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Development and validation of a sensitive ultra performance liquid chromatography tandem mass spectrometry method for the analysis of fentanyl and its major metabolite norfentanyl in urine and whole blood in forensic context

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ABSTRACT

Fentanyl and its major metabolite norfentanyl often occur in low doses in biological samples. Therefore, a highly sensitive liquid chromatography tandem mass spectrometry (LC–MS/MS) method has been developed and fully validated. Sample preparation was performed on a mixed-mode cation exchange solid phase extraction (SPE) cartridge with an additional alkaline wash step to decrease matrix effects and thus increase sensitivity. Ionization of fentanyl and norfentanyl with electrospray ionization (ESI) was more efficient than atmospheric pressure chemical ionization (APCI). The use of a mobile phase of high pH resulted in higher ESI signals than the conventional low pH mobile phases. In the final method, gradient elution with 10 mM ammonium bicarbonate (pH 9) and methanol was performed. A comparison of columns with different internal diameter and/or smaller particles showed optimal resolution and sensitivity when an Acquity C18 column (1.7 μm, 2.1 mm × 50 mm) was used. Deuterium labeled internal standards were used, but with careful evaluation of their stability since loss of deuteriums was seen. With limits of detection of 0.25 pg/ml for fentanyl and 2.5 pg/ml for norfentanyl in urine and 5 pg/ml for fentanyl and norfentanyl in whole blood the presented method is highly appropriate for the analysis of fentanyl and norfentanyl in forensic urine and blood samples.

1. Introduction

Fentanyl is a synthetic narcotic analgesic of high potency and with short duration of action [1]. It is used in the treatment of chronic pain and as a supplement to surgical anesthesia [1]. Being an opioid, the literature describes the possible toxic effects of fentanyl: severe respiratory depression, cardiovascular collapse and arrest which can result in a sudden death [1]. Reports indicate that 0.4–20% of administered fentanyl is eliminated via urine as unchanged drug; the rest is excreted as the main metabolite norfentanyl and some small amounts of despropionylfentanyl, hydroxyfentanyl and hydroxynorfentanyl [1–3]. None of the metabolites has pharmacological activity [2]. Because of fentanyl's high lipid solubility, rapid distribution into tissues occurs after intake leading to low blood concentrations [2]. Moreover, because of its high potency, effective doses are very low, making fentanyl difficult to quantify in biological samples. Therefore, a highly sensitive and selective detection technique is required for analysis of fentanyl and its metabolites. Thus far analysis of fentanyl and norfentanyl in biological samples has been achieved by immunoassays [4–7], liquid chromatography with ultraviolet detection (LC–UV) [8–10], gas chromatography with nitrogen phosphorous detection (GC–NPD) [11–13], gas chromatography mass spectrometry (GC–MS) [3,4,12,14–37], GC–MS/MS [38,39] and LC–MS/MS [28,40–58]. In urine, LC–MS/MS methods have identified concentration levels as low as 3 pg/ml fentanyl and 21 pg/ml norfentanyl [50,51]. In whole blood, lowest reported limits of detection are 80 pg/ml fentanyl and 330 pg/ml norfentanyl, using LC–MS/MS [53,59]. Recent developments in the domain of LC–MS/MS which include the use of smaller column particles (so-called ultra performance LC (UPLC) or ultrafast LC (UFLC)) and the use of high pH mobile phases instead of the conventional low pH mobile phases can increase selectivity and sensitivity even more [60–65].

To develop an optimal method for the analysis of fentanyl and norfentanyl in biological samples, the following approach was used. (1) Two factors with an influence on ionization were studied: mobile phase composition and source type. (2) Sample preparation and chromatography were thoroughly optimized to minimize matrix effects and maximize recovery and sensitivity. (3) The optimized method was fully validated for both urine and whole blood.

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Table 1
Analytes, MRM transitions and the voltage settings declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE) and collision cell exit potential (CXP). The collision gas (CAD) was set at medium. Dwell times were set at 100 ms and at 25 ms when using the column with 1.7 μm particle size in order to obtain enough data points. Underlined ions were used as quantifier.

Analyte	Q1 mass	Q3 mass	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
Fentanyl	337	188	46	4.5	16	31	4
	337	105	46	4.5	16	51	4
Fentanyl-d_5	342	105	46	4.5	16	51	4
Norfentanyl	233	84	36	4.5	20	25	4
	233	55	36	4.5	18	51	4
Norfentanyl-d_5	238	84	33	3.5	17	25	4

2. Materials and methods

2.1. Chemicals

Fentanyl, norfentanyl, the internal standards (IS) fentanyl-d_5 and norfentanyl-d_5 were purchased from LGC (Molsheim, France). Dichloromethane, isopropanol, methanol, monopotassium phosphate and ammonium hydroxide were purchased from Merck (Darmstadt, Germany). Water (H_2O) was obtained from a Milli Q Water Purification System (Millipore, Brussel, Belgium). LC–MS grade acetonitrile (ACN) and methanol (MeOH) were purchased from Biosolve (Valkenswaard, The Netherlands). All LC–MS grade mobile phase additives (formic acid, ammonium formate, acetic acid, ammonium acetate, ammonium bicarbonate and ammonium hydroxide) were obtained from Sigma–Aldrich (Bornem, Belgium). Glassware was silanized using AcquaSil Sliconizing Fluid (Thermo Scientific, Breda, The Netherlands), which deactivates the silanol groups on glass. 1.5 ml screw cap vials and 100 μl vial inserts (deactivated glass and polypropylene inserts) were purchased from Agilent (Diegem, Belgium).

2.2. Standard solutions

Methanolic stock solutions were purchased with following concentrations: 1 mg/ml fentanyl; 1 mg/ml norfentanyl; 0.1 mg/ml fentanyl-d_5 and 0.1 mg/ml norfentanyl-d_5. Standard solutions were prepared by diluting the stock solutions with methanol to several concentration levels. Each standard solution contained 1 ng/ml fentanyl-d_5 and 5 ng/ml norfentanyl-d_5. All solutions were stored at −20 °C.

2.3. Instrumentation

LC–MS/MS analysis was carried out using a UFLC Shimadzu system consisting of a LC-20ADXR pump, a SIL-20ACXR autosampler, a DGU-20A3 degasser and a CTO-20A oven (Shimadzu Prominence, Antwerpen, Belgium) in combination with a 3200 QTRAP (Applied Biosystems, Halle, Belgium) and Analyst software (version 1.5).

