Analysis of Fentanyl and its Metabolite, Norfentanyl by CESI-MS

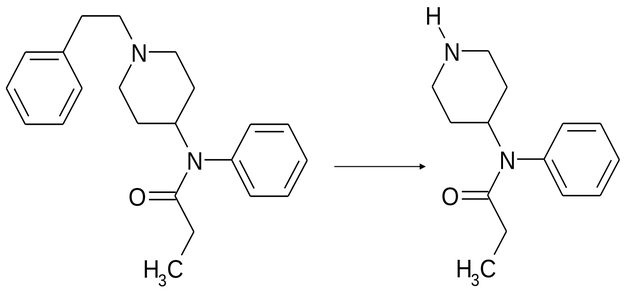
John C. Hudson, Beckman Coulter Canada
Regina, Saskatchewan, S4V 0R4 Canada

Introduction

There is an on-going requirement in clinical and forensic casework to detect and quantify low levels of potent drugs and their metabolites. This need arises especially when there has been extended periods of time between administration of the drug and collection of the sample for analysis. Forensic cases include Drug-Facilitated Sexual Assault (DFSA) and Driving Under the Influence of Drugs (DUID) where there may be a lengthy delay in the assault being reported or samples from an impaired driver being obtained.

In this work, one such drug, fentanyl, and its metabolite, norfentanyl, were analyzed at challenging sub-therapeutic levels with both samples and unknowns processed as a blind trial. For this purpose, we have used a new technique developed in our laboratory, called CESI-MS, where capillary electrophoresis (CE) and electrospray ionization (ESI) have been integrated into a single dynamic process, to significantly improve the efficiency of analyte ionization.

Fentanyl (Figure 1), a potent synthetic narcotic analgesic, surgical anesthetic and recreational drug, is commonly administered at low dosages of 25 µg/patch, resulting in very low therapeutic levels of 0.3 to 1.2 ng/mL of serum after 24 hours. It is reported to be 50-100 times more potent than morphine (1). Its main metabolite, norfentanyl can be detected for up to 72 hours after administration.



Fentanyl Norfentanyl

Figure 1: Fentanyl Metabolism in Biological Fluids

Material and Methods

Chemicals:
All chemicals were reagent grade and were purchased on-line from VWR International.

Drug and Metabolite Standards:
Standards were purchased from Cerilliant Corporation, Round Rock, TX, USA. Stock solutions were prepared at a concentration of 1 mg/mL and further diluted for spiking serum with methanol. Standard solutions for mass spectrometry and extractions were prepared at 5, 1 or 0.1 ng/µL in 5 to 50 mM Ammonium Formate (pH 2.85).

Serum Calibration Standards:
Serum was provided and the samples, including blind controls, were prepared by an external agency (Pharmalytics Inc., Saskatoon, SK, Canada). Fentanyl and norfentanyl solutions were prepared, spiked into blank human serum and extracted as per the protocol (Figure 2). The samples were kept frozen until the time of analysis.

CESI-MS Conditions

OptiMS* Porous Capillary Interface	92.4 cm Surface^{+} (covalent cation) coated prototype 150 µm OD, 30 µm ID with porous emitter tip
Separation	30kV, 216 V/cm, 1.6 µamps
Temperatures	Capillary and Sample Storage = 20ºC
Background Electrolyte (BGE)	50 mM Ammonium Formate, pH 2.85
Sample Introduction	Hydrodynamic 5 psi for 10 s
CE Instrument	CESI 8000* Prototype
MS Instrument	Waters Xevo TQ with MassLynx 4.1
Conductive Liquid	0.7% Formic Acid
ESI Voltage	1.25 kV
Capillary Conditioning	The capillaries were initially conditioned with MeOH, water, 1N NaOH, water and BGE.

Biological Fluid Extraction Protocol

To 1 mL of whole blood, serum or urine:

1. Add 0.2 mL conc. NH_4OH and vortex.
2. Add 5 mL of 1-chlorobutane and shake for 10 min.
3. Centrifuge at 0-4ºC for 10 min. at 3000 rpm.
4. Evaporate carefully just to dryness with N_2 or in a SpeedVac₮.
5. Add 100 µL of 5 mM BGE to tube, vortex, heat to dissolve extract.
6. Transfer to a 200 µL Microfuge tube.
7. Centrifuge at 14,000 rpm for 20 min.
8. Pressure-inject the sample at 10 seconds at 5 psi.

Figure 2: Liquid-Liquid Extraction Protocol for Biofluids

Results

1. Serum calibration samples for fentanyl and norfentanyl using doxapram as the internal standard were prepared and analyzed using a liquid-liquid extraction protocol (Figure 2).

2. An OptiMS CESI interface (Figure 3) was used to interface CE and MS, providing the required sensitivity on injections of only 7 nL of extract dissolved in 100 µL.

3. Samples were analyzed by CESI-MS and the separation is shown in Figure 4.

4. Multiple Reaction Monitoring (MRM) was used in the quantitative processing (Fentanyl: 337.4 → 188.2, Norfentanyl: 233.3 → 84.1, Doxapram IS: 379.4 → 292.4).

