Improved Method for the Analysis of 31 Drugs of Abuse/Pain Management Panel in Oral Fluid Samples using the Thomson eXtreme® Filter Vials by LC-MS/MS

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Abstract

The goal of this study was to improve the sample preparation for the analysis of drugs of abuse/pain management panels in oral fluids. The oral fluid samples were collected with Intercept® i2he™ Oral Fluid Collection Devices. The diluted oral fluid samples were filtered using Thompson Filter Vials, followed by LC/MS/MS analysis. The most critical aspects of reliable Oral Fluid analysis are the reduction of interferences from the sample matrix and analyte recovery. Traditionally, SPE, SLE and centrifugation have been used to reduce matrix interference prior to MS analysis. However, these techniques are time consuming, adversely impact recovery, require expensive consumables and equipment and use large amounts of solvent. Thomson eXtreme® Filter Vials (patented) offer multi-layer filtration for viscous samples and samples containing up to 30% solid particulates.

Experimental

Equipment:

Improved Method: 31 drugs

- Orasure Technologies Intercept® i2he™ Oral Fluid Collection Device (p/n 3001-2673)
- Orasure Technologies Intercept i2he Diluent (p/n 1001-0384)
- Thomson eXtreme|FV® 0.2µm PVDF (p/n 85531)
- Thomson 48 position Vial Filter Press (p/n 35010)
- Thomson 48 position Vial Filter Press (Part # 35010)
- Eppendorf MixMate®
- AB Sciex 4500 Mass Spectrometer
- Shimadzu Prominence HPLC equipped with:
  - Autosampler: SIL-20AC HT
  - Pumps A, B: LC-20AD
  - Communication Bus Module: CBM-20A
  - Column Oven: CTO-20A
  - Degasser: DGU-20A$_5$R
- 31 Analytes, see Table 1:
  - Column: Restek Ultra Biphenyl Columns (5µm, 50 x 2.1 mm)
    - Mobile Phases:
      - A: 0.1% Formic Acid in HPLC Water
      - B: 0.1% Formic Acid in Methanol
    - Flow Rate: 0.5 mL/min
    - Injection Volume: 15µL
    - Run-Time: 8 minutes

Obsolete Method: 4 drugs

- Caliper Life Sciences Turbo-Vap® Concentration Workstation
- Rapid Trace® Solid Phase Extraction Workstation
- Vortex Mixer
- AB Sciex 3200 Mass Spectrometer
- Shimadzu Prominence HPLC
  - Autosampler: SIL-20AC HT
  - Pumps A & B: LC-20AD
  - Communication Bus Module: CBM-20A
  - Column Oven: CTO-20AC
  - Degasser: DGU-20A$_5$
- Morphine and BZE
  - Column: Restek Ultra Biphenyl (5µm, 50 x 2.1mm)
    - Mobile Phases:
      - A: 0.1% Formic Acid in HPLC Grade Water
      - B. 0.1% Formic Acid in HPLC Grade Methanol
    - Flow rate: 0.5 mL/min
    - Injection Volume = 30µl
- PCP and Methadone
  - Column: Restek Allure Biphenyl (5µm 50 x 2.1mm)
    - Mobile Phases (Isocratic 10% Water in Methanol):
      - A: 0.1% Formic Acid in HPLC Grade Water
      - B. 0.1% Formic Acid in HPLC Grade Methanol
    - Flow rate: 0.3 mL/min
    - Injection Volume: 30µl

Sample Preparation

Improved Sample Preparation:

1. Allow standards, specimens and control to come to room temperature.
2. Add 100 µL of 10% Methanol / Water
3. Add 100 µL of Standard (Intercept i2he Diluent)/ Control/oral fluid sample + 10uL Internal Standard
4. Place Thomson Filter Plunger on top of the Thomson vial, Thomson vials -eXtreme/FV® 0.2µm PVDF, w/Pre-Slit Red Cap (p/n #85531)
5. Press filter plunger down approximately ¼ of the way into each of the Thomson Vial outer shells.
6. Vortex for 10 seconds using the Eppendorf MixMate®.
7. Press Filter plunger the rest of the way down using the Thomson 48 position Vial Filter Press.