2.4. MS/MS conditions

The best MS-parameters were automatically obtained by the software Analyst 1.5. Source-dependent MS-parameters were optimized for a flow rate of 0.5 ml/min. Compound-dependent MS-parameters were optimized for fentanyl, norfentanyl, fentanyl-d_5 and norfentanyl-d_5. The Turbo V ion source equipped with ESI probe used following settings: gas 1: nitrogen, 55 psi; gas 2: nitrogen, 55 psi; ion-spray voltage: 5000 V; ion-source temperature: 550 °C; curtain gas: nitrogen, 25 psi. The APCI probe operated with following parameters: gas 1: nitrogen, 40 psi; needle current: 4 μA; ion-source temperature: 475 °C; curtain gas: nitrogen, 15 psi. The mass spectrometer was operated in multiple reaction monitoring mode (MRM) with settings listed in Table 1.

2.5. LC conditions

The used LC columns (XBridge C18 (2.5 μm particle size, 3.0 mm × 50 mm), XBridge C18 (2.5 μm particle size, 2.1 mm × 50 mm), Acquity C18 (1.7 μm particle size, 2.1 mm × 50 mm)) were purchased from Waters (Zellik, Belgium). The columns with 2.5 μm particle size were fitted with a precolumn ultra low dead volume filter 0.5 μm (Upchurch Scientific, obtained through Achrom, Machelen, Belgium). The 1.7 μm column was fitted with a guard filter 0.2 μm (Waters, Zellik, Belgium).

Tested mobile phases were: ACN + 0.1% formic acid and H_2O + 25 mM ammonium formate set at pH 3.0 with formic acid; MeOH + 0.1% formic acid and H_2O + 25 mM ammonium formate set at pH 3.0 with formic acid; ACN + 0.1% acetic acid and H_2O + 25 mM ammonium acetate set at pH 4.0 with acetic acid; MeOH + 0.1% acetic acid and H_2O + 25 mM ammonium acetate set at pH 4.0 with acetic acid; ACN and H_2O + 10 mM ammonium bicarbonate set at pH 9.0 with ammonium hydroxide and finally MeOH and H_2O + 10 mM ammonium bicarbonate set at pH 9.0 with ammonium hydroxide. The used flow rate was 0.5 ml/min. The autosampler temperature was set at 10 °C, the column oven at 30 °C. The final optimized method had following gradient conditions using H_2O + 10 mM ammonium bicarbonate at pH 9.0 (solvent A) and MeOH (solvent B): 0–2 min: 25–90%B; 2–3.5 min: 90%B; 3.5–4 min: 90–25%B; 4–6 min: 25%B, with solvent A daily prepared because of pH instability.

2.6. Sample preparation

Both Bond Elut Certify cartridges (130 mg, 10 ml) and Bond Elut Plexa PCX cartridges (60 mg, 3 ml) were purchased from Varian (Sint-Katelijne-Waver, Belgium). Both are mixed-mode SPE cartridges with hydrophobic and cation exchange capabilities. All extractions were carried out on a Vac Elut SPS 24 (Varian, Sint-Katelijne-Waver, Belgium) at 0.2–0.4 bar. If blockage of the SPE cartridge occurred, a syringe was fitted on the SPE column and by pressing the plunger the fluid was forced through the cartridge. The final optimized protocol is described here.

2.6.1. Conditioning

The SPE cartridge was conditioned with 2 ml MeOH, 2 ml H_2O and 1 ml 0.1 M phosphate buffer pH 6.

2.6.2. Sample application

In a silanized glass tube, 2 ml urine was mixed with 1 ml 0.1 M phosphate buffer pH 6 and 100 μl of IS containing 1 ng/ml fentanyl-d_5 and 5 ng/ml norfentanyl-d_5. The mixture was vortexed and centrifuged (12 min at 2500 rpm). The supernatant was loaded on the SPE cartridge. When analyzing whole blood, 1 ml of blood mixed with 100 μl IS containing 1 ng/ml fentanyl-d_5 and 5 ng/ml norfentanyl-d_5 was sonicated for 15 min to fragment red blood cells. Next, 5 ml of 0.1 M phosphate buffer pH 6 was added. The

mixture was vortexed and centrifuged (12 min at 2500 rpm). The supernatant was loaded on the SPE cartridge.

2.6.3. Wash

The loaded cartridge was washed with 2 ml H_2O, 2 ml 1 M acetic acid, 2 ml MeOH and finally 2 ml MeOH–H_2O–ammonium hydroxide (68.5:29.5:2), which was daily prepared. The SPE column was then dried for 10 min.

2.6.4. Elution

The analytes were eluted in a 1.5 ml vial with 1.5 ml isopropanol–dichloromethane–ammonium hydroxide (20:78:2). The elution solvent was daily prepared. The eluates were evaporated to dryness at room temperature using a SpeedVac Plus SC110A (Savant, Thermo Scientific, Breda, The Netherlands). The residues were reconstituted in 100 μl MeOH–H_2O (25:75), since this corresponds to the starting mobile phase. The solution was transferred to a 100 μl polypropylene insert and 5 μl was injected into the LC–MS/MS system.

2.7. Method validation

The method was validated according to internationally accepted recommendations [66,67]. Full validation was performed for both urine and whole blood. Urine was spiked with 100 μl of standard solution to obtain seven concentration levels: 0.0025 (LOQ); 0.005; 0.05 (LOW); 0.5 (MED); 2.5 (HIGH); 5; 50 (above calibration range sample, ACR) ng/ml fentanyl and 0.005 (LOQ); 0.05; 0.5 (LOW); 2.5 (MED); 5 (HIGH); 10; 100 (ACR) ng/ml norfentanyl. For blood, the concentrations were as follows: 0.01 (LOQ); 0.1 (LOW); 1 (MED); 5 (HIGH); 10; 20; 200 (ACR) ng/ml fentanyl and 0.01 (LOQ); 0.1; 1 (LOW); 5 (MED); 10 (HIGH); 20; 200 (ACR) ng/ml norfentanyl. All statistical analyses were performed with GraphPad Prism (version 5.02, La Jolla, US).

