Extraction of Buprenorphine and Norbuprenorphine from Oral Fluid Using EVOLUTE® EXPRESS CX Prior to LC-MS/MS Analysis

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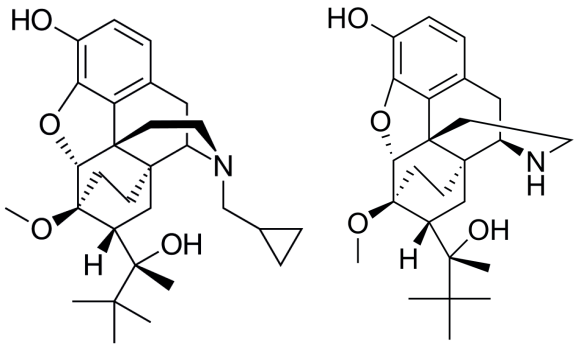

Figure 1. Structures of Buprenorphine and Norbuprenorphine.

Introduction

This application note describes a simple and robust mixed-mode cation exchange solid phase extraction protocol using EVOLUTE® EXPRESS CX 96-well plates for the extraction of buprenorphine and norbuprenorphine from oral fluid. Excellent extract cleanliness allows quantitation of the analytes across the range 0.1 ng/mL to 100 ng/mL using LC-MS/MS.

EVOLUTE EXPRESS solid phase extraction products combine powerful EVOLUTE sorbent chemistry with enhanced 'EXPRESS' components. EVOLUTE EXPRESS products dramatically improve flow characteristics, and enhance sample preparation productivity. By truly eliminating the need for column conditioning and equilibration, samples can be prepared using a simple, fast **load-wash-elute** procedure.

Analytes

Buprenorphine, Norbuprenorphine

Internal Standards

Buprenorphine D_4, Norbuprenorphine D_3

Sample Preparation Procedure

Format:

EVOLUTE® EXPRESS CX 10 mg fixed well plate, part number 601-0010-PX01

Sample Pre-treatment

Add 100 µL of sample (calibrator, QC or patient) to 900 µL of 0.1% aqueous formic acid. Either add an appropriate amount of internal standard separately or mix internal standard into formic acid pre-treatment solution prior to adding to sample.

Sample Loading

Load a 1 mL volume of pre-treated sample into each well at a flow rate of 1 mL/min (~10–12 drops/min).

Wash 1

Elute interferences with acetone:water (50: 50, (v:v), 2 x 1 mL)

Wash 2

Elute interferences with HPLC grade water (1 mL). Dry thoroughly (e.g. 30–40 psi, 2–5 minutes, using Biotage® PRESSURE+96) to remove any excess water.

Analyte Elution

Elute analytes with isopropanol: acetonitrile: conc. ammonium hydroxide solution (42:55:3, (v/v), 600 µL) at a flow rate of 1 mL/min (~10–12 drops/min).

Post Extraction

Dry the extract in a stream of air or nitrogen using a SPE Dry (40 °C at 60 L/min) or TurboVap (40 °C at 1.0 bar).

Reconstitution

Add methanol:water (60/40, (v:v)) with 0.1% formic acid (100 µL) to each well and let sample equilibrate for 15 minutes. Vortex the samples for 2–5 minutes.

HPLC Conditions

Instrument
Agilent 1260 Liquid Handling System (Agilent, Santa Clara, CA.)

Column
Restek Raptor Biphenyl (50 mm x 2.1 mm, 3 µm)

Mobile Phase
A: Water with 0.1% aqueous formic acid

B: Methanol with 0.1% formic acid

Flow Rate
0.3 mL/min

Injection Volume
10 µL

Column Temperature
35 °C

Table 1. HPLC Gradient Conditions

Step	Time (min)	Flow Rate (µL/min)	%A	%B
1	0.0	300	40	60
2	0.20	300	40	60
3	0.6	300	15	85
4	1.0	300	15	85
5	1.1	300	40	60
6	5.0	300	40	60

MS Conditions

A Sciex 4000 Q-Trap triple quadrupole mass spectrometer (Sciex, Foster City, CA.) was used equipped with a Turbo Ionspray® interface for mass analysis. Positive ions were acquired in the multiple reaction monitoring (MRM) mode with the ion source temperature at 500 °C.

