Quantitation of Clinical Research Pain Panel Analytes from Oral Fluid Utilizing Microelution Solid Phase Extraction Coupled with LC-MS/MS

Shahana Huq and Daniel Buchan | Phenomenex, Inc., 411 Madrid Ave., Torrance, CA 90501, USA

Introduction

Oral fluid has emerged as a popular biological matrix for analysis due to its non-invasive nature and ease of sample collection. It has wide applicability for drug testing and screening in clinical research. However, the analysis of these compounds in oral fluid becomes challenging due to the presence of the excipients, surfactants, and preservatives in the collection buffer of commercially available oral fluid collection (OFC) devices. These additives are necessary to ensure the stability and authenticity of the sample during collection and transport. However, the presence of these additives can foul the optics of the mass spectrometer and diminish the signal response if the samples are not cleaned up adequately before injection. In this communication we present an effective sample cleanup method for oral fluid analysis that targets 32 pain panel analytes, utilizing a mixed mode strong cation exchange Strata™-X-C microelution 96-well plate. A Kinetex™ core-shell 2.6 µm Biphenyl, 50 x 4.6 mm column was employed for fast chromatographic separation.

Materials and Methods

Reagents and Chemicals

Analytical reference standards and internal standards were purchased from Cerilliant® (Round Rock, TX, USA). Human saliva was obtained from GoldenWest (Temecula, CA, USA). The Intercept® i2he™ OFC device was obtained from OraSure Technologies, Inc. (Bethlehem, PA, USA). Ultrapure D.I. water was obtained via Sartorius® arium® Comfort II from Sartorius Corporation (Bohemia, NY, USA). All other chemicals were obtained from Sigma-Aldrich® Company (St. Louis, MO, USA).

LC Conditions – Quantitative Analysis for Pain Panel Analytes

Column: Kinetex 2.6 µm Biphenyl
Dimensions: 50 x 4.6 mm
Part No.: 00B-4622-E0
Mobile Phase: A: 10 mM Ammonium formate
B: Methanol
Gradient: 15 to 70 % B over 1 min, 95% B in 2 min, Hold 2.5 min
Flow Rate: 0.6 mL/min
Injection Volume: 5 µL
Temperature: Ambient
LC System: Agilent® 1260 Infinity
Detection: MS/MS
Detector: SCIEX® 4500 Triple Quad

MS/MS Conditions

Ion Source: ESI
Polarity: Positive
Source Temperature: 650 °C
GS1: 70
GS2: 70
CUR: 25
IS: 5000

LC Conditions – Qualitative Q1 Scan (200-2000 Da) of Preservative Buffer in OFC Device

Column: Kinetex 2.6 µm Biphenyl
Dimensions: 50 x 2.1 mm
Part No.: 00B-4462-AN
Mobile Phase: A: 0.1 % Formic acid in water
B: 0.1 % Formic acid in Methanol
Gradient: 10 to 95 % B over 5 min, Hold 1.5 min
Flow Rate: 0.5 mL/min
Injection Volume: 1 µL
Temperature: Ambient
LC System: Agilent 1260 Infinity
Detection: MS/MS
Detector: SCIEX 4500 Triple Quad

MS/MS Conditions

Ion Source: ESI
Polarity: Positive
Source Temperature: 650 °C
GS1: 70
GS2: 70
CUR: 25
IS: 5000

Solid Phase Extraction (SPE) Sample Preparation

Sample Pretreatment:	Drug free human saliva was spiked (conc. used as per Table 1) with standards. 1 mL of oral fluid was pipetted onto the cellulose pad and allowed to absorb until the indicator window turned blue. The saturated pad was placed into a transport tube containing buffer solution and allowed to sit overnight. The plastic nipple at the end of transport tube was removed, and the tube was placed in a centrifuge at 6000 rpm for 10 minutes. The supernatant was collected.	
	Strata-X-C 2 mg/well 96-well Microelution plate (8M-S029-4GA)	Strata-X-C 30 mg/well 96-well plate (8E-S029-TGB)
Condition:	200 µL Methanol	1 mL Methanol
Equilibrate:	200 µL Water	1 mL Water
Load:	150 µL supernatant diluted with 150 µL 1 % Formic acid in water Total volume: 300 µL	0.5 mL supernatant diluted with 0.5 mL 1 % Formic acid in water Total volume: 1 mL
Wash 1:	200 µL water	1 mL water
Wash 2:	200 µL 50 % Acetone in 1 % Formic acid	1 mL 50 % Acetone in 1 % Formic acid
Dry down 1:	30 sec at high vacuum (15-20 in. Hg)	5 min at high vacuum (15-20 in. Hg)
Elute:	2 x 50 µL Methanol / Acetonitrile / Ammonium Hydroxide (5:5:2, v/v/v)	2 x 0.5 mL Methanol / Acetonitrile / Ammonium Hydroxide (5:5:2, v/v/v)
Dry down 2:	Evaporate to dryness under a gentle stream of nitrogen at 40-45 °C Time: ≅ 4-5 min Total dry down time: ≅ 5 min	Evaporate to dryness under a gentle stream of nitrogen at 40-45 °C Time: ≅ 20 min Total dry down time: ≅ 25 min
Reconstitute:	100 µL initial mobile phase	0.35 mL initial mobile phase