8. Extracts are ready for LC/MS/MS analysis using the Shimadzu / AB Sciex 4500

Obsolete Sample Preparation:

1. Allow standards, specimens and control to come to room temperature.
2. To appropriately labeled 13 x 100 mm tubes add 3 mL of 50mM Phosphoric Acid.
3. Prepare the 13 x 100 mm tubes for analysis. Standards/Controls/Patient Samples
4. Vortex for 10 seconds.
5. The tubes are now ready for automated extraction using on the Caliper Life Sciences Turbo-Vap® Concentration Workstation
6. After the elution is complete on the Rapid Trace®, remove the racks with the tubes intact.
7. Add 50µL of 1% HCL in Methanol to each tube.
8. Vortex for 15 seconds.
9. The original sample tubes and the used SPEC DAU Columns can be discarded.
10. Take to dryness at 55°C in the Caliper Life Sciences Turbo-Vap®.
11. Reconstitute samples by adding 1 mL of 10% HPLC Grade Methanol in Water to all tubes.
12. Vortex for 15 seconds.
13. Extracts are ready for LC/MS/MS analysis using the Shimadzu / AB Sciex 3200

Result

The improved method utilizes the Thomson eXtreme Filter Vials for sample clean-up significantly reducing the cost and time of per sample analysis. This method was validated for all the analytes in Table 1. Mass spectrum of all the analytes in Table 1 can be seen in Fig. 5. Table 2 shows the 4 drugs that were analyzed in oral fluid by the obsoleted method. Table 3 shows the transitions used to validate the improved method. Linearity of the assay for the drugs listed in Table 1 is displayed in Table 4. Table 5 shows ion suppression and drug recovery for analytes in table 1. Unextracted standards (neats) were run along with 3 different negative patient samples, extracted and spiked with standard and internal standard post extraction at the cutoff concentration to access ion suppression and drug recovery. To calculate drug recovery, the mean area counts of the extracted samples was compared to the mean area counts of the unextracted samples. To determine ion suppression, the mean concentration of the extracted samples was compared to the mean concentration of the post-extracted samples.

Table 1. The following 31 drugs in oral fluid will be analyzed by this "Improved Method":

6-Monoacetylmorphine (6-MAM)	7-Aminoclonazepam (7AMINO)	Alprazolam (ALPR)
Amphetamine (AMPH)	Benzoylecgonine (BE)	Buprenorphine (BUP)
Carisoprodol (CARIS)	Clonazepam (CLONZ)	Cocaine
Codeine (CODE)	Diazepam (DIAZ)	Fentanyl (FENT)
Hydrocodone (HCOD)	Hydromorphone (HMOR)	Lorazepam (LOR)
Meprobamate (MEPRO)	Methadone (MTHD)	Methamphetamine (MAMP)
Methylenedioxyamphetamine (MDA)	Methylenedioxymethamphetamine (MDMA)	Morphine (MORP)
Norbuprenorphine (NBUP)	Nordiazepam (NDIAZ)	Norfentanyl (NFENT)
Oxazepam (OXAZ)	Oxycodone (OCOD)	Oxymorphone (OMOR)
Phencyclidine (PCP)	Temazepam (TEM)	Zolpidem (ZOLP)
α-hydroxy-Alprazolam (OH-AL)		

Table 2. The following analytes were analyzed in the "Obsolete Method"

Benzoylecgonine (BE)
Phencyclidine (PCP)
Methadone (MTHD)
Morphine (MORP)

Table 3. Final concentrations for the various analytes are as follows

	AMPH* MAMP MDA MDMA (ng/mL)	7-AMINO CLONZ ALPR OH-AL DIAZ NDIAZ TEM** OXAZ** LOR** ZOLP (ng/mL)	CODE MORP HCOD HMOR OCOD OMOR MTHD (ng/mL)	COKE BZE (ng/mL)	PCP THC (ng/mL)	6MAM FENT NFENT (ng/mL)	CARIS MEPRO (ng/mL)	BUP NBUP** (ng/mL)
Level 1	10	0.5	5	2	0.25	0.5	20	1
Level 2	20	1	10	4	0.5	1	40	2
Level 3	50	2.5	25	10	1.25	2.5	100	5
Level 4	100	5	50	20	2.5	5	200	10
Level 5	500	25	250	100	12.5	25	1000	50
Level 6	2500	125	1250	500	62.5	125	5000	250
Level 7	5000	250	2500	1000	125	250	10000	500

* Cutoff concentration for Amphetamine is 20ng/mL
** Cutoff concentration for Temazepam, Oxazepam, Lorazepam and Norbuprenorphine are 5ng/mL
All units are in diluted oral fluid concentrations. Multiply results by three to convert to neat oral fluid.

