Determination of Opioids by Capillary HPLC-MS/MS

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Abstract

This application note demonstrates the use of the compact Axcend Focus LC capillary chromatograph coupled to an Agilent Ultivo triple quadrupole mass spectrometer for quantitative analysis of opioids in model aqueous samples. Components of a test mixture containing 8 opioids were identified, and calibration curves were constructed in the concentration range of 1-1000 ng/mL for fentanyl, norfentanyl oxalate (corresponding to 0.721-721 ng/mL norfentanyl free base), oxycodone, meperidine, and methadone, 10-1000 ng/mL for heroin, and 30-1000 ng/mL for codeine and desomorphine.

The method was used to monitor in-tip solid phase extraction of fentanyl and norfentanyl oxalate in aqueous samples containing these opioids at a concentration of 1 ng/mL each.

Introduction

Abuse of opioids is an ongoing social and public health problem. In recent years, it has become extremely acute because of the spread of illicitly manufactured fentanyl and its analogs [1, 2]. These readily available and inexpensive compounds are highly potent and easily result in accidental overdose [1, 2]. Very sensitive analytical methods are required for the detection and quantification of fentanyl-like compounds, since they occur in biological samples in low concentrations [2].

The current trend in the development of new chromatographic analytical methods is to miniaturize the sample preparation [3] and the separation procedures [4]. This satisfies the principles of green chemistry, which require dramatically reduced consumption of toxic organic solvents [5]. Using capillary chromatography for the detection and quantification of opioids in biological samples is in line with this trend [6]. In addition, the low flow rates used in capillary chromatography are beneficial for sensitive mass spectrometry detection [7].

Materials and Methods

Instrumentation

An Axcend Focus LC (2.2.0 Axcend Drive software, Axcend, Provo, UT, USA) was used in this application. A triple quadrupole mass spectrometer (G6465B Ultivo LC/TQ equipped with a Jet Stream Electrospray Ionization ion source, Agilent, Santa Clara, CA, USA) was interfaced to the compact capillary LC. MassHunter software was used for instrument control and data processing (acquisition: v1.1; qualitative and quantitative analysis: v10.0). Direct sample infusion into the mass spectrometer was performed with a Model 22 syringe pump from Harvard Apparatus (Holliston, MA, USA). Centrifugation in solid phase extraction experiments was carried out using a desktop centrifuge MagFuge with 12-tube 1.5-2.0 mL rotor from Heathrow Scientific (Vernon Hills, IL, USA).

LC to MS Interface

To accommodate mobile phase microflow rates, the regular nebulizer of the ion source was replaced with a microflow nebulizer (Part No. G1946-67260, Agilent). The transfer line from the column cartridge to the microflow nebulizer (25 cm long, 360 µm OD, 25 µm ID PEEKsil tubing, Part No. 0624374, Trajan, Melbourne, Victoria, Australia) was attached to the column using a zero-dead-volume 360 µm union with a 50 µm bore hole (Part No. C360UPK2, VICI Valco Instruments, Houston, TX, USA). The microflow nebulizer was attached to the end of the transfer line using a 1/16" to 360 µm zero-dead-volume reducing union with a 100 µm bore hole (Part No. C360RUS64, VICI Valco Instruments).

Chemicals and Solvents

Fentanyl and norfentanyl oxalate standard compounds (both 1 mg/mL in methanol) were purchased from Restek (Bellefonte, PA, USA). Codeine (1 mg/mL in methanol), desomorphine (1 mg/mL in acetonitrile), heroin (1 mg/mL in acetonitrile), meperidine (1 mg/mL in methanol), methadone (1 mg/mL in methanol), and oxycodone (1 mg/mL in methanol) were purchased from Cerilliant (Round Rock, TX, USA). D5-Fentanyl and D5-norfentanyl (both 100 µg/mL in methanol) used as internal standards were purchased from Sigma-Aldrich (MilliporeSigma, St. Louis, MO, USA). LC-MS grade water and acetonitrile were also obtained from Sigma-Aldrich while LC-MS grade formic acid was purchased from ThermoFisher (Waltham, MA, USA). Mixed mode solid phase extraction tips (Empore C18/SCX, 200 µL) were purchased from CDS (Oxford, PA, USA).