2.7.1. Selectivity

Samples from different sources (n = 10, half of the samples were post-mortem samples, the other 50% was derived from living persons) were extracted as described in Section 2.6 and checked for peaks that might interfere with the detection of the analytes or IS. Two zero samples (blank samples including one post-mortem sample were spiked with 100 μl IS containing 1 ng/ml fentanyl-d_5 and 5 ng/ml norfentanyl-d_5) were analyzed to check the absence of analyte ions in the peaks of the IS. The used samples were analysed as routine forensic samples and were negative for fentanyl and norfentanyl but some contained other drugs and/or alcohol.

2.7.2. Matrix effect (ME), extraction efficiency or recovery (RE) and process efficiency (PE)

Three sets of samples (at both LOW and HIGH concentrations) were prepared for determination of ME, RE and PE: set 1 consisted of pure standard dissolved in 25% MeOH and 75% H_2O. For set 2, blank matrices (n = 5 at each concentration level with three post-mortem samples) from different sources were first extracted as unknown samples. The dry residues were then dissolved in pure standard. For the samples in set 3, blank matrices (n = 5) from the same sources as set 2 were spiked with pure standard. Thereafter, they were extracted and dry residues were dissolved in 25% MeOH and 75% H_2O. From the resulting peak areas or peak area ratios, if using an IS, one can calculate ME, RE and PE using following equations:

$$\text{ME\%} = \left(\frac{B}{A}\right) \times 100\% \quad (1)$$

$$\text{RE\%} = \left(\frac{C}{B}\right) \times 100\% \quad (2)$$

$$\text{PE\%} = \left(\frac{C}{A}\right) \times 100\% \quad (3)$$

where A is the peak area or peak area ratio from set 1 (pure standard), B from set 2 (post-extraction spiked sample) and C from set 3 (pre-extraction spiked sample). A ME% value smaller than 100% can be interpreted as ion suppression, a value above as ion enhancement. ME%, RE% or PE% of 100% reflects the perfect situation.

2.7.3. Processed sample stability

The stability of extracted samples (at LOW and HIGH concentrations, n = 8 at each concentration level) was evaluated. The extracts at each concentration level were transferred into one vial which was placed in the cooled autosampler. At time intervals of 3 h, 5 μl of the sample was injected onto LC–MS/MS over a total time of 9 h, which is the maximum time of storage in the cooled autosampler under conditions of analysis. The peak area of the analytes at each concentration was plotted versus injection time. Next, regression analysis was performed to check for a significantly negative slope, which would indicate instability.

2.7.4. Linearity

Blank matrices (n = 6 at each concentration level) were spiked to obtain calibration standards with concentrations of 0.0025; 0.005; 0.05; 0.5; 2.5; 5 ng/ml fentanyl in urine, 0.005; 0.05; 0.5; 2.5; 5; 10 ng/ml norfentanyl in urine and 0.01; 0.1; 1; 5; 10; 20 ng/ml fentanyl and norfentanyl in blood. Each calibrator was also spiked with 100 μl IS containing 1 ng/ml fentanyl-d_5 and 5 ng/ml norfentanyl-d_5. The calibration standards were extracted as unknown samples. The peak area ratio of analyte and IS was plotted versus analyte concentration. The regression line was calculated using a weighted [1/(concentration)^2] least-squares linear regression model. Daily calibration curves using the same concentrations (n = 1 at each concentration level) were prepared for all following experiments.

2.7.5. Limit of detection (LOD) and limit of quantification (LOQ)

The LOQ was defined as the lowest point of the calibration curve which fulfilled the criteria for LOQ based on precision and accuracy data (<20% for precision and ±20% for accuracy). The LOD was calculated based on a specific calibration curve in the range of the LOD. A linear calibration curve without weighting factor containing samples spiked with IS (n = 2), the LOQ (n = 6) and the second lowest calibrator (n = 6) was established. The LOD was calculated using following formula:

$$\text{LOD} = 3 \times \left(\frac{\text{SD}_{\text{intercept}}}{S}\right) \quad (4)$$

where $\text{SD}_{\text{intercept}}$ is the standard deviation of the intercept of the calibration curve and S is the slope of the calibration curve.

2.7.6. Precision and accuracy

Quality control samples (at LOQ, LOW, MED, HIGH and ACR concentrations, n = 2 at each concentration level) were extracted as described in Section 2.6 and analyzed on each of 8 days. The concentrations of the analytes were calculated via the daily calibration curves to include the possible influence of the daily variations of the calibration curve. Accuracy, expressed as bias, and precision, expressed as repeatability (rep%, within-day) and time-different intermediate precision (int. prec%, combination of within- and between-day effects) were calculated using following equations:

$$\text{Bias\%} = \left(\frac{X - \mu}{\mu}\right) \times 100\% \quad (5)$$

$$\text{Rep\%} = \left(\frac{\text{MS}_w^{0.5}}{X}\right) \times 100\% \quad (6)$$

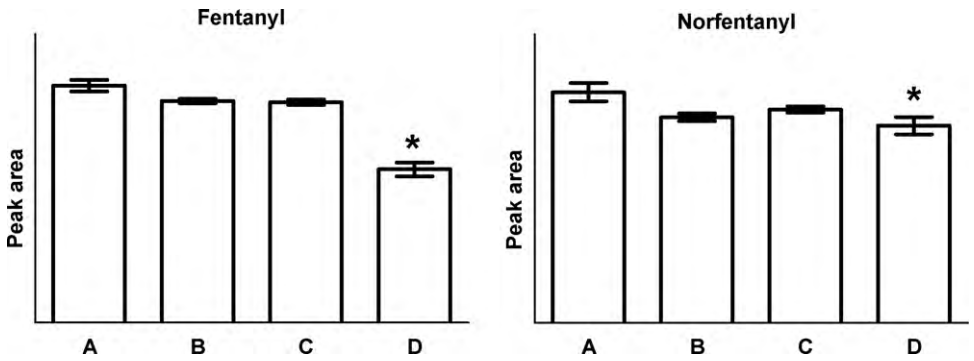

Fig. 1. Comparison of different materials used during analysis of fentanyl and norfentanyl. A 25% methanolic, 75% aqueous stock solution containing 10 ng/ml fentanyl and 10 ng/ml norfentanyl was made in a 1.5 ml vial (A), 40 μl was transferred from the vial into a polypropylene insert (B), a manually silanized deactivated glass insert (C) and a deactivated glass insert (D). The sample was vortexed and 5 μl was injected into LC–MS/MS. Transfer of stock solutions in each of the inserts was repeated five times. The peak areas indicated by an asterisk are significantly different from A (one-way ANOVA, Dunnett test, $p < 0.05$).

$$\text{Int. prec\%} = \left(\frac{((\text{MS}_\text{B} + (n-1) \times \text{MS}_\text{W})/n)^{0.5}}{X} \right) \times 100\% \quad (7)$$

where X is the mean calculated concentration, μ is the nominal concentration, MS_w is the mean square within days calculated by one-way ANOVA, MS_B is the mean square between days calculated by one-way ANOVA and n is the number of observations each day. The acceptance limits were <15% for precision and within 15% of the nominal value for bias, except at LOQ where <20% for precision and within 20% of the nominal value for bias were accepted.