Total time difference in processing samples using the SPE 96-well Microelution plate: ≅ 20 mi

Results

Figure 1. Representative Chromatogram of 32 Pain Panel Analytes in Oral Fluid Extracted using a Strata-X-C 96-well Microelution Plate and Analyzed by a Kinetex 2.6 µm Biphenyl Column.

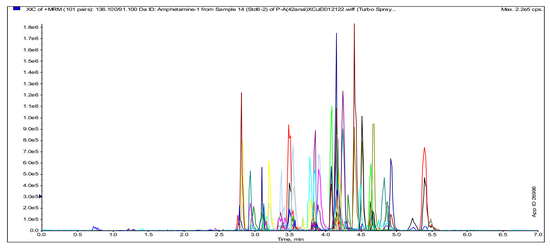


Figure 2. Representative Q1 Scan Chromatogram for Cleanup of Intercept i2he Preservative Oral Fluid Buffer Extracted using a Strata-X-C 96-well Microelution Plate and Analyzed by a Kinetex 2.6 µm C18 Column.

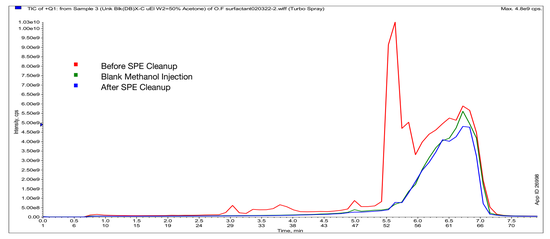


Figure 3. Ion Suppression/Enhancement Study by Post-column Infusion of Codeine to Evaluate Relative Cleanliness of Oral Fluid Sample Extract With or Without Using a Strata-X-C 96-well Microelution Plate.

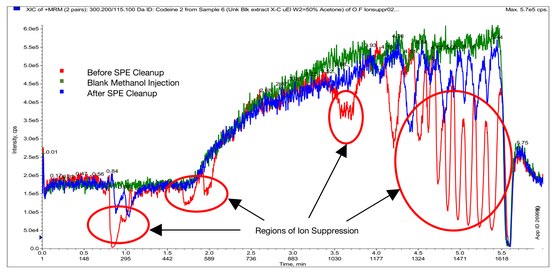
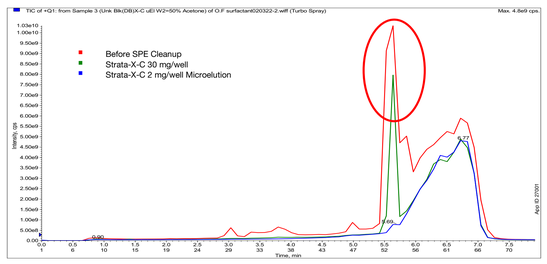


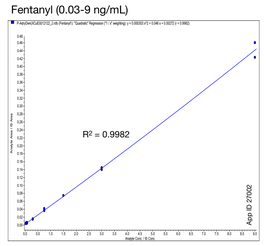
Figure 4. Parallel Comparisons of Q1 Scan (200-2000 m/z) for Cleanup of Intercept i2he Preservative Oral Fluid Buffer Extracted using a Strata-X-C 30 mg/well 96-well Plate versus a Strata-X-C 2 mg/well 96-well Microelution Plate.



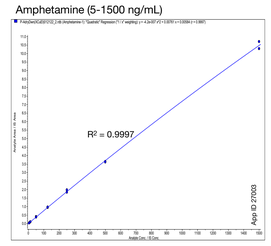
Results

Figure 5. Linearity Curves for Selected Analytes in Oral Fluid Sample Extracted using a Strata-X-C 96-well Microelution Plate over a 300-fold Dynamic Concentration Range.