Table 4. Ion Suppression and Drug Recovery

	Ion Suppression (%)		Drug Recovery (% Neat)	
	Collected Sample	Calibrator	Collected Sample	Calibrator
Amphetamine	7	3	70	76
Methamphetamine	3	1	69	52
3,4-Methylenedioxyamphetamine	5	5	79	85
3,4-Methylenedioxy-methamphetamine	4	5	69	73
7-Aminoclonazepam	3	-6	77	80
Clonazepam	-11	0	72	75
Alprazolam	12	0	41	46
OH-Alprazolam	7	-1	66	72
Diazepam	24	10	30	40
Nordiazepam	4	3	47	51
Temazepam	12	-1	40	51
Oxazepam	-3	-4	77	77
Lorazepam	-7	-5	85	86
Zolpidem	11	-2	50	48
Cocaine	7	9	38	45
Benzoylecgonine	8	2	78	76
Methadone	31	18	36	36
Codeine	10	5	109	115
Morphine	7	7	83	97
Hydrocodone	8	6	85	94
Hydromorphone	7	6	109	110
Oxycodone	6	-1	92	100
Oxymorphone	6	7	100	103
6-Acetylmorphine	5	2	100	125
Phencyclidine	5	7	47	51
Buprenorphine	3	6	60	76
Norbuprenorphine	5	-1	74	94
Fentanyl	10	2	50	54
Norfentanyl	4	3	86	86
Carisoprodol	-15	-1	70	78

Fig. 1 Calibration curves for 3,4-Methylenedioxyamphetamine, 3,4-Methylenedioxy-methamphetamine, Amphetamine, Methamphetamine . Correlation Coefficients are > 0.99.

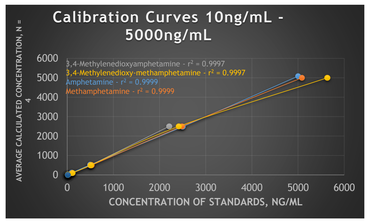


Fig. 2 Calibration curves for 7-Aminoclonazepam, Alprazolam, Clonazepam, Diazempam, OH-Alprazolam , . Correlation Coefficients are > 0.99.

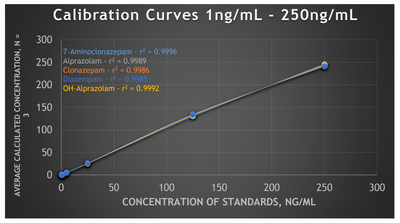


Fig. 3 Calibration curves for Lorazepam, Nordiazepam, Oxazepam, Temazepam. Correlation Coefficients are > 0.99.