2.7.7. Freeze/thaw stability and bench top stability

The stability of the analytes during several freeze/thaw cycles and at ambient temperature (bench top stability) was evaluated with following experiment. Processed samples (at LOW and HIGH concentration level, $n = 6$ at each concentration level) were frozen at −20 °C for 21 h and kept at room temperature for 3 h. After three of these cycles, the samples were injected again. The concentrations of the analytes were calculated via the daily calibration curves. Stability was assumed when the ratio of the means (stability samples versus control samples) was within 90–110% and the 95% confidence interval of the stability sample mean was within 80–120% of the control mean.

3. Results and discussion

3.1. Optimal glassware

Glassware is silanized to prevent adsorption of analytes to glassware. No significant loss of fentanyl and norfentanyl was seen when using silanized tubes or the 1.5 ml Agilent vials. However, when using the commercially available silanized glass inserts, significant and variable loss of both fentanyl and norfentanyl was seen (Fig. 1D). Despite being labeled as deactivated glass inserts, adsorption of fentanyl and norfentanyl on the insert seemed to occur. When we applied our silanization protocol as applied to glass tubes onto these inserts, no loss was seen (Fig. 1C). The commercially available polypropylene inserts also worked fine (Fig. 1B). These results suggest a careful use of glassware: even when inactivated one should check for possible loss of analytes.

3.2. Choice of internal standard

Variability in LC–MS/MS analysis can be caused by sample preparation or matrix effects. Matrix effects are caused by co-eluting compounds which have an influence on the ionization efficiency of the analyte resulting in ion suppression or ion enhancement [68]. Internal standards are known to tackle variations due to sample preparation and matrix effects [68–71]. The

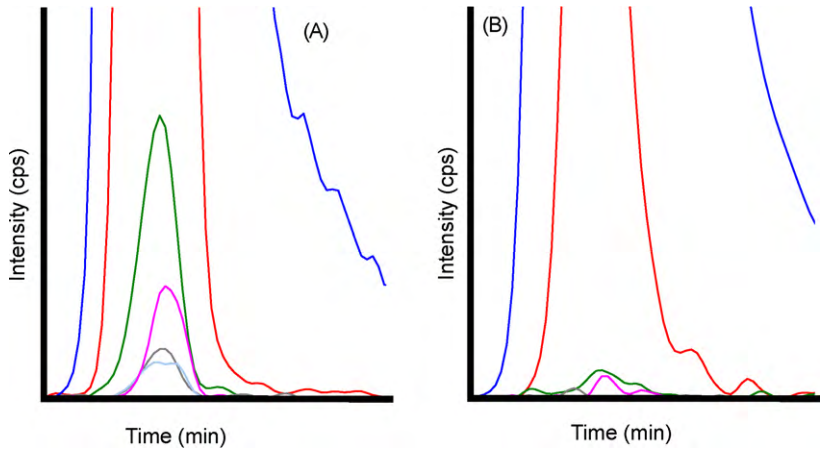

Fig. 2. Analysis of fentanyl-d_5 and norfentanyl-d_5. 5 μl from a solution containing 100 ng/ml fentanyl-d_5 and 100 ng/ml norfentanyl-d_5 was injected onto LC–MS/MS. For both fentanyl (A) and norfentanyl (B) compounds with five deuteriums (dark blue trace), four deuteriums (red trace), three deuteriums (green trace), two deuteriums (grey trace), one deuterium (light blue trace) and unlabeled form (pink trace) were analyzed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

ideal internal standard possesses equal physicochemical properties as the analyte, therefore isotopically labeled standards are preferred [69]. Both fentanyl and norfentanyl have deuterated analogues which were already used as internal standard [43,45,50,52–54]. Our data confirmed similar behavior for fentanyl and fentanyl-d_5 and for norfentanyl and norfentanyl-d_5, making the deuterated compounds appropriate internal standards. However, the stability of the deuterated substances needs to be carefully evaluated. Injection of fentanyl-d_5 and norfentanyl-d_5, both stored in MeOH for 2 years at −20 °C revealed the presence of analogues containing 4, 3, 2, 1 or no deuteriums (Fig. 2). The most obvious explanation seems that the deuteriums are being exchanged with hydrogens, which has also been reported for other substances [72]. When using fresh internal standard containing 1 ng/ml fentanyl-d_5 and 5 ng/ml norfentanyl-d_5, the stability problem was not seen.

3.3. Optimal mobile phase and ionization interface

Initially, six mobile phases and two ionization interfaces (ESI and APCI) were investigated to determine which combination resulted in highest ionization efficiency. Tested mobile phases include high pH mobile phases (using ammonium bicarbonate and ammonium hydroxide) and the classical low pH mobile phases (using ammonium formate/acetate and formic/acetic acid) which are commonly used for analysis of basic compounds because acid mobile phases are thought to increase sensitivity because of increased ionization of bases at low pH. ESI and APCI were compared. As expected, more organic modifier results in more ionization due to changing solvents properties [73,74]. ESI clearly gave better results than APCI (Fig. 3). Considering the results for both fentanyl and norfentanyl, four mobile phases combined with ESI can be seen as most effective for ionization: ACN + 0.1% formic acid and H_2O + 25 mM ammonium formate at pH 3.0, MeOH + 0.1% formic acid and H_2O + 25 mM ammonium formate at pH 3.0, ACN and H_2O + 10 mM ammonium bicarbonate at pH 9.0 and finally MeOH and H_2O + 10 mM ammonium bicarbonate at pH 9.0 (Fig. 3). Moreover, the classical low pH mobile phases seem to be less efficient than the high pH mobile phases. This opposes to the simple view of electrospray ionization theory that states that the pH of the mobile phase determines the ionization of the analyte in ESI. Other reports have also described this so-called 'wrong-way-round' electrospray ionization [62–64,75–79]. Different theories have been described, but the exact mechanism is still unclear [63,78,79]. However, since several data show excellent results using high pH mobile phases and more and more compatible columns are available, the use of high pH mobile phases can become common in LC–MS/MS.