4. Samples including blanks prepared by the external agency, were extracted in duplicate bracketing the blind controls (Figure 5).

5. The calibrations were linear with R^2 >0.99 for both fentanyl and norfentanyl from 0.1 to 50 ng/mL (Figure 6).

6. LOD and LOQ calculations showed that the protocol can provide detection of fentanyl and norfentanyl at low picogram per milliliter levels in serum (Figure 7).

7. The results for the externally prepared blind controls compared to the target values were acceptable at less than 15% (Figure 8).

OptiMS ESI Interface

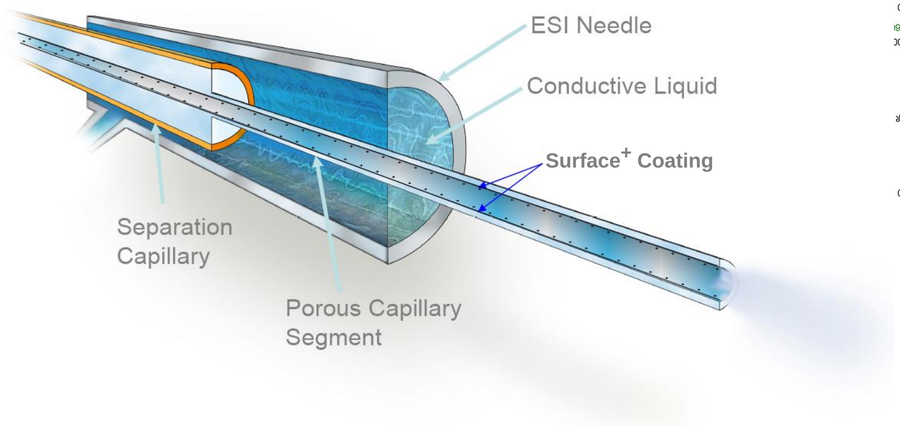


Figure 3: OptiMS CESI Interface Schematic

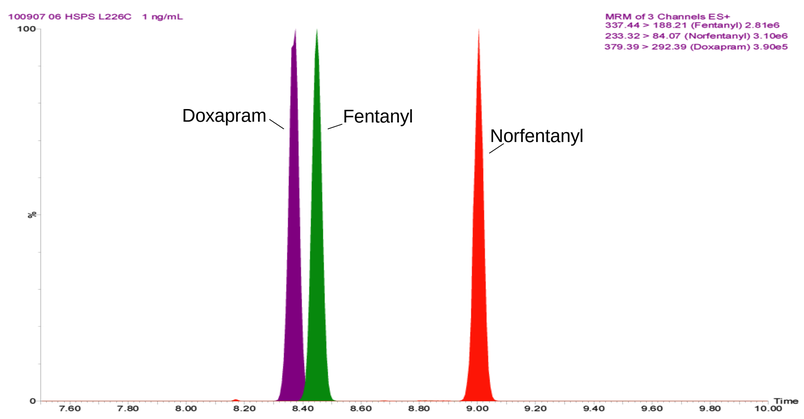


Figure 4: Separation of Fentanyl, Norfentanyl and Doxapram IS

	File Name	File Text	Conc A	Sample Type	MS File
1	100907 01 HSPS L226C	BK-1	0	Blank	Fentanyl_Norfentanyl
2	100907 02 HSPS L226C	BKIS-1	0	Blank	Fentanyl_Norfentanyl
3	100907 03 HSPS L226C	50-1	50	Standard	Fentanyl_Norfentanyl
4	100907 04 HSPS L226C	10-1	10	Standard	Fentanyl_Norfentanyl
5	100907 05 HSPS L226C	5-1	5	Standard	Fentanyl_Norfentanyl
6	100907 06 HSPS L226C	1-1	0	Standard	Fentanyl_Norfentanyl
7	100907 07 HSPS L226C	0.5-1	0.5	Standard	Fentanyl_Norfentanyl
8	100907 08 HSPS L226C	0.1-1	0.1	Standard	Fentanyl_Norfentanyl
9	100907 09 HSPS L226C	BK-1	0	Blank	Fentanyl_Norfentanyl
10	100907 10 HSPS L226C	BKIS-1	0	Blank	Fentanyl_Norfentanyl
11	100907 11 HSPS L226C	A-1		Analyte	Fentanyl_Norfentanyl
12	100907 12 HSPS L226C	A-2		Analyte	Fentanyl_Norfentanyl
13	100907 13 HSPS L226C	B-1		Analyte	Fentanyl_Norfentanyl
14	100907 14 HSPS L226C	B-2		Analyte	Fentanyl_Norfentanyl
15	100907 15 HSPS L226C	C-1		Analyte	Fentanyl_Norfentanyl
16	100907 16 HSPS L226C	C-2		Analyte	Fentanyl_Norfentanyl
17	100907 17 HSPS L226C	BK-2	0	Blank	Fentanyl_Norfentanyl
18	100907 18 HSPS L226C	BKIS-2	0	Blank	Fentanyl_Norfentanyl
19	100907 19 HSPS L226C	50-2	50	Standard	Fentanyl_Norfentanyl
20	100907 20 HSPS L226C	10-2	10	Standard	Fentanyl_Norfentanyl
21	100907 21 HSPS L226C	5-2	5	Standard	Fentanyl_Norfentanyl
22	100907 22 HSPS L226C	1-2	1	Standard	Fentanyl_Norfentanyl
23	100907 23 HSPS L226C	0.5-2	0.5	Standard	Fentanyl_Norfentanyl
24	100907 24 HSPS L226C	0.1-2	0.1	Standard	Fentanyl_Norfentanyl
25	100907 25 HSPS L226C	BK-2	0	Blank	Fentanyl_Norfentanyl
26	100907 26 HSPS L226C	BKIS-2	0	Blank	Fentanyl_Norfentanyl

