetyl norfentanil and LLOQ of thiofentanyl, respectively. Table 2 shows the results of imprecision and bias.

The extraction yield was higher than 58% (fentanyl, at high quality control concentration), and matrix effect was greater than 11.1% (acetyl fentanyl, at high quality control concentration). The results of imprecision, bias, matrix effect and extraction yield for each analyte are described in Table 3.

**Analysis of authentic forensic toxicology cases**

This method was applied in the Forensic Toxicology Laboratory of the São Paulo State Police for four months (n = 1,359 blood samples), with fentanyl being the most common opioid found in postmortem blood samples. Fentanyl was detected in 79 samples (45.4% of positive identifications among opioids), in concentrations between 1.0-18.7 ng mL\(^{-1}\). Figure 2 shows a positive sample for fentanyl.

Only 1 case of acetyl norfentanyl was identified (11.8 ng mL\(^{-1}\)), and no authentic sample was above the therapeutic concentration. Fentanyl is used as anesthetic in hospital surgeries, and most of the samples are also positive for midazolam, a common combination used for sedations and rapid sequence intubation. Since we observed only concentrations within the therapeutic range, we believe that we cannot classify the 79 positive samples as abuse of this substance. Despite the few cases of blotter papers containing fentanyl and the report of cocaine mixed with carfentanil in Argentina,\(^{33,34}\) the most consumed drugs in South America are cannabis, followed by cocaine, while opioids and opiates occupy fifth and sixth place, respectively, in prevalence of consumption.\(^{35}\)

Our work evaluated the profile of opioid consumption...
in postmortem cases of the São Paulo State since there is a lack of epidemiological data about consumption, addiction or fatal intoxications by these NPS. Besides, Bastos and Krawczyk recently published a comment addressing the growing use of fentanyl in present-day Brazil, expressing concern, but suggesting that a crisis similar to that in the US can still be prevented. With this method, the Forensic Toxicology Laboratory of the São Paulo State Police, which is responsible for the toxicological analysis of all 645 cities that are part of the State, is previously capable of detecting and quantifying these substances. Other methods were described for the analysis of fentanyl and its metabolites in biological samples, but using higher volumes of sample or needed more steps, including dryness...
of extraction solvent or solid-phase extraction (SPE).\textsuperscript{4,18-20} Both steps increase the time spent on sample preparation and could be laborious, besides increasing the costs of the analysis. Moreover, compared to other studies, our method quantifies more fentanyl analogues.\textsuperscript{18,19}

### Conclusions

A sensitive method was developed to quantify fentanyl and 7 of its analogues in postmortem blood samples by LC-MS/MS. The efficiency of the method was

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Bias (n = 15) / %</th>
<th>Matrix effect (n = 6) / %</th>
<th>Extraction yield (n = 6) / %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LLOQ Low Medium High</td>
<td>Low CV / % High CV / % Low High</td>
<td></td>
</tr>
<tr>
<td>Acetyl norfentanyl</td>
<td>–3.8 –10.7 –7.9 –7.5</td>
<td>–1.7 4.0 3.9 4.4</td>
<td>62.4 58.9</td>
</tr>
<tr>
<td>Acetylfentanyl</td>
<td>4.1 4.9 2.2 2.9</td>
<td>1.4 8.0 11.1 3.5</td>
<td>58.9 60.2</td>
</tr>
<tr>
<td>Thiofentanyl</td>
<td>8.0 2.8 –0.8 –1.6</td>
<td>3.0 3.2 7.2 2.5</td>
<td>61.4 58.3</td>
</tr>
<tr>
<td>Acrylfentanyl</td>
<td>0.5 0.9 5.2 3.4</td>
<td>8.4 3.5 7.3 2.7</td>
<td>63.5 60.6</td>
</tr>
<tr>
<td>Furanyl fentanyl</td>
<td>11.3 0.6 5.3 3.9</td>
<td>6.1 11.7 7.3 4.7</td>
<td>61.0 60.1</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>6.6 2.5 3.8 4.4</td>
<td>6.8 3.4 9.9 1.8</td>
<td>61.3 58.0</td>
</tr>
<tr>
<td>Carfentanil</td>
<td>4.7 4.5 2.3 1.5</td>
<td>5.7 12.2 9.1 2.4</td>
<td>63.8 61.8</td>
</tr>
<tr>
<td>Valeryl fentanyl</td>
<td>2.5 1.0 3.2 0.2</td>
<td>7.9 4.2 7.4 2.8</td>
<td>57.7 58.5</td>
</tr>
</tbody>
</table>

LLOQ: lower limit of quantitation; CV: coefficient of variation; LLOQ = 1 ng mL\(^{-1}\); low = 3 ng mL\(^{-1}\); medium = 25 ng mL\(^{-1}\); high = 75 ng mL\(^{-1}\).
demonstrated through analysis of samples from forensic toxicology cases, being applied for 4 months in the routine of the Forensic Toxicology Laboratory, Institute of Legal Medicine of the São Paulo State Police, showing that fentanyl was the opioid mostly found.

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Author Contributions

Taís B. Rodrigues was responsible for the formal analysis of the data, performing the experiments to validate the analytical method, data collection and writing the original draft; Márcio H. Matsubara for the data collection and sample preparation of authentic samples of forensic toxicology cases; Damila R. Morais for the development of the analytical method; Victor A. P. Gianvecchio for the project administration and acquisition of laboratory resources such as reagents, materials and instrumentation; Elvis M. Aquino donated and prepared the authentic samples of forensic toxicology cases; José Luiz Costa was responsible the conceptualization of the work, funding acquisition, project administration and supervision. All co-authors contributed to the review and editing of the manuscript.

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