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Special Issue

A Dilute-and-Shoot LC–MS Method for Quantitating Opioids in Oral Fluid

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Opioid testing represents a dominant share of the market in pain management clinical testing facilities. Testing of this drug class in oral fluid (OF) has begun to rise in popularity. OF analysis has traditionally required extensive clean-up protocols and sample concentration, which can be avoided. This work highlights the use of a fast, 'dilute-and-shoot' method that performs no considerable sample manipulation. A quantitative method for the determination of eight common opioids and associated metabolites (codeine, morphine, hydrocodone, hydromorphone, norhydrocodone, oxycodone, noroxycodone and oxymorphone) in OF is described herein. OF sample is diluted 10-fold in methanol/water and then analyzed using an Agilent chromatographic stack coupled with an AB SCIEX 4500. The method has a 2.2-min LC gradient and a cycle time of 2.9 min. In contrast to most published methods of this particular type, this method uses no sample clean-up or concentration and has a considerably faster LC gradient, making it ideal for very high-throughput laboratories. Importantly, the method requires only 100 μL of sample and is diluted 10-fold prior to injection to help with instrument viability. Baseline separation of all isobaric opioids listed above was achieved on a phenyl-hexyl column. The validated calibration range for this method is 2.5–1,000 ng/mL. This 'dilute-and-shoot' method removes the unnecessary, costly and time-consuming extraction steps found in traditional methods and still surpasses all analytical requirements.

Introduction

Opioids are among the most prescribed drugs in the USA and dependence on them is a major national health risk and a continual burden on the American healthcare system. For this reason, ongoing monitoring of pain management patients is a necessary practice. Traditionally, urine drug testing has been the most common means of monitoring possible patient adherence or potential risk due to other non-disclosed drug use. However, many physicians have begun to re-evaluate this protocol with the advent of oral fluid (OF) testing devices. This technological advance, albeit a simplistic one, has had a considerable impact on the ever-changing clinical laboratory drug testing landscape. Despite a lesser influence at the current time (when compared with urine), the growth of OF sample collection and analysis in the near future is projected by various clinical chemistry practitioners (1).

OF testing is less invasive than urine testing as it does not require a private area or restroom facility to collect the specimen. Because of this feature, OF samples are also less prone to adulteration, which may occur with the collection of a urine sample that cannot be directly observed. In OF sample collection, the specimen is often stabilized immediately through the addition of phosphate buffer, surfactants and other additives, and then shipped by mail to the testing facility. From an analytical chemistry standpoint, OF samples present more of a challenge than an improvement compared with urine samples. The chemical components of the Quantisal™ buffer [<1% Proclin 300, <1% sodium phosphate monobasic, <1% dibasic sodium phosphate, <1% sodium chloride and >90% water (2)], for example, have traditionally proven difficult for chemical analyzers to tolerate without some sort of sample clean-up. The relatively low concentrations of drugs in the oral cavity coupled with the further dilution in stabilization buffer challenge the sensitivity limits of modern chemical analyzers. OF offers some conveniences however; namely, the lack of the extensive network of metabolic combinations that often occur in a urine matrix (e.g., glucuronidation), thus often negating the necessity for chemical or enzymatic hydrolysis.

The most common form of chemical analysis in the present clinical laboratory is liquid chromatography–mass spectrometry (LC–MS). Although a powerful technique, LC–MS is not without its drawbacks—namely, a severe sensitivity to buffers and surfactants (3). Hence, initial work on LC–MS analysis of OF depended heavily on analyte extraction from this matrix and, at times, a concentration step due to the relatively low levels of analytes in OF (4). Since these early studies, advances in instrumentation and hardware have made