HPLC Method

A C18 capillary column (10 cm x 150 µm i.d., 1.8 µm particle size, CoAnn, Richland, WA, USA) was used. UV absorption was monitored at 235 nm using an on-column detector (Axcend). A binary gradient was generated from Solvent A (97:3:0.1 water/acetonitrile/formic acid, v/v) and Solvent B (97:3:0.1 acetonitrile/water/formic acid, v/v). The mobile phase program included an isocratic step at 3% B (0-0.5 min), linear gradients 3-42% B (0.5-4.5 min) and 42-97% B (4.5-6.5 min), and finally an isocratic step at 97% B (6.5-10 min). The flow rate was 1 µL/min and the injection volume was 250 nL (full loop).

MS Method

Specific molecular ions and ion fragments were identified for the opioid analytes by infusing a diluted test mixture (each component at 10 µg/mL) using a syringe pump at a flow rate of 1 µL/min and performing the analysis in product ion positive polarity mode. The identified ion transitions "molecular Ion → fragment Ion" were subsequently used to monitor the analytes in multiple reaction monitoring (MRM) mode. Deuterated fentanyl and norfentanyl were used as internal standards. The MRM method was optimized using MassHunter optimizer software by introducing a mixture of opioids (100 ng/mL each at 1 µL/min) into the mass spectrometer. Table 1 shows the identified optimum fragmentor voltage and collision energy values for the quantifier and qualifier ions of the individual analytes and internal standards. Other MS settings were as follows: capillary voltage, 3000 V; gas temperature, 200 ^{o}C; gas flow rate, 5 L/min; nebulizer pressure, 10 psi; dwell time, 50 ms.

Results and Discussion

Analyte Identification

The chemical structures of the analyzed opioids are presented in Figure 1 and chromatographic separation of a mixture of 8 opioids with UV detection at 235 nm is shown in Figure 2. Each analyte in the UV chromatogram was identified based on MS data as shown in the extracted ion chromatograms in Figures 3 and 4.

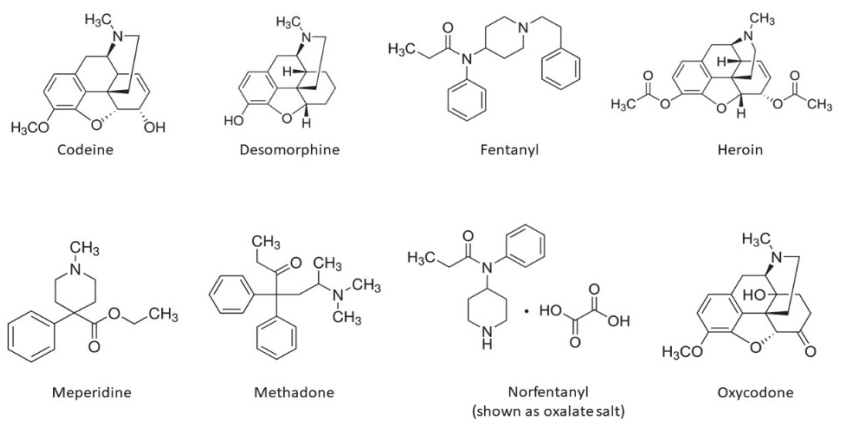

Figure 1. Chemical structures of the analyzed opioids.