Table 2. MRM transitions for target analytes

Analyte	MRM Transition	Declustering	Collision Energy (CE)
Buprenorphine	468 > 396.2	40	70
Norbuprenorphine	414 > 83	40	70
Buprenorphine-D_4	472.1 > 58.9	40	80
Norbuprenorphine-D_3	417.1> 83.0	40	80

Results/Discussion

Chromatography
Buprenorphine and norbuprenorphine (analyte and internal standard) were chromatographically separated on a biphenyl column using a linear gradient of methanol and water, both with 0.1% formic acid. The extracted ion chromatogram (see Figure 2) shows baseline separation of the two analytes and their corresponding deuterated internal standards.

Extraction Recoveries and Matrix Suppression
The analytes were spiked into blank oral fluid at three different concentration levels (10 ng/mL, 50 ng/mL and 100 ng/mL) and allowed to equilibrate for a minimum of 30 minutes. The spiked oral fluid samples were then extracted using the EVOLUTE® EXPRESS CX 10 mg 96-well plate using the protocol outlined above. The extracted solutions were clear in color. The recoveries of buprenorphine and norbuprenorphine were subsequently calculated at each of the concentration levels.

The impact of increased matrix load volumes on analyte recovery and measured matrix effects was evaluated. Load volumes of 100 µL, 200 µL and 500 µL of spiked oral fluid were pre-treated with 0.1% formic acid (final overall volume of 1mL) and loaded onto the EVOLUTE EXPRESS CX plate.

The samples were extracted, dried down and reconstituted as described. All of the extracted samples were clear in appearance. Analyte recoveries were determined using fortified blanks with the same amount of extracted matrix at each level. Figure 3 shows the recoveries for both analytes at the three different extracted volumes across a concentration range of 10 ng/mL to 100 ng/mL. The samples were extracted in replicates of 3 with RSDs less than 10%. It was observed that the best recovery was obtained for the smallest extracted amount of oral fluid matrix (i.e. 100 µL).

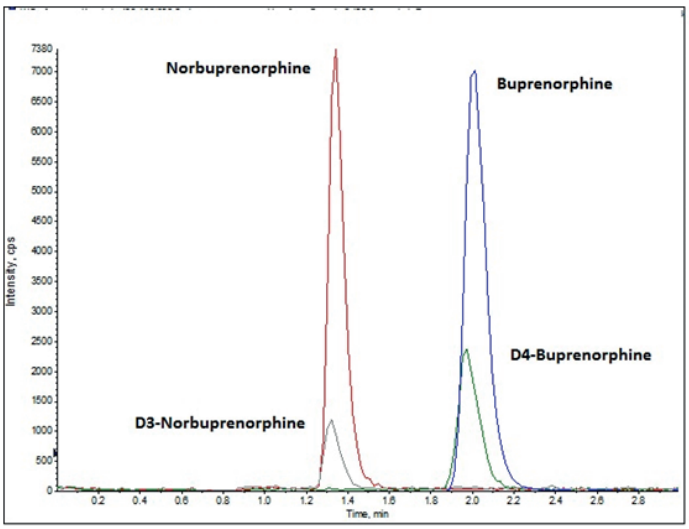

Figure 2. Extracted Ion Chromatogram for Buprenorphine and Norbuprenorphine with internal standards extracted from oral fluid spiked at 10ng/mL.

