Development of a Whole Blood Microsampling Bioanalytical Method for the Analysis of Opiates with a Goal of Point-of-Care Therapeutic Drug Monitoring

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Introduction

With the ever increasing sensitivity of high performance triple quadrupole and QTOF mass spectrometers, quantification of most drugs (non-biological) does not require large volumes of collected blood or serum but convention dictates large blood volume collection by trained phlebotomists. The vast majority of bioanalytical labs utilize very small aliquots of the copiously collected blood, serum or plasma and even introduce significant sample dilution during the extraction and reconstitution steps prior to LC/MS/MS analysis.

Some alternatives to invasive vena-puncture that have been considered for quantification of drugs (therapeutic drug monitoring) include oral fluids and urine. Oral fluids theoretically serve as a surrogate matrix for plasma drug concentration. Urine as a drug testing matrix is convenient, simple and cost-effective for assessing the presence or absence of a drug but is not particularly useful for guiding individual therapy. Blood is by far the preferred collection matrix for measuring pharmacologically active drug levels. However, standard methods for blood collection techniques are costly and invasive and do not offer convenience to the patient. Thus, alternative blood sampling techniques that provide comparable therapeutic drug concentrations are desirable.

One such alternative is whole blood microsampling, as it provides a direct measure of blood/serum concentrations. Finger pricks produce small blood volume (100µl or less) samples that may be collected either into capillary tubes or onto DBS cards. The finger prick method offers a less invasive and particularly attractive alternative for some patient groups (e.g., neonatal screening, geriatric populations). Until recently, DBS has been somewhat overlooked as an obvious alternative to vena-puncture, because of concerns of the limited amount of blood volume available, thereby limiting the number of replicates and analytes capable of being analyzed in a given time.

A number of groups have published on the potential of DBS for therapeutic drug monitoring. However, they cite some of the main challenges of DBS are correlation of blood concentrations to serum concentrations as well as hematocrit bias.

In this poster, we present two methods that overcome some of the DBS challenges– micro-serum processing and a new whole blood microsampler method, Mitra, that overcomes hemaotcrit bias. These methods are evaluated for their potential to aid in personalized medicine and individual therapeutic drug monitoring. Opiates are chosen for this evaluation as the model analyte class. In the arena of illicit drug use, therapeutic drug monitoring can provide a mechanism for improved compliance or more appropriate titrating of dose to achieve the pharmacological effect.

Instrumentation

LC/MS/MS System

API 4000 Qtrap Triple Quadrupole Mass Spectrometer (AB SCIEX)
Symbiosis HPLC (Spark Holland)

HPLC Conditions

Mobil Phase:
A. Water + 0.1% formic acid
B. Acetonitrile + 0.1% formic acid

Column: Kinetex C18 2.6 µm 50 x 2.1 mm column (Phenomenex, Inc.); 0.4 ml/min flow rate

Gradient:
2% B hold for 0.10 minutes; 2% B → 10% B in 0.25 minutes; 10% B → 30% B in 1.75 minutes; 30% B → 90% B in 1.5 minutes

Opiates Evaluated

Table 1. MRM Transitions

- MRM 300.0/152.0 Codeine 1
- MRM 300.0/115.0 Codeine 2
- MRM 337.0/188.0 Fentanyl1
- MRM 337.0/105.0 Fentanyl 2
- MRM 300.0/199.0 Hydrocodone 1
- MRM 300.0/128.0 Hydrocodone 2
- MRM 286.0/185.0 Hydromorphone 1
- MRM 286.0/128.0 Hydromorphone 2
- MRM 310.0/265.0 Methadone1
- MRM 310.0/223.0 Methadone 2
- MRM 286.0/152.0 Morphine 1
- MRM 286.0/165.0 Morphine 2
- MRM 316.0/241.0 Oxycodone 1
- MRM 316.0/256.0 Oxycodone 2
- MRM 302.0/227.0 Oxymorphone 1
- MRM 302.0/198.0 Oxymorphone 2
- MRM 468.3/55.0 Buprenorphine 1
- MRM 468.3/396.3 Buprenorphine 2
- MRM 342.3/188.0 Fentanyl-D5 i.s.
- MRM 306.3/202.0 Hydrocodone-D6 i.s.
- MRM 305.3/230.0 Oxymorphone –D6 i.s.
- MRM 289.3/201.0 Morphine-D3 i.s.
- MRM 292.3/185.2 Hydromorphone-D6 i.s.
- MRM 313.3/268.2 Methadone-D3 i.s.
- MRM 472.3/400.3 Buprenorphine-D4 i.s.