Table 1. Detected Precursor and Product Ions of Opioids and Optimized Acquisition Parameters (MRM Positive Polarity Mode)

Compound Name	Precursor Ion (m/z)	Quantifier Product Ion (m/z)	Qualifier Product Ion (m/z)	Fragmentor Voltage (V)	Collision Energy (eV)
Codeine	300.1	165.1		153	57
Codeine	300.1		215.2	50	25
Desomorphine	272.1	195.1		153	33
Desomorphine	272.1		167.0	50	35
Fentanyl	337.2	188.1		147	21
Fentanyl	337.2		105.0	50	35
Heroin	370.1	268.2		50	25
Heroin	370.1		328.2	50	35
Meperidine	248.1	220.2		50	25
Meperidine	248.1		70.1	50	35
Methadone	310.2	265.2		50	10
Methadone	310.2		105.0	50	10
Norfentanyl	233.1	84.0		50	15
Norfentanyl	233.1		233.1	50	15
Oxycodone	316.1	298.2		50	20
Oxycodone	316.1		241.1	50	25
D5-Fentanyl (Internal Standard)	342.2	188.1	-	147	21
D5-Norfentanyl (Internal Standard)	238.2	84.0	-	50	15

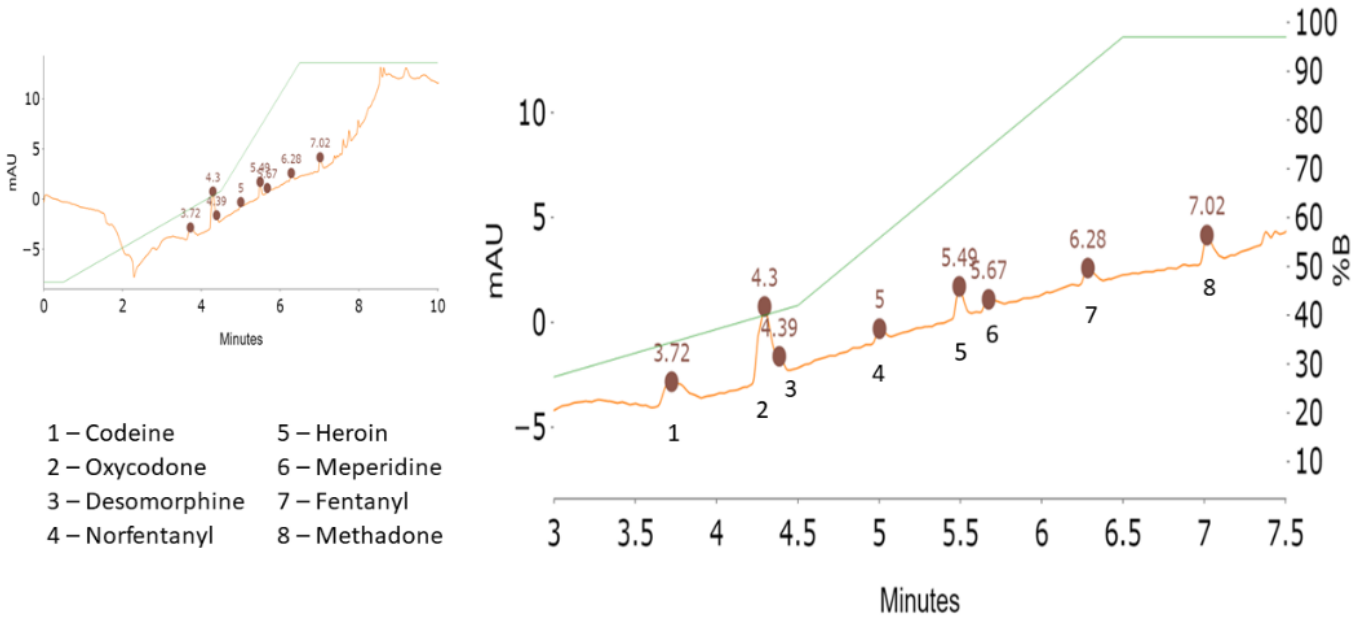

Figure 2. Analysis of a mixture of opioids (1 µg/mL each, except norfentanyl at 721 ng/mL corresponding to 1 µg/mL of norfentanyl oxalate) with UV detection at 235 nm. The identifications are based on mass spectrometry data (see Figure 3).