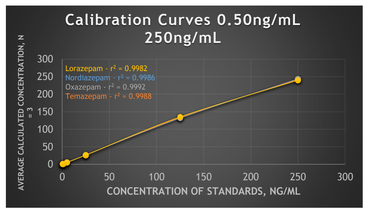


Fig. 4 Calibration curve for Cocaine. Correlation Coefficients are > 0.99

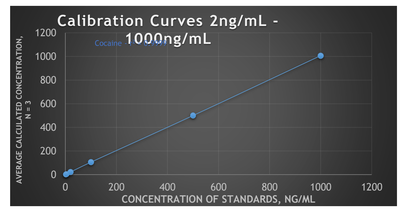
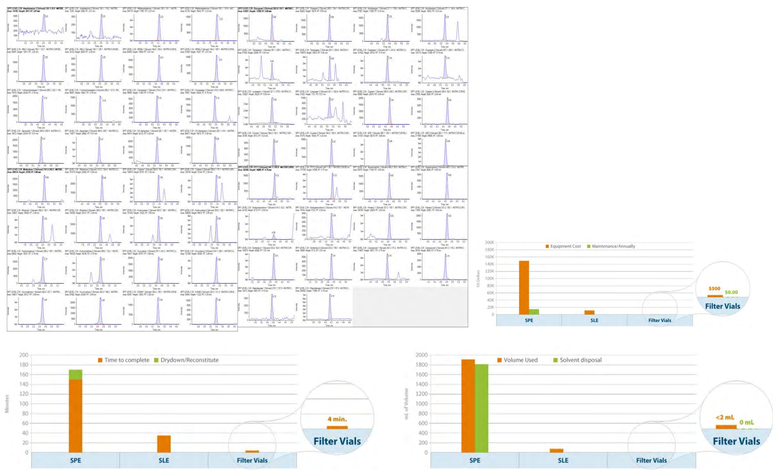


Fig 5. Mass Spectrum - Level 2



Conclusion

This validated method alleviates the need for sample clean-up by SPE or SLE thereby reducing the amount of equipment required, solvent usage and sample preparation time. Samples are filtered by pipetting the sample into the filter vial shell, inserting the plunger into the shell, and then pushing the plunger into the shell. The filtration process from sample pipetting to autosampler ready only requires 15 seconds. Benefits to the use of Thomson eXtreme® Filter Vials include lower cost, faster sample preparation time, less use and disposal of organic solvents:

High Throughput Screening and confirmation of 41 Pain Panel Drugs in Oral Fluid by an Integrated On-Line Extraction UHPLC-MS/MS System

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Abstract

A high throughput, cost effective and sensitive procedure for screening and confirmation of Pain Panel Drugs (PPDs). Synthetic Saliva samples were prepared using Thomson eXtreme|FV for and using an integrated On-Line Extraction (OLE)-UHPLC-MS/MS System for sample analysis.

Sample Preparation

- Transfer 200 µL of 60% Methanol/water containing 5 ppb internal standard into Thomson vial.
- Add 200 µL of drug standard in synthetic saliva (Immunalysis Corp.) to the vial and mix.
- Place Thomson Filter Plunger on top of the Thomson vial, Thomson vials-eXtreme/FV 0.2 um PVDF, w/Pre-Slit Red Cap
- Press filter plunger down approximately ¼ of the way into each of the Thomson Vial outer shells.
- Vortex for 10 sec
- Press Filter plunger the rest of the way down using Thomson Vial Filter Press.

Equipment

EVOQ Elite Triple Quadrupole Mass Spectrometer
Spray Voltage (ESI positive): 4000 v
Cone Gas Flow: 30 units
Cone Temperature: 350 ˚C
Heated Probe Gas Flow: 40 units
Heated Probe Temperature: 400 ˚C
Nebulizer Gas Flow: 65 units
Exhaust Gas: on
q2 pressure: 2.0 mTorr (Argon

Bruker Integrated On-Line Extraction-UHPLC
CTC Autosampler
Trap Column: YMC-Pack Pro ODS-AQ, 3 µm, 10 mm x 3.0 mm I.D.
Mobile Phase C: 0.1%formic acid (FA), 0.05% TFA in water
Equilibration flow: 600µL (3.0 min)
Loading Flow:600 µL
Analytical Column: YMC-Triart pfp, 1.9 µm, 50mm × 2.0 mm (I.D.)
Column Temperature: 40 ˚C
Injection Volume: 30 µL
Mobile Phase A: 0.1% FA in water
Mobile Phase B: 2 mM Ammonium formate and 0.1% FA in MeOH/Acetonitril=50/50

Gradient:

Time	%A	%B	Flow (uL/min)
0.0	80	20	350
0.2	80	20	350
3.5	5	95	350
3.9	5	95	350
4.0	80	20	350
6.0	80	20	350

Results

The sample preparation time was less than a minute by transferring saliva sample to filter vial and diluting with same volume of 60% methanol/water containing internal standard (IS) followed by mixing and press filtering. Forty one pain drugs were evaluated. Two MRM transitions were used for each compound. The first peak and last peak were eluted at 0.9 minutes and 3.3 minutes, respectively. Thirteen isotope labeled drugs were used as IS that had retention time spreading from 0.9 minutes to 3.27 minutes. The total method run time was 8.5 min including re-equilibration. The time for the entire procedure was less than 10 minutes.