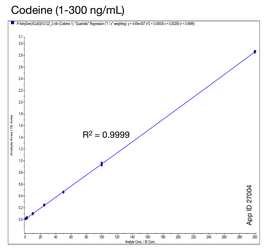
Fentanyl (0.03-9 ng/mL)



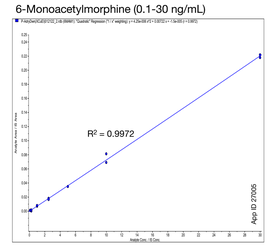
Amphetamine (5-1500 ng/mL)



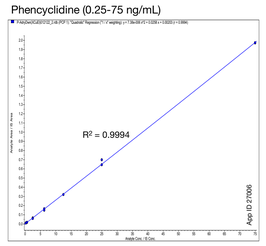
Codeine (1-300 ng/mL)



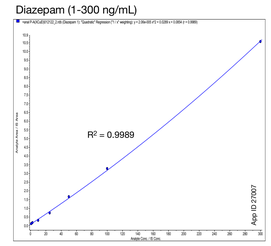
6-Monoacetylmorphine (0.1-30 ng/mL)



Phencyclidine (0.25-75 ng/mL)



Diazepam (1-300 ng/mL)



Discussion

The Kinetex 2.6 µm Biphenyl column provides fast chromatographic separation and good selectivity for critical isomeric (codeine/hydrocodone, morphine/hydromorrphone, 6-Monoacetylmorphine/Naloxone) pairs (**Table 1**). To remove the harmful effect of the components of the OFC device on the LC-MS/MS, an aggressive organic wash was necessary. The prescribed SPE method resulted in a clean oral fluid extract with minimal interference as observed in the Q1 scan monitored from 200 to 2000 Da (**Figure 2**).

A qualitative matrix effect experiment by post-column infusion was conducted. Upon continuous infusion of codeine, multiple suppression zones were revealed for the injection of unextracted preservative buffer. The microelution SPE successfully removed most of those interferences that were responsible for ion suppression (**Figure 3**). The total ion current experiment by Q1 scan demonstrates the relative cleanliness of the extracted samples using the 2 mg microelution over traditional 30 mg bed mass. The poor retention of the microelution SPE for the unwanted excipients results in a cleaner extract and an effective sample prep choice for oral fluid analysis (**Figure 4**).

The QC samples for replicate extraction at 3 different concentration levels showed precision and accuracy data between 1.4 to 20.3 % and 80 to 118 %, respectively, which are within acceptable industry standard (**Table 2**). The dynamic range of this method was tested with seven calibrators over a 300-fold concentration range with linearity values of R2 ≥ 0.995 (**Figure 5**, **Table 1**). The simplified microelution sample extraction method provides the ideal combination of automatability and high throughput with minimum solvent usage. The workflow is 20 minutes faster than conventional format.

Conclusion

The prescribed sample prep method utilizing microelution SPE resulted in a simple, rapid extraction for identification and quantitation of 32 pain panel analytes from oral fluid which is cost effective and can efficiently be incorporated in clinical workflow analysis.

Results

Table 1. MRM Transitions and Linearity Data for 32 Pain Panel Anayltes Extracted from Oral Fluid using the Strata-X-C 96-well Microelution Plate.