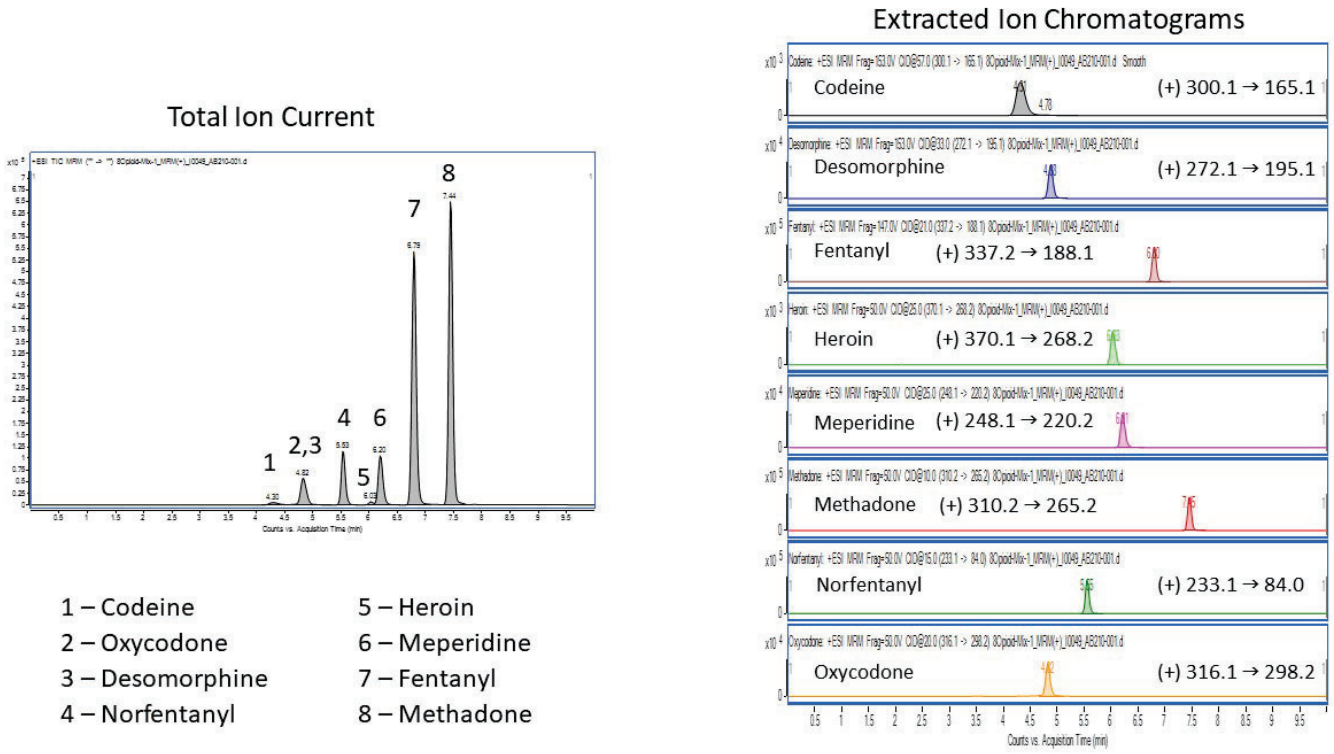

Figure 3. Analysis of a mixture of opioids (1 μg/mL each, except norfentanyl at 721 ng/mL corresponding to 1 μg/mL of norfentanyl oxalate) with MS detection in multiple reaction monitoring positive polarity mode.