Fig. 1. Selected chromatograms at 0.2 ng/mL PPDs in Synthetic Saliva.

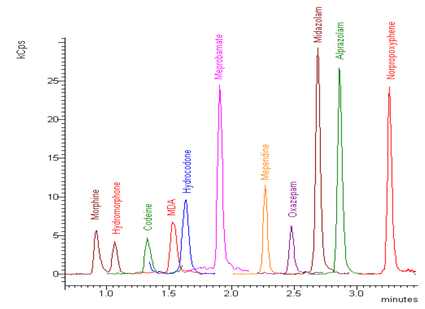

Table 1. 6MAM-d_6, Alprazolam-d_5, Buprenorphine-d_4, Clonazepam-D_4, Codeine-d_6, Fentanyl-d_5, Meperidine-d_4, Methadone-d_3, Morphine-d_6, Norbuprenorphine-d_3, Norfentanyl-d_5, Oxymorphone-d_3, Tramadol ^{13}C-d_3 were used as internal standard for above data.

Name	Linear Range (ng/mL)	R^2	Response Factor % RSD	Name	Linear Range (ng/mL)	R^2	Response Factor % RSD
6-MAM	0.02-100	0.999	13.3	Meprobamate	0.05-100	0.998	9.1
Alprazolam	0.01-100	1.000	3.5	Methadone	0.01-100	1.000	4.7
Amphetamine	0.02-100	0.999	7.2	Methamphetamine	0.10-100	1.000	8.0
Benzoylecgonine	0.02-100	1.000	10.3	Midazolam	0.01-100	0.999	10.0
Buprenorphine	0.02-100	0.999	8.0	Morphine	0.02-100	1.000	5.0
Carisoprodol	0.05-100	0.999	9.0	Naloxone	0.02-100	0.999	11.2
Clonazepam	0.05-100	1.000	5.7	Naltrexone	0.02-100	1.000	11.0
Codeine	0.02-100	1.000	6.6	Norbuprenorphine	0.20-100	1.000	3.6
Diazepam	0.02-100	0.998	8.1	Nordiazepam	0.02-100	1.000	9.1
EDDP	0.01-100	0.997	6.5	Norfentanyl	0.01-100	1.000	6.1
Fentanyl	0.01-100	1.000	5.0	Normeperidine	0.05-100	0.999	5.8
Flunitrazepam	0.02-100	1.000	5.8	Norpropoxyphene	0.02-100	0.999	8.7
Flurazepam	0.01-100	1.000	2.0	Oxazepam	0.02-100	1.000	12.6
Hydrocodone	0.02-100	0.997	6.3	Oxycodone	0.02-100	0.996	13.8
Hydromorphone	0.02-100	1.000	4.9	Oxymorphone	0.01-100	1.000	4.4
Hydroxyalprazolam	0.02-100	1.000	4.3	PCP	0.01-100	1.000	7.4
Lorazepam	0.10-100	1.000	14.6	Propoxyphene	0.01-100	0.999	4.9
MDA	0.02-100	0.996	9.9	Sufentanil	0.01-100	0.998	9.1
MDEA	0.05-100	0.998	14.4	Temazepam	0.01-100	1.000	6.1
MDMA	0.02-100	1.000	4.3	Tramadol	0.01-100	1.000	6.2
Meperidine	0.02-100	1.000	2.9				

Conclusion

- **Simple** (diluted, filter and shoot), **Fast** (less than 10 min) and **Sensitive** (LOQ at 0.01-0.2 ng/mL)
- Bruker LC/MS/MS coupled with integrated On-Line Extraction-UHPLC is a system of choice for high throughput PPDs analysis for clinical research needs.
- Lower limit of quantitation (LLOQ) is 0.01-0.2 ng/mL
- Upper limit of quantitation (ULOQ) is 100 ng/mL.
- Linearity regression coefficient R^2 was >0.99.
- Blanks show no interference of the analysis at the LLOQ level.
- Sub ng/mL level PPDs detection with about three orders of dynamic detection range will cover the clinical research needs.