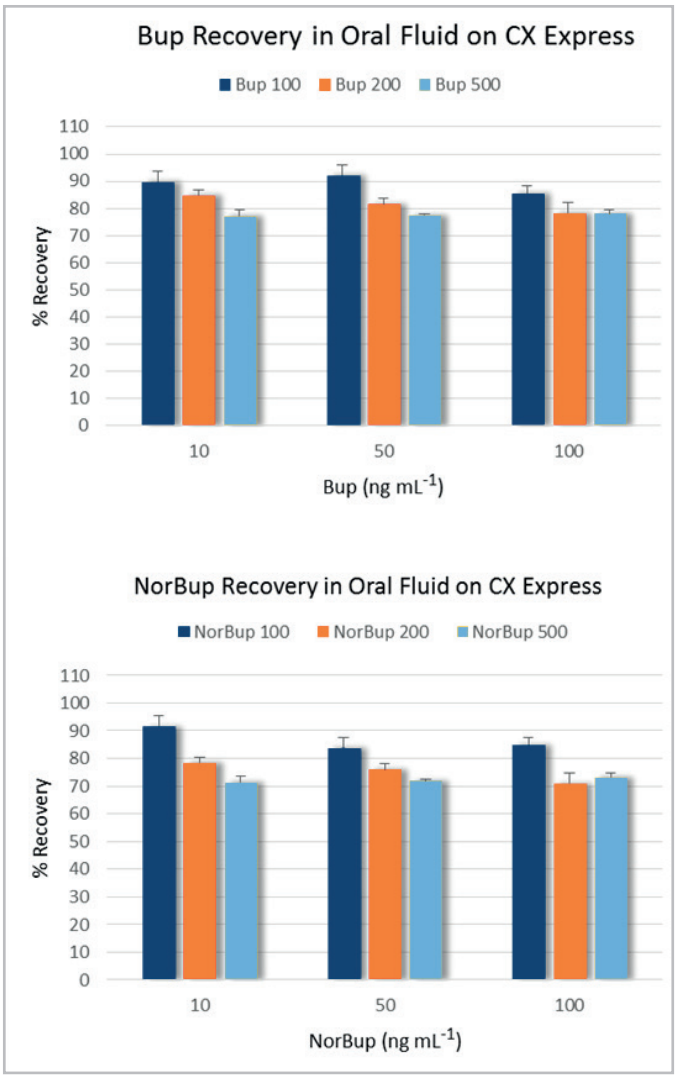

Figure 3. Percent recovery for Buprenorphine and Norbuprenorphine spiked into oral fluid at 10 ng/mL, 50 ng/mL and 100 ng/mL and extracted at matrix load volumes of 100 µL, 200 µL and 500 µL.

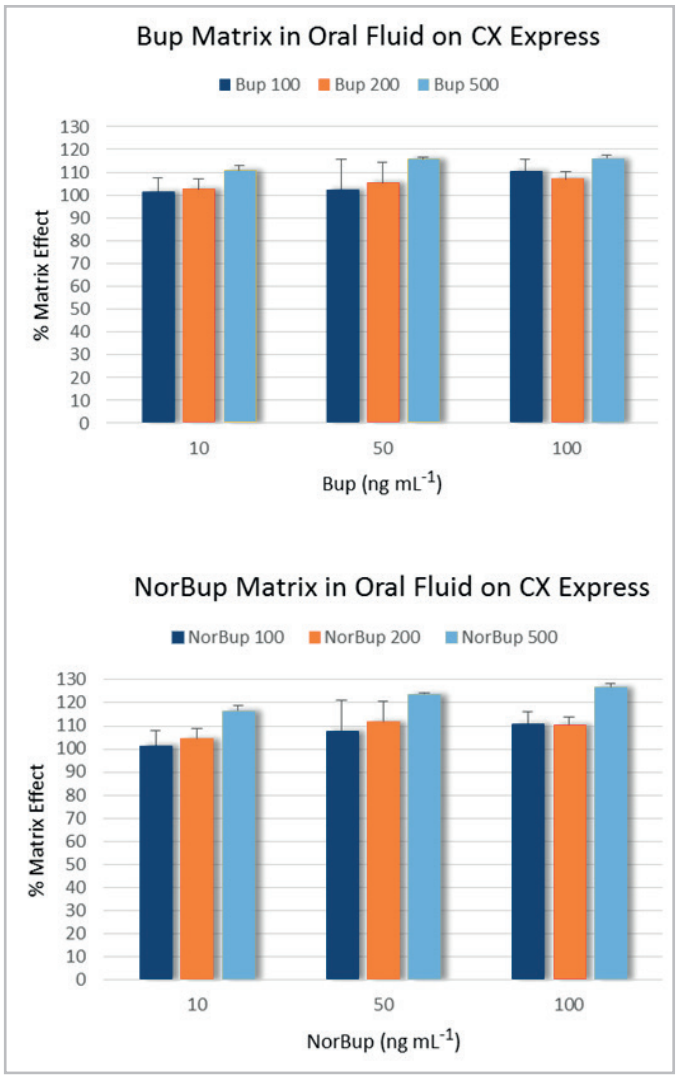

Figure 4. Plot of measured matrix effect for oral fluid spiked at 10 ng/mL, 50 ng/mL and 100 ng/mL at total matrix load volumes of 100 µL 200 µL and 500 µL

Matrix effects were determined at the three concentration ranges of 100 µL, 200 µL and 500 µL extracted samples (Figure 4). It was observed that the no matrix suppression was occurring with the samples but matrix enhancement was observed ranging from 1 –10% for samples extracted at 100 µL load volumes. The enhancement increased with higher matrix load volumes (> 20%). The matrix effect data correlates well with the recovery data suggesting that lower load volumes would be ideal for optimal sample extraction efficiencies.