Analyte Quantification

To normalize the intensities of MS signals, deuterated analogs of fentanyl and norfentanyl (D5-fentanyl and D5-norfentanyl) were added as internal standards to all test samples to give a concentration of 10 ng/mL each. These internal standards improved the results not only for fentanyl and norfentanyl, but for the other analytes as well. The best calibration curves for fentanyl, codeine, meperidine, and methadone were obtained with D5-fentanyl as an internal standard, and the best calibration curves for norfentanyl, desomorphine, heroin, and oxycodone were obtained with D5-norfentanyl as an internal standard. The linear coefficient of determination R^2 was above 0.99 in the following concentration ranges: 1-1000 ng/mL for fentanyl, norfentanyl oxalate (corresponding to 0.721-721 ng/mL of norfentanyl free base), oxycodone, meperidine, and methadone, 10-1000 ng/mL for heroin, and 30-1000 ng/mL for codeine and desomorphine. Calibration curves for fentanyl and norfentanyl are presented in Figures 5 and 6, respectively.

In-tip Solid Phase Extraction of Fentanyl and Norfentanyl

Based on fentanyl and norfentanyl structures and properties, a mixed mode solid phase C18/SCX capable of both hydrophobic and cation-exchange interactions was selected for testing the extraction of these opioids from aqueous samples. Loading was performed in acidified water to enhance both types of interaction, and elution was conducted using methanol containing ammonia to suppress hydrophobic and ionic interactions. An in-tip solid phase extraction format was chosen due to its convenience and small solvent consumption. Quantification based on the HPLC-MS/MS method described above was used for monitoring the extraction process. Calibration curves for this purpose were constructed using calibration standards at two concentration levels (within the linear concentration ranges verified earlier) with three replicates at each level.

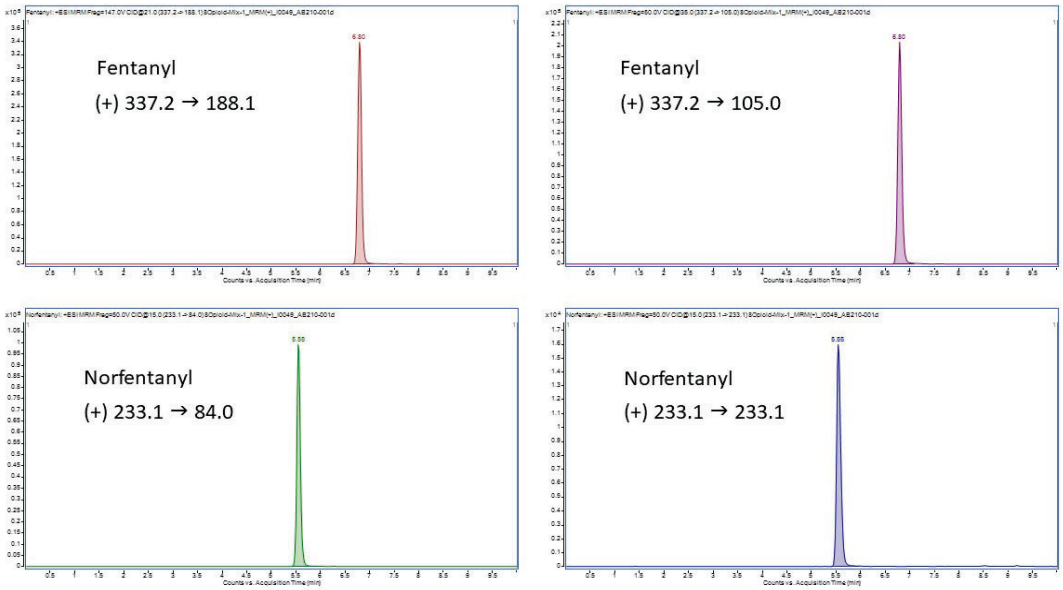

Figure 4. Extracted ion chromatograms of fentanyl at 1 µg/mL and norfentanyl at 721 ng/mL (corresponding to 1 µg/mL of norfentanyl oxalate) with MS detection in multiple reaction monitoring positive polarity mode.