Materials & Methods

Preparation of spiked whole blood

Opiates were spiked into whole blood in one of two ways. A). Preparation of opiates stock solutions in methanol followed by fixed volume (5µl) transfer to whole blood aliquots (95µl) or B) Spike of highest methanol calibrator stock solution into whole blood followed by serial dilution into blank whole blood. The concentration range for these studies was 2 ng/ml to 500 ng/ml for all opiates except buprenorpine and fentanyl, which were prepared over the concentration range of 200 pg/ml to 50 ng/ml.

Microsampling Collection and Extraction Method 1.

Human whole blood (Bioreclamation, Inc.) or human whole blood, derived by finger prick, from test subjects, was collected into Sarstedt 100µl serum collection tubes. Whole blood was processed to serum by centrifugation at 11,000 rpm for 5 minutes.

Sarstedt Sample Extraction Protocol

20µl of serum removed from Sarstedt tube, Extracted with 100µl of cold acetonitrile containing 10 ng/ml internal standards. Samples vortexed, centrifuged for 10 minutes at 8600 rpm. Extract removed, evaporated to dryness under heated nitrogen stream (37°C). Following evaporation, samples reconstituted in 100 µl of mobile phase A and analyzed by LC/MS/MS.

Microsampling Collection and Extraction Method 2.

Human whole blood (Bioreclamation, Inc.) or whole blood derived by finger prick from test subjects was collected onto Mitra Tips (Neoteryx, Inc., Torrance, CA). Mitra tips were dried for a minimum of 3 hours or for a maximum of 5 days prior to sample extraction. Samples were collected onto Mitra in duplicate.

Mitra Sample Extraction Protocol

The duplicate Mitra tips were placed into an Eppendorf tube containing 150µl of water + 0.1% formic acid. Samples were vortexed at 1000 rpm for 1 hour. Aqueous extract (deep-red in color) was transferred to a new tube and 600µl of acetonitrile containing 1.6 ng/ml internal standards was added. Samples were vortexed, centrifuged for 10 minutes at 8600 rpm and the supernate transferred to a deep well microtiter plate. The tip was subjected to a 2nd extraction, this time with 100µl of acetonitrile for 30 minutes at 1000 rpm. The 2nd extract was then combined with the supernate of the 1st extract into the deep well plate. The sample was dried under heated N2 stream and then reconstituted in 100µl of MP-A prior to LC-MS-MS analysis.

Results and Discussion

Microsampled Whole Blood Processed to Serum

Our preliminary evaluation of microsampling to support the concept of opiate therapeutic drug monitoring involved processing small volumes of whole blood directly to serum followed by simple protein precipitation by addition of organic solvent (acetonitrile) followed by LC/MS/MS quantitation. From a 50µl whole blood sample collected into Sarstedt tubes (see Figure 3), 20µl of serum was readily retrieved following centrifugation. Standard curves for all opiates evaluated were found to have R values > 0.99, Examples for methadone (Fig. 1A) and Hydrocodone (Fig. 1B) from replicate serum samples (n=3) processed on the same day are shown below. Precision was evaluated (see Table 2) over a course of 4 days. Quantitation results are shown in Table 2 below for a subset of the opiates tested.