The XBridge C18 column (2.5 μm particle size, 3.0 mm × 50 mm) was next tested with the four most efficient mobile phases. As expected when using reversed phase chromatography, the elution of basic compounds at high pH results in longer retention times than elution at low pH because of their charge state (Table 2). Because of the difference in solvent strength, ACN as the organic modifier gave less retention than MeOH (Table 2). Peak shape was similar for all mobile phases. A final selection was based on peak area (sensitivity), retention (because of possible advantage of avoiding matrix effects, which are mostly seen in the solvent-front) and the cost of the solvent (considering the current ACN shortage, MeOH is preferred). Based on these criteria, MeOH and H_2O + 10 mM ammonium bicarbonate at pH 9.0 was selected as final mobile phase (Table 2).

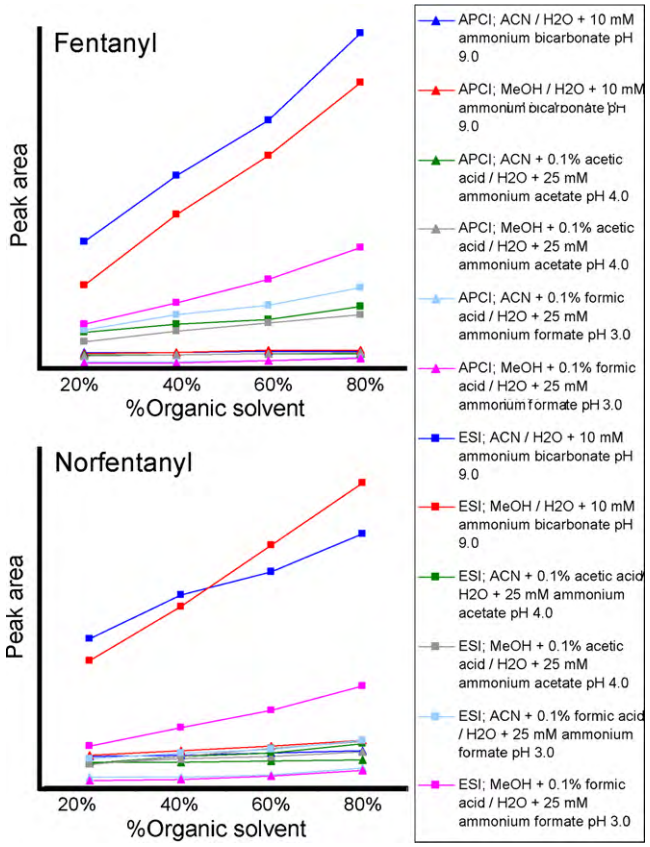

Fig. 3. Comparison of different combinations of mobile phase and source. Flow injection analysis was performed for each combination of source and mobile phase at 20, 40, 60 and 80% organic modifier. Measurements were made in triplicate and the mean peak area of fentanyl quantifier and norfentanyl quantifier was compared for the different combinations of mobile phase and source. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.4. Optimal SPE method and LC method

The gradient using H_2O + 10 mM ammonium bicarbonate at pH 9.0 (solvent A) and MeOH (solvent B) on the XBridge C18 column (2.5 μm particle size, 3.0 mm × 50 mm) was shortened to reduce analysis time: 0–2 min: 25–90%B; 2–3.5 min: 90%B; 3.5–4 min: 90–25%B; 4–6 min: 25%B.

As sample preparation, mixed-mode SPE was selected since it has already proven to be successful for the analysis of fentanyl

Table 2
Comparison of mobile phases on XBridge C18 column (2.5 μm particle size, 3.0 mm × 50 mm). A gradient with total run time of 11 min was used: 0–5 min: 10–90%B; 5–7 min: 90%B; 7–9 min: 90–10%B; 9–11 min: 10%B. Six replicate injections of a standard were analyzed on two separate days. Retention time (RT) and peak area (maximum peak area was set at 100%) were measured for fentanyl quantifier and norfentanyl quantifier.

Mobile phase	Fentanyl		Norfentanyl	
	RT (min)	Peak area	RT (min)	Peak area
MeOH 0.1% HCOOH/H_2O 25 mM NH_4COOH pH 3.0	3.99	45.0%	3.24	44.3%
ACN 0.1% HCOOH/H_2O 25 mM NH_4COOH pH 3.0	3.10	42.9%	2.24	24.0%
MeOH/H_2O 10 mM NH_4HCO_3 pH 9.0	5.77	100.0%	3.76	100.0%
ACN/H_2O 10 mM NH_4HCO_3 pH 9.0	4.72	77.0%	2.47	63.0%

Table 3
Comparison of matrix effects when changing chromatographic conditions and sample preparation in the analysis of urine. Except if differently mentioned, used LC conditions were: gradient elution with H_2O + 10 mM ammonium bicarbonate at pH 9.0 (solvent A) and MeOH (solvent B) on a XBridge C18 column (2.5 μm particle size, 3.0 mm × 50 mm): 0–2 min: 25–90%B; 2–3.5 min: 90%B; 3.5–4 min: 90–25%B; 4–6 min: 25%B with flow rate 0.5 ml/min. ME was calculated with Eq. (1), by using peak area or ratio of peak areas of analyte and internal standard. For each condition, two independent extractions of urine obtained from drug free volunteers were performed and injected into LC–MS/MS.

	Fentanyl		Norfentanyl	
ME%	Peak area	Ratio analyte/IS	Peak area	Ratio analyte/IS
Start	69	96	18	66
Less steep gradient[a]	79	94	56	100
ACN as solvent B	71	100	25	105
Alkaline wash	86	100	65	100
Alkaline wash 2.5 μm, 2.1 mm × 50 mm column	87	100	76	100
Alkaline wash 1.7 μm, 2.1 mm × 50 mm column	83	100	73	100

[a] A gradient with total run time of 12 min was used: 0–6 min: 25–40%B; 6–7 min: 40–90%B; 9–10 min: 90–25%B; 10–12 min: 25%B with flow rate 0.5 ml/min.