Analyte Name	RT (min)	Reference conc. (ng/mL)	Q1 (m/z)	Q3 (m/z)	Linearity Range (ng/mL)	Linear regression (R2)
Hydroxyalprazolam	4.1	100	325.1	297	1-300	0.998
Amphetamine	2.8	500	136.1	91.1	5-1500	0.999
Benzoylecgonine	2.8	150	290.1	168.1	1.5-450	0.999
Codeine	3.8	100	300.2	152.1	1-300	0.999
Diazepam	4.6	100	285	193.2	1-300	0.998
3,4-Methylenedioxymethamphetamine	3.2	250	194.1	105.1	2.5-750	0.999
Methamphetamine	2.99	500	150.1	91	5-1500	0.998
Oxymorphone	3.3	100	302.1	227	1-300	0.997
Phencyclidine	4.9	25	244.3	91	0.25-75	0.999
Sufentanil	4.9	3	387.2	238.1	0.03-9	0.995
6-Monoacetylmorphine	3.5	10	328.1	165.1	0.1-30	0.998
Clonazepam	3.8	100	316.1	270.1	1-300	0.995
2-Ethylidene-1,5-dimethyl-3,3-dipehnylpyrrolidine	5.2	100	278.2	234.2	1-300	0.997
Fentanyl	4.6	3	337.3	105.1	0.03-9	0.998
Flunitrazepam	4.3	100	314.1	268.2	1-300	0.998
Flurazepam	4.7	100	388.2	315.2	1-300	0.996
Hydrocodone	4.2	100	300.2	199	1-300	0.999
Hydromorphone	3.4	100	286.1	185.1	1-300	0.999
3,4-Methylenedioxyamphetamine	2.9	250	180.1	133	2.5-750	0.999
Methyl diethanolamine	3.2	250	208.2	163	2.5-750	0.998
Meperidine	3.9	250	248.2	220.2	2.5-750	0.999
Methadone	5.4	100	310	265	1-300	0.999
Midazolam	4.5	100	326.1	291.1	1-300	0.999
Morphine	3.1	100	286.1	152.1	1-300	0.997
Naloxone	4.17	100	328.2	212	1-300	0.995
Naltrexone	4.19	100	342.2	267.1	1-300	0.996
Nordiazepam	4.15	100	271	140	1-300	0.997
Normeperidine	3.5	100	234.1	160.1	1-300	0.995
Oxycodone	4.2	100	316.1	241.2	1-300	0.999
Temazepam	4.3	100	301.1	255.1	1-300	0.996
Tramadol	3.5	100	264.1	58.1	1-300	0.999
Cocaine	4.2	100	304.2	150	1-300	0.998

Table 2. Precision and Accuracy Data for 32 Pain Panel Analytes Extracted from Oral Fluid using the Strata-X-C 96-well Microelution Plate.

Analyte Name	QC-1 (5% of Reference) (ng/mL)		QC-2 (40% of Reference) (ng/mL)		QC-3 (2x Reference) (ng/mL)	
	% Accuracy	% CV (N=4	% Accuracy		% CV (N=4	
Hydroxyalprazolam	104.2	6.4	105.9	7.4	108.4	13.1
Amphetamine	103.7	13.5	99.3	5.2	94	6.2
Benzoylecgonine	111.3	11.3	114.3	13.4	105.5	2.9
Codeine	108.9	12.3	108.6	6.2	103.2	3.3
Diazepam	101.3	6.2	105.3	9.9	104.9	9.9
3,4-Methylenedioxymethamphetamine	109.4	10.1	99.8	4.7	99.9	2.2
Methamphetamine	99.1	6.4	95.6	3.7	93.4	3.6
Oxymorphone	106.8	5.3	109	7	100.6	4.8
Phencyclidine	95	4.8	100.3	7.6	97	1.5
Sufentanil	103.9	18.1	118.1	10.9	89.6	11.3
6-Monoacetylmorphine	99.3	16.7	104.9	4.3	103.8	4.2
Clonazepam	108.3	16.4	99.6	11	95.2	7
2-Ethylidene-1,5-dimethyl-3,3-dipehnylpyrrolidine	114.3	15.6	99.8	12.8	105.4	10.9
Fentanyl	107.4	14.9	117	8.8	98.8	1.9
Flunitrazepam	107.3	18.1	113	15.6	115	10.2
Flurazepam	98.5	20.3	114.4	14.9	117.8	1.9
Hydrocodone	112.4	8.8	107.4	7.4	95.7	1.8
Hydromorphone	116.3	13.5	112.9	3.4	108.7	1.6
3,4-Methylenedioxyamphetamine	100	6.7	109.4	4.9	94.9	5.3
Methyl diethanolamine	110.8	10.6	102.3	4.5	101.1	5.4
Meperidine	91.6	15.3	98.6	3.4	91.3	3.2
Methadone	86.5	9.3	89.5	9	92.5	5.9
Midazolam	12.2	13	111.1	7.6	105.7	8.5
Morphine	102.6	7.1	102.5	4.3	93.9	5.3
Naloxone	113.5	19.3	102.7	14.9	98.7	6
Naltrexone	105.2	8.5	109	10.2	97.6	6.5
Nordiazepam	93.6	14.5	118	15.5	97.1	14.4
Normeperidine	80.8	18.3	90.2	13.2	95.2	11.2
Oxycodone	115.5	5.1	112.1	8.1	97.9	1.4
Temazepam	96.1	10.7	116.9	11.2	109.3	12.5
Tramadol	92.3	9.6	109.1	10.3	85	3
Cocaine	102.7	5.3	114.8	7.7	91	15.1