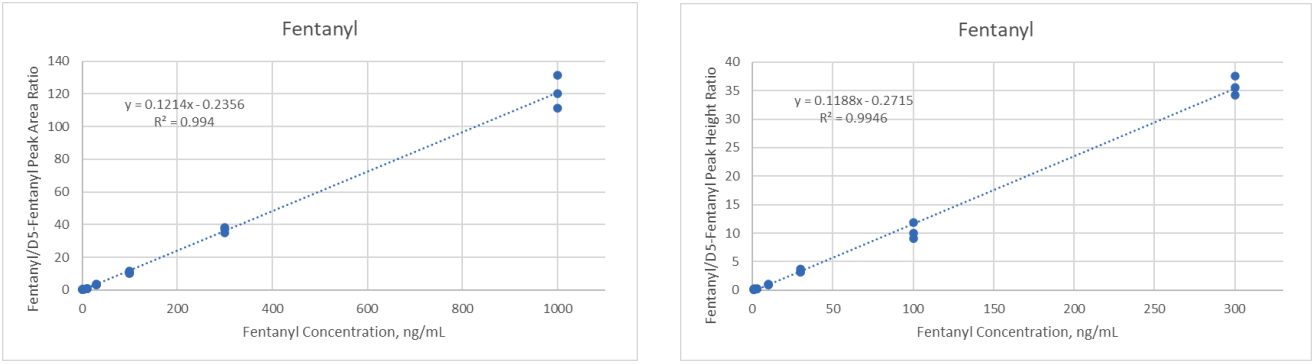

Figure 5. Calibration curves for fentanyl based on peak areas in the concentration range of 1-1000 ng/mL (left) and peak heights in the concentration range of 1-300 ng/mL (right).

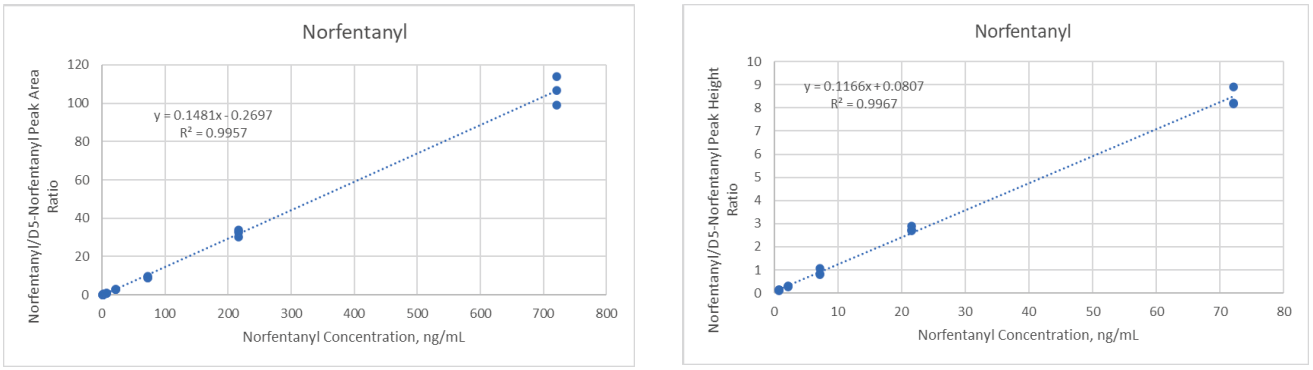

Figure 6. Calibration curves for norfentanyl based on peak areas in the concentration range of 0.721-721 ng/mL (corresponding to 1-1000 ng/mL of norfentanyl oxalate, left) and peak heights in the concentration range of 0.721-72.1 ng/mL (corresponding to 1-100 ng/mL of norfentanyl oxalate, right).

The solid phase extraction process consisted of the following steps:

1. Conditioning with 150 µL of methanol.
2. Activation with 150 µL of 0.1% formic acid in water.
3. Loading of the sample.
4. Washing with 150 µL of methanol.
5. Elution with 100 µL of 5% aqueous ammonia (29% NH_4OH) in methanol (3 portions in the initial experiments, 1 portion thereafter).
6. Evaporation to dryness by leaving the sample vials open in a fume hood overnight.
7. Reconstitution in 50 µL of 0.1% formic acid in water containing D5-fentanyl and D5-norfentanyl (10 ng/mL each).