[34,35,41,80]. A simple protocol (conditioning as described in Section 2.6.1, sample application, washes with H_2O, acid and MeOH and elution with isopropanol–dichloromethane–ammonium hydroxide (20:78:2)) was used on the silica-based mixed-mode Bond Elut Certify SPE cartridges. The results were not repeatable (coefficient of variation% of peak area was >30%). When the elution solvent was evaporated to dryness, a white residue was seen. It seems that the high pH of the elution solvent dissolves some of the silica of the sorbent which traps some of the fentanyl and norfentanyl, even when resolving the residue with MeOH–H_2O (25:75). This theory was confirmed by the use of Bond Elut Plexa PCX cartridges. These have the same selectivity properties as the Bond Elut Certify SPE cartridges, but are polymeric and not silica-based. The results were repeatable and no white residue was seen.

3.4.1. ME, RE and PE

When calculating ME, RE and PE by using peak areas of the extracts of urine on the polymeric SPE cartridges, strong ion suppression was seen for norfentanyl (Table 3). As expected, when calculating the same values by using the ratios analyte/IS, less ion suppression was noticed. Internal standardization brings ME, RE and PE values closer to 100%, but cannot compensate the loss of sensitivity associated with matrix suppression or poor extraction. Therefore, sample preparation and chromatography was further optimized [81,82].

We started by adapting the chromatographic conditions, which can easily be changed. In order to separate the interferences from the analytes, the slope of the gradient was made less steep. A decrease in ion suppression was seen but the analysis time was longer (Table 3). Another option to change selectivity was substitution of MeOH by ACN, but the effect was minimal (Table 3). Changes in chromatography did not seem to be very successful to avoid matrix effects, therefore we decided to improve sample preparation. To decrease the amount of co-eluting compounds in the extract, an additional alkaline wash step was added to the SPE protocol. The optimal organic content of the alkaline wash solvent was determined by several washes with increasing amounts of MeOH in the alkaline solvent (10, 20, 30, 40, 50, 60, 70, 80, 90% MeOH). Elution of the analytes was first seen when washing with solvent containing 70% MeOH, therefore, the alkaline wash step was set at MeOH–H_2O–ammonium hydroxide (68.5:29.5:2). Ion suppression was lower for the SPE procedure where an alkaline wash step was added (Table 3).

To obtain a further decrease in signal suppression and increase in sensitivity, columns with different internal diameter and/or particle size were evaluated. Columns with smaller internal diameters have a smaller column volume and therefore increased sensitivity since the analyte will be more concentrated in the smaller peak volume. As can be deducted from the Van Deemter curve, the use of smaller particles improves efficiency and thus sensitivity [60,61,65]. Since the MS-parameters were optimized for a flow rate of 0.5 ml/min, we decided to keep the flow rate the same for all columns. An equal separation would be reached by keeping the ratio of gradient volume to column volume constant. However, since we wanted to improve separation (to decrease matrix effects), we kept the same LC conditions for all columns: the gradient is then less steep for the columns with a smaller column volume. Injection volume was also kept constant, since peak shape was good when injecting 5 μl on each column. Ion suppression of norfentanyl significantly decreased when using the 2.5 μm, 2.1 mm × 50 mm column or 1.7 μm, 2.1 mm × 50 mm column instead of the 2.5 μm, 3.0 mm × 50 mm column (Table 3). This can be explained by the smaller peaks produced by the 2.5 μm, 2.1 mm × 50 mm column and the 1.7 μm, 2.1 mm × 50 mm column: analytes will less co-elute with interferences during ionization, so matrix effects are lower. Other values (ME for fentanyl, RE and PE) were comparable for all three columns.

Compared to urine, the extraction of drugs from whole blood has an extra problem: blood (especially post-mortem blood) is more viscous than urine and red blood cells are present which can block the SPE cartridge [83]. Sonication of blood to fragment the red blood cells and dilution with a buffer to reduce viscosity have been reported to solve this problem and were therefore added to our protocol [83,84]. Considering the success of the alkaline wash and column with smaller internal diameter and particle size for the analysis of urine, we decided to use the same sample preparation and chromatography for whole blood.

3.4.2. Sensitivity

When comparing sensitivity a clear difference was seen between the three columns (Fig. 4). As expected, sharpest and highest peaks were obtained when using the column with 1.7 μm particle size. However, this column had also the highest backpressure (a maximum pressure of 600 bar versus 280 bar for the 2.5 μm, 2.1 mm × 50 mm column and 180 bar for the 2.5 μm, 3.0 mm × 50 mm column). To cope with this backpressure, adapted hardware (i.e. a UPLC- or UFLC-system) is necessary, since a classical HPLC will only tolerate maximum pressures around 300 bar.

3.4.3. Final LC–MS/MS method

The most sensitive and efficient method was selected. This method includes an alkaline wash step in the protocol on the Bond Elut Plexa PCX cartridges. A column with a smaller internal diameter (XBridge C18, 2.5 μm, 2.1 mm × 50 mm) decreased ion suppression and increased sensitivity. An even greater improve-

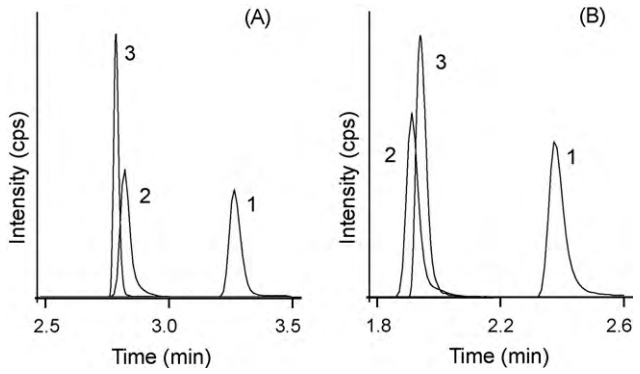

Fig. 4. Comparison of columns with different dimensions. 5 μl of a stock solution containing 10 ng/ml fentanyl and 10 ng/ml norfentanyl was injected in triplicate on each column: XBridge C18 (2.5 μm, 3.0 mm × 50 mm; peak 1), Xbridge C18 (2.5 μm, 2.1 mm × 50 mm; peak 2), Acquity C18 (1.7 μm, 2.1 mm × 50 mm; peak 3). Used LC conditions were: gradient elution with H_2O + 10 mM ammonium bicarbonate at pH 9.0 (solvent A) and MeOH (solvent B): 0–2 min: 25–90%B; 2–3.5 min: 90%B; 3.5–4 min: 90–25%B; 4–6 min: 25%B with flow rate 0.5 ml/min. The resulting chromatograms are shown for fentanyl quantifier (A) and norfentanyl quantifier (B).

ment was seen when using a column with a smaller diameter and smaller particle size (Aqcuity C18, 1.7 μm, 2.1 mm × 50 mm). Therefore, the method on the latter column was selected and fully validated for both urine and whole blood. Since the main focus was on sensitivity and not speed, the rather long analysis time of 6 min was not considered as a problem.

3.5. Method validation

3.5.1. Selectivity

The method was found to be selective for fentanyl, norfentanyl and their deuterated analogues: analysis of 10 urine extracts and 10 blood extracts showed no interferences with the detection of fentanyl, norfentanyl or the internal standards. No analyte peaks were observed in the zero samples, showing good stability of the internal standards.

3.5.2. ME and RE

To evaluate matrix effects, two approaches can be used: a post-extraction addition approach and post-column infusion [66]. Since the post-extraction addition approach yields more quantitative and thus more objective information, this method was used for determination of ME and RE [66,71]. As shown in Table 4, some degree of ion suppression was seen for fentanyl and norfentanyl in both urine and blood when calculating ME based on absolute peak areas. The variation on the degree of ion suppression can be explained by the samples included in method validation: post-mortem samples and samples derived from living persons, both containing various drugs. Post-mortem samples are known to be dissimilar because of redistribution and several other post-mortem processes which result in the presence of putrefactive and decomposition compounds [85]. Forensic samples, both ante- and post-mortem samples may contain a variety of drugs and their metabolites in a very wide concentration range. The variability in the composition of forensic samples is reflected in the variability of the matrix effect. The ME values based on peak area are also lower than those obtained in the method optimization part (Table 3). Again this can be explained by the use of forensic samples in the method validation. For the method optimization on the other hand, urine from living, drug free volunteers was used, which contains less interferences resulting in a higher value for ME. As the goal of the method optimization was simply to compare different chromatographic conditions and sample preparation protocols, the use of urine from drug free volunteers is justified. As described above, the utilization of the deuterated compounds effectively eliminates the matrix effects and variability is minimized. The ME values calculated with peak area ratios show no relevant matrix effects (Table 4). For recoveries, the same trend was seen as for ME when using calculations based on absolute peak areas (Table 4). Likewise, values based on peak area ratios are excellent (Table 4). For both matrices, there was no significant difference in ME or RE between LOW and HIGH concentrations levels independent of calculation method (one-way ANOVA, Bonferroni test, $p > 0.05$). For both compounds, there is an obvious difference in RE based on absolute peak areas between blood and urine (one-way ANOVA, Bonferroni test, $p < 0.05$), indicating that the method will be less sensitive for whole blood.

3.5.3. Processed sample stability

Plotting of the absolute peak areas of each analyte against injected time resulted in linear curves with slopes not significantly different from zero ($p > 0.05$) for all analytes at all concentration levels in both urine and whole blood. Therefore there was no indication of instability of processed samples over a time period of 9 h, which is the maximum time of storage in the cooled autosampler when performing a regular analytical run.

3.5.4. Linearity

In linearity experiments, a weighted least-squares linear regression model was used for calculation of calibration curves. The inverse of the squared concentration was found to be the appropriate weighting factor to account for unequal variances (heteroscedasticity) across the calibration range. The calibration curves showed good linearity from 0.0025 to 5 ng/ml for fentanyl in urine, 0.005–10 ng/ml for norfentanyl in urine and 0.01–20 ng/ml for both fentanyl and norfentanyl in blood ($R^2 > 0.998$). The back-calculated

Table 4
Matrix effects and recovery of the final LC–MS/MS method. ME and RE were calculated with Eqs. (1) and (2), by using absolute peak areas or the ratios of peak areas of analyte and IS. ME values below 100% indicate ion suppression, above 100% ion enhancement. Coefficients of variation (CV%) were calculated by dividing the mean by the standard deviation multiplied by 100%.

	Fentanyl				Norfentanyl			
	ME%	CV%	RE%	CV%	ME%	CV%	RE%	CV%
Absolute peak areas								
Urine LOW ($n = 5$)	77.7	10.4	83.5	12.2	62.4	14.1	62.5	9.8
Urine HIGH ($n = 5$)	71.6	13.7	90.9	12.1	60.7	12.0	58.9	6.1
Blood LOW ($n = 5$)	63.8	14.8	35.6	19.6	75.8	17.1	35.3	18.8
Blood HIGH ($n = 5$)	72.0	22.6	39.7	19.6	77.8	13.1	32.8	3.3
Peak area ratios								
Urine LOW ($n = 5$)	93.0	5.1	103.5	6.5	94.7	3.7	99.0	6.8
Urine HIGH ($n = 5$)	98.8	5.0	98.6	3.1	95.3	5.2	101.8	5.7
Blood LOW ($n = 5$)	104.8	6.0	96.8	8.3	101.0	4.0	103.6	3.6
Blood HIGH ($n = 5$)	96.8	7.5	93.4	10.1	105.1	4.8	100.1	3.5

Table 5
Precision and accuracy of the final LC–MS/MS method. Each sample was analyzed on each of 8 days. Accuracy, expressed as bias% and precision, expressed as repeatability and intermediate precision were calculated using Eqs. (5)–(7).

	Fentanyl			Norfentanyl		
	Bias%	Rep%	Int. prec%	Bias%	Rep%	Int. prec%
Urine LOQ ($n=2$)	13.6	14.8	12.1	7.0	13.8	14.2
Urine LOW ($n=2$)	14.1	6.8	8.6	−2.0	6.0	9.0
Urine MED ($n=2$)	3.0	4.6	6.1	3.1	6.8	7.7
Urine HIGH ($n=2$)	6.8	5.0	6.8	1.4	4.4	6.4
Urine ACR ($n=2$)	5.8	6.0	6.9	4.3	4.1	5.4
Blood LOQ ($n=2$)	3.8	8.2	9.4	1.9	10.5	10.4
Blood LOW ($n=2$)	13.4	12.0	12.7	1.0	8.3	8.4
Blood MED ($n=2$)	5.5	6.6	6.6	5.3	5.0	7.5
Blood HIGH ($n=2$)	0.6	8.4	7.5	8.6	4.8	7.1
Blood ACR ($n=2$)	3.1	5.2	5.8	0.1	3.5	5.4

values of the calibration points agreed closely with the theoretical values (all within 10% of theoretical value).