Figure 1A. Methadone Standard

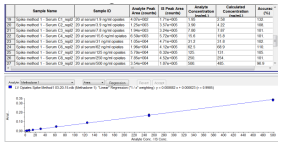


Figure 1B. Hydrocodone Standard

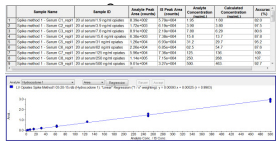


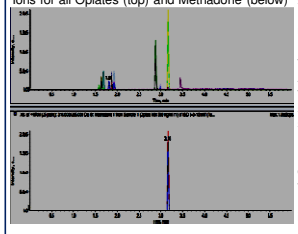
Table 2. Partial Summary of Intra-Day / Inter-Day Precision for Opiates spiked into 50µl WB and processed to 20µl of serum

Expected Concentration ng/ml	Hydrocone		Oxycodone		Methadone		Codeine		Fentanyl*	
	Mean Conc	SD	Mean Conc	SD	Mean Conc	SD	Mean Conc	SD	Mean Conc	SD
1.95 (0.195)	1.81	0.55	1.46	0.61	1.71	0.82	1.77	1.17	0.14	0.07
3.9 (0.39)	4.25	0.96	4.11	0.95	3.65	1.23	4.17	1.53	0.45	0.12
7.8 (0.78)	7.82	1.30	8.71	1.60	7.97	1.86	7.97	0.82	0.82	0.12
15.6 (1.56)	15.68	1.46	16.93	1.60	16.50	4.77	16.43	3.40	1.69	0.16
31.2 (3.12)	30.55	2.70	32.58	4.38	31.58	6.53	30.22	5.66	3.07	0.33
62.5 (6.25)	58.58	7.27	59.98	7.29	63.21	7.36	60.36	11.08	6.08	0.52
125 (12.5)	134.42	14.43	127.75	14.80	139.92	28.31	129.17	20.99	12.88	0.83
250 (25)	245.09	32.48	236.73	13.43	249.55	26.17	240.64	34.62	24.68	2.54
500 (50)	499.36	72.41	509.09	60.39	482.64	48.38	507.00	68.70	49.95	5.14

20µl serum samples, derived from 50µl of human whole blood, and extracted with 100µl of acetonitirile containing 10 ng/ml internal standard mixture and analyzed in quadruplicate over a period of 4 days. For the higher concentrations standards, intra-day variability was ~10-15% where as at the two lowest concentrations, the variability was slightly higher (~ 25%). Fentanyl concentration range was 0.195 ng/ml – 50 ng/ml.

Microsampled Whole Blood → Direct Collection onto MITRA

Figure 2. MRM Chromatograms for Quantifier Ions for all Opiates (top) and Methadone (below)



Next, we evaluated microsampling onto a new sampling device, called MITRA. This device, manufactured by Neoteryx, is particularly easy to use. A whole blood sample of 30µl volume is required to transfer 10µl of blood quantitatively to the tip of the MITRA device, as shown in Figure 4. After the tips have dried (drying time ≥ 3 hours), tips are extracted with aqueous and organic solvents, as described in the materials/methods section. Data quality for opiates extracted from MITRA are shown in Figure 2 to the left (methadone highlighted). Whole blood concentrations for the 9 opiates tested compared very favorably to the serum drug concentrations. Results for Codeine and Hydrocodone are shown in Table 3 below.

Sample Information	Expected Concentration	Measured Concentration	Accuracy (%)
Mitra Calibrator 1 repl 1	1.95	0.775	39.7
Mitra Calibrator 2 repl 1	3.9	3.96	102
Mitra Calibrator 3 repl 1	7.8	9.41	121
Mitra Calibrator 4 repl 1	15.6	19.7	126
Mitra Calibrator 5 repl 1	31.2	36.5	117
Mitra Calibrator 6 repl 1	62.5	69.9	112
Mitra Calibrator 7 repl 1	125	160	128
Mitra Calibrator 8 repl 1	250	238	95.3
Mitra Calibrator 9 repl 1	500	576	115
Mitra Calibrator 1 repl 2	1.95	1.74	89.1
Mitra Calibrator 2 repl 2	3.9	2.71	69.5
Mitra Calibrator 3 repl 2	7.8	8	103
Mitra Calibrator 4 repl 2	15.6	21	135
Mitra Calibrator 5 repl 2	31.2	40.1	129
Mitra Calibrator 6 repl 2	62.5	66.7	107
Mitra Calibrator 7 repl 2	125	137	110
Mitra Calibrator 8 repl 2	250	280	112
Mitra Calibrator 9 repl 2	500	484	96.7