In each step, the solid phase extraction tips were loaded with the corresponding liquid (solvent or sample) through the upper opening, and the liquid was forced through the layer of solid phase absorbent by centrifugation (7 min at 500 g).

In the initial experiments, the starting concentrations were 10 ng/mL of fentanyl and 7.21 ng/mL of norfentanyl (10 ng/mL of norfentanyl oxalate). Ten aliquots of 150 µL were loaded onto a single tip in the loading step. The total loaded volume was 1500 µL and the total loaded amount of fentanyl and norfentanyl oxalate was 15 ng each. This is well below the binding capacity of the tip, which is 40 µg according to the product specifications. The theoretical concentration factor (assuming 100% yield) was 30 (1500 µL / 50 µL = 30, where 1500 µL is the total loaded volume and 50 µL is the final reconstitution volume). The first fraction in the elution step contained 81% of the total loaded fentanyl and 67% of norfentanyl, indicating concentration factors of 24 and 20 for fentanyl and norfentanyl, respectively. Additional amounts of fentanyl and norfentanyl were found in the second elution fraction (0.8% and 1.1% of the loaded amount, respectively) and in the third elution fraction (0.2% and 0.3%, respectively). The unretained fraction contained 1.4% of the total loaded fentanyl and 1.7% of norfentanyl. Finally, the wash fraction contained 0.04% of the total loaded fentanyl and no quantifiable norfentanyl. Thus, under the test conditions, fentanyl and norfentanyl binding was quite efficient, the washing step did not cause significant losses of the target analytes, and a single 100 µL portion of the elution solvent was sufficient for acceptable recovery.

In the following experiments, a two-step extraction process was evaluated. The initial concentrations were 1.0 ng/mL of fentanyl and 0.721 ng/mL of norfentanyl (corresponding to 1.0 ng/mL of norfentanyl oxalate). In the first step 12 solid phase extraction tips were used. Each tip was loaded with 12 portions of 200 µL of starting solution, corresponding to 2.4 mL per tip and 28.8 mL total loaded volume. The elution fractions from each tip were evaporated to dryness by leaving the vials open in a fume hood overnight and then reconstituted in 0.1% formic acid in water. All reconstituted samples were combined and extracted a second time on a single solid phase extraction tip using the same procedure. The final reconstituted volume was 50 µL. The maximal theoretical concentration factor for the two-step process was 576 (28.8 mL/0.050 mL). The measured concentrations of fentanyl and norfentanyl in the final reconstituted fraction were 390 ng/mL and 177 ng/mL, corresponding to recoveries of 68% and 43% and concentration factors of 390 and 245, respectively.

To achieve higher concentration factors using the one- or two-step procedures described, the loading volumes would have to be increased. In principle, this is possible as only a small portion of the binding capacity of the solid phase extraction tips was used. However, this manual process is quite laborious. Therefore, an automated procedure based on this approach will be further developed using an automated sample preparation system.

Conclusions

The efficiency of detection and quantification using the compact Axcend Focus LC coupled to an Agilent Ultivo triple quadrupole mass spectrometer with microflow nebulizer was demonstrated.

Simultaneous quantification of eight opioids in model aqueous samples based on compact capillary HPLC – tandem mass spectrometry (HPLC-MS/MS) was successfully accomplished.

Linear calibration curves with a regression coefficient R^2 above 0.99 were obtained in the concentration ranges of 1-1000 ng/mL for fentanyl, norfentanyl oxalate (corresponding to 0.721-721 ng/mL norfentanyl), oxycodone, meperidine, and methadone; 10-1000 ng/mL for heroin; and 30-1000 ng/mL for codeine and desomorphine.

The developed HPLC-MS/MS method was applied for monitoring in-tip solid phase extraction of fentanyl and norfentanyl in a mixed reversed phase/cation exchange mode.

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