3.5.5. LOD and LOQ

For the determination of LOQ, different methods are available: LOQ can be based on precision and accuracy data, on the signal-to-noise ratio, on the standard deviation of the response from blank samples or on a specific calibration curve in the range of the LOQ [66]. We preferred the precision and accuracy approach, since this required no extra experiments (samples of precision and accuracy experiments could be used). The LOQ was fixed to the lowest concentration used for the calibration curve which fulfilled the criteria for LOQ based on precision and accuracy data. This gives a LOQ of 2.5 pg/ml for fentanyl in urine, 5 pg/ml for norfentanyl in urine and 10 pg/ml for both fentanyl and norfentanyl in blood. At these concentrations, precision was smaller than 20% and accuracy was within 20% of the nominal value (Table 5).

Except for the method based on precision and accuracy, the same methods can be used for calculation of the LOD [66]. In forensic samples, the signal-to-noise is quite variable and also heavily dependent on when and how long the noise is measured. The response of blank forensic samples is also variable, again because of the variable composition of forensic samples. Therefore, we opted for the third approach. A linear calibration curve in the range of the LOD was successfully established. The LOD was calculated by multiplying the ratio of the standard deviation of the intercept and the slope of the calibration curve by three. This resulted in a LOD of 0.3 pg/ml fentanyl in urine, 2.0 pg/ml norfentanyl in urine, 4.7 pg/ml fentanyl in blood and 5.0 pg/ml norfentanyl in blood. To evaluate these calculated LODs, we extracted two urine samples (spiked with 0.25 pg/ml fentanyl and 2.5 pg/ml norfentanyl) and two blood samples (spiked with 5 pg/ml fentanyl and norfentanyl). Fentanyl and norfentanyl could be identified at these concentration levels, so the estimated LODs by use of a calibration curve give an adequate result (Fig. 5). However, this is only an estimation of a general LOD. Every forensic sample can be different, so deviation of this LOD can be expected. A good way to control this is to evaluate the signal of the IS. By comparing the IS signal of a standard solution and the extracted sample, PE can be calculated. Since analyte and IS behave in the same way, the PE will be the same for the analyte. If the PE is comparable to the values obtained in this method validation, a LOD of 0.25 pg/ml for fentanyl and 2.5 pg/ml for norfentanyl in urine and 5 pg/ml for fentanyl and norfentanyl in whole blood can be assumed. In previous reports, LODs were

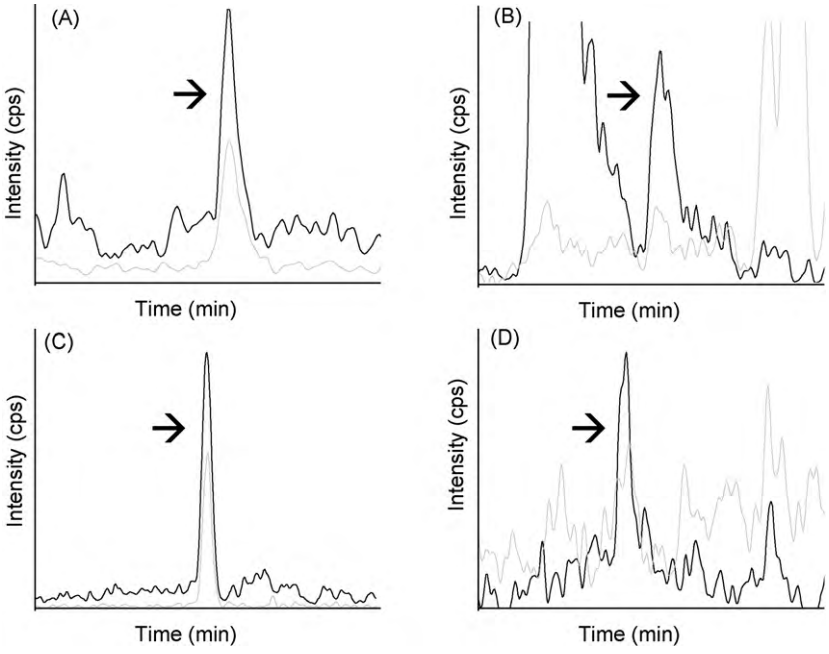

Fig. 5. Analysis of fentanyl and norfentanyl around LOD. 5 μl from an extracted urine sample containing 0.25 pg/ml fentanyl (A), 2.5 pg/ml norfentanyl (B), an extracted blood sample containing 5 pg/ml fentanyl (C) and 5 pg/ml norfentanyl (D) was injected onto LC–MS/MS. The arrow indicates the peak of the analyte at the right retention time. Black traces are quantifier ions, grey ones are qualifier ions.

3 pg/ml fentanyl and 21 pg/ml norfentanyl in urine and 80 pg/ml fentanyl and 330 pg/ml norfentanyl in whole blood [50,51,53,59]. Compared with these previous reports, this LC–MS/MS method is clearly superior.

3.5.6. Precision and accuracy

A minimum injection volume of 5 μl was required for the autosampler to have reproducible results. All accuracy data lay within the acceptance interval of 15% (20% for LOQ) of the nominal value and the precision values were smaller than 15% (20% for LOQ), indicating good accuracy and precision for both urine and whole blood (Table 5). Samples containing concentrations outside the calibration range (ACR) could be successful quantified by dilution with water prior to extraction. Dilution with blank matrix is more correct, but since matrix effects are compensated by the use of internal standards, dilution with water is in this study also appropriate.

3.5.7. Freeze/thaw stability and bench top stability

In freeze/thaw and bench top stability experiments all the acceptance criteria as described in Section 2.7.7 were fulfilled for fentanyl and norfentanyl at both concentrations (LOW and HIGH) in both matrices, indicating good stability of the samples.

4. Conclusions

To the best of our knowledge, the UFLC–MS/MS method presented here is the most sensitive method available for the analysis of fentanyl and norfentanyl in biological samples. Three factors contributed to the high sensitivity: (1) an alkaline wash step in the SPE protocol which removed interferences, thereby decreasing matrix effects, (2) the use of mobile phase with a high pH instead of the conventional low pH which resulted in a higher electrospray signal and (3) the use of columns with smaller particle size (1.7 μm versus 2.5 μm). The method was fully validated and successfully used for the analysis of both ante- and post-mortem forensic samples.

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