Figure 5: Serum Calibration with Bracketed Blind Controls

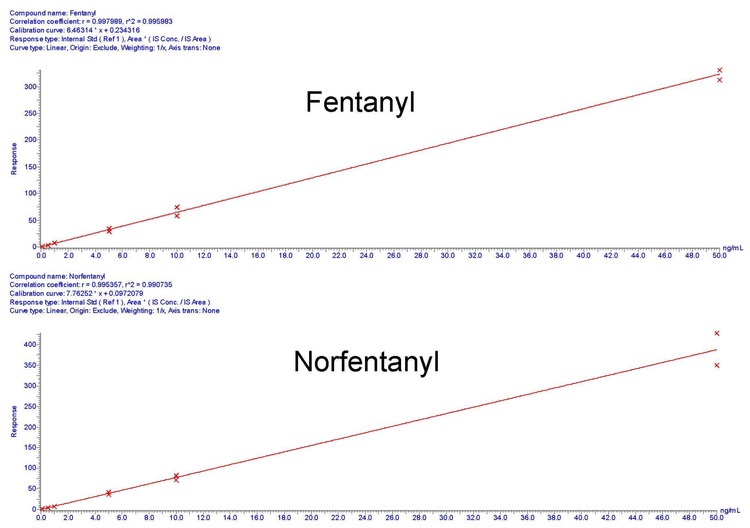


Figure 6: Linear Regression for Fentanyl and Norfentanyl

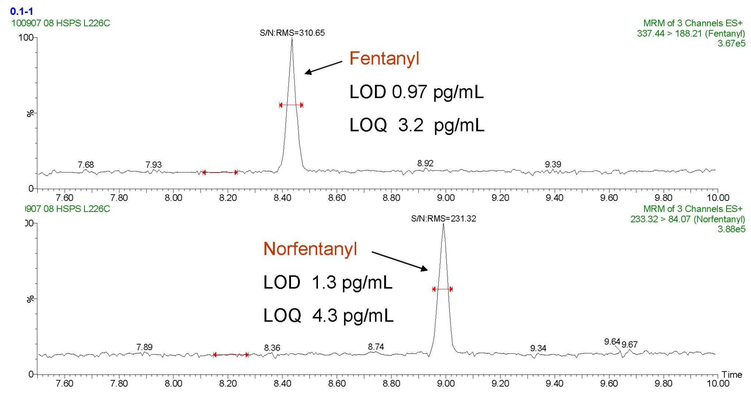


Figure 7: Calculated LOD and LOQ

		Fentanyl		Norfentanyl	
Controls:	Spiked Amt.	Found:	%Dev.	Found:	%Dev.
A	30	34.1	13.7	26.2	12.7
B	7.5	7.9	5.3	6.6	12
C	1.5	1.65	10	1.35	10

Figure 8: Blind Control Results

Conclusions

CESI, which is the integration of CE and ESI into a single dynamic process, interfaced to mass spectrometry provides the resolution and sensitivity required to analyze one of the most challenging drugs and its metabolite, fentanyl and norfentanyl. The small sample volumes required, in this case, 7 nL injected, create a significant advantage to forensic scientists who, very often, deal with inadequate specimen submission. This important advancement in separation and ionization technology permits replicate quantitative analysis on less than 100 microliters of biofluid and detection of many challenging drugs and their metabolites at sub-therapeutic levels. The process is robust and automated and will be of great value to analytical laboratories that work with charged analytes.

Acknowledgements

Dr. Gordon McKay, CEO, Pharmalytics Inc., Saskatoon, SK, Canada.

Jeff Chapman, Chitra Ratnayake and Scott Mack of

Beckman Coulter Inc., Brea, CA, USA.

* In development.

₮ All trademarks are the property of its respective owners.

References

1. Baselt, R.C. Disposition of Toxic Drugs and Chemicals in Man, pp. 616-619, 8th Edition, Biomedical Publications, Foster City, California, 94404 (2008).

2. H. Copley, “Validation of Capillary Electrophoresis in Forensic Toxicological Analyses: Screening Whole Blood for Common Basic Drugs”, A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfilment of the Requirements for the Degree of Master of Science in the Toxicology Graduate Program University of Saskatchewan, Saskatoon. (2004).

3. Shah, V.P. *et.al.*, “Bioanalytical Method Validation – A Revisit with a Decade of Progress”, Pharmaceutical Research 17(12) 1551-57 2000.