Sample Information	Expected Concentration	Measured Concentration	Accuracy (%)
Mitra Calibrator 1 repl 1	1.95	2.39	123
Mitra Calibrator 2 repl 1	3.9	2.99	76.5
Mitra Calibrator 3 repl 1	7.8	8.12	104
Mitra Calibrator 4 repl 1	15.6	20.6	132
Mitra Calibrator 5 repl 1	31.2	32.8	105
Mitra Calibrator 6 repl 1	62.5	68	109
Mitra Calibrator 7 repl 1	125	145	116
Mitra Calibrator 8 repl 1	250	266	107
Mitra Calibrator 9 repl 1	500	552	110
Mitra Calibrator 1 repl 2	1.95	1.1	56.3
Mitra Calibrator 2 repl 2	3.9	4.69	120
Mitra Calibrator 3 repl 2	7.8	6.07	77.8
Mitra Calibrator 4 repl 2	15.6	17.3	111
Mitra Calibrator 5 repl 2	31.2	39.8	128
Mitra Calibrator 6 repl 2	62.5	65	104
Mitra Calibrator 7 repl 2	125	134	107
Mitra Calibrator 8 repl 2	250	234	93.7
Mitra Calibrator 9 repl 2	500	541	108

Table 3. Quantification of Hydrocodone (left panel above) and Codeine (right panel above) after collection onto Mitra. Samples were quantified against the serum calibration curve. Reasonable correlation between serum concentration and whole blood concentration was observed.

Mitra Sample Stability

Mitra tips were processed 3 hours after whole blood sampling and compared with Mitra tips stored for 5 days at room temperature. The results between the two sets of tips was comparable. Differences were observed in the color of the tips extracted 3 hours post-dipping vs. tips extraced after sitting for 5 days, however, the data for the opiates was consistent across hydrophilic and lipophilic molecules.

Microvolume Collection

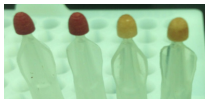


Figure 3. Mitra tips before extraction (left two tips) and after extraction (right two tips). The discoloration of tips indicates successful extradction of whole blood from the tip as a result of the extraction procedure.

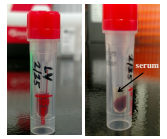


Figure 4. Sarstedt microvette whole blood/serum collection tube. Blood is collected onto the fixed volume capillary and concentrated onto the bottom of the collection tube prior to centrifugation. Serum generated shown on right hand side of photo.

Conclusions

Standard curves generated by spiking whole blood over a wide range of opiate concentrations followed by dipping, drying and extracting Mitra tips was comparable to standard curves generated by processing whole blood to serum using microvette serum collection tubes. Whole blood processed to serum generally provided a cleaner matrix and a slightly lower LLOQ than the MITRA extraction procedure, where there was need for additional clean up steps to remove particulates and matrix interferents, especially after aqueous extraction. The Mitra procedure requires less blood (i.e.,, to achieve a 10µl collection onto Mitra requires approximately 30µl of whole blood whereas to achieve a 20µl serum sample requires approximately 50µl of whole blood). Both approaches are straight-forward, offer a path forward to in-office blood sampling with a goal of providing insights into compliance, abuse and therapeutic drug monitoring to aid in patient care. Future work will focus on refining the collection, storage and extraction procedures with a goal towards point-of-care testing in the clinic.

Acknowledgements

The authors are grateful to Bobby Virasingh and Stuart Kushon, Neoteryx, Inc., Torrance, CA for supplying MITRA tips, extraction plates and most importantly, helpful guidance on optimization of extraction conditions of drugs from the MITRA tips.

The authors are also grateful to Sarstedt, Inc. for providing the 100µl serum collection tubes as well as helpful suggestions regarding blood collection and processing to serum.

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