Method Limit of Quantitation

A series of calibrators were prepared from negative oral fluid stock across a range of 0.1 ng/mL to 100 ng/mL for both buprenorphine and norbuprenorphine extracted at total load volumes of 100 µL. The analytes were spiked into the negative oral fluid matrix and allowed to equilibrate for no less than an hour. The calibrators were extracted as described above. The area response for each calibrator was plotted to demonstrate linearity of response across the concentration range as well as an apparent limit of quantitation for buprenorphine and norbuprenorphine. The observed limit of quantitation for buprenorphine and norbuprenorphine was 0.1 ng/mL and 0.5 ng/mL, respectively. Accuracy determinations for buprenorphine and norbuprenorphine are listed in Tables 3 and Table 4, respectively. Typical calibration curves generated from the data in the calibrator table had linear regression values of > 0.99 with and without weighting. A typical calibration curve observed for both norbuprenorphine and buprenorphine is shown in Figure 5.

Table 3. Buprenorphine calibrator table from 100 µL of extracted oral fluid samples.

Buprenorphine	Sample Type	Area (cps)	Target Conc (ng/mL)	Calculated Conc. (ng/mL)	Accuracy (%)
0.5 ng/mL	Standard	1.314e+03	0.50	0.4429	88.57
1.0 ng/mL	Standard	3.091e+03	1.00	0.956	95.60
5.0 ng/mL	Standard	1.494e+04	5.00	5.247	104.93
10 ng/mL	Standard	2.625e+04	10.00	9.673	96.73
25 ng/mL	Standard	6.691e+04	25.00	23.94	95.75
50 ng/mL	Standard	1.266e+05	50.00	50.8	101.59
100 ng/mL	Standard	2.880e+05	100.00	100.4	100.43

Table 4. Norbuprenorphine calibrator table from 100 µL of extracted oral fluid samples.

Buprenorphine	Sample Type	Area (cps)	Target Conc (ng/mL)	Calculated Conc. (ng/mL)	Accuracy (%)
0.5 ng/mL	Standard	1.391e+03	0.50	0.4753	95.05
1.0 ng/mL	Standard	2.983e+03	1.00	1.099	109.90
5.0 ng/mL	Standard	9.925e+03	5.00	4.805	96.10
10 ng/mL	Standard	2.042e+04	10.00	10.42	104.17
25 ng/mL	Standard	5.273e+04	25.00	23.3	93.21
50 ng/mL	Standard	9.701e+04	50.00	50.17	100.34
100 ng/mL	Standard	2.231e+05	100.00	101.2	101.23

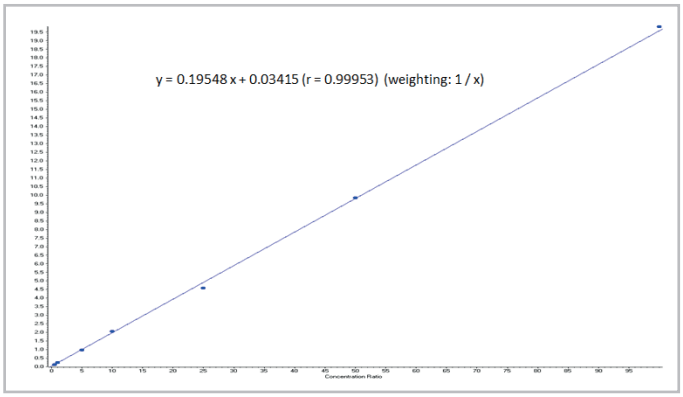

Figure 5. Calibration plot for Norbuprenorphine extracted on EVOLUTE® EXPRESS CX at a total matrix load volume of 100 µL.

Processing Notes

- As a guideline, sample flow rates of 1 mL/min (~10-12 drips/min) can be achieved at pressure of ~1 psi using EVOLUTE EXPRESS CX 10 mg fixed well plates, when processed using a Pressure+96 system
- Interference wash steps were loaded at a pressure of 5–10 psi

Biotage®

Ordering Information

Part Number	Description	Quantity
601-0010-PX01	EVOLUTE® EXPRESS CX 10 mg Fixed Well Plate	1
414001	Biotage® Extrahera™ Sample Processing Workstation	1
SD-9600-DHS-EU	Biotage® SPE Dry Sample Concentrator System 220/240 V	1
SD-9600-DHS-NA	Biotage® SPE Dry Sample Concentrator System 100/120 V	1
C103264	TurboVap® 96, Evaporator 220/240V	1
C103263	TurboVap® 96, Evaporator 100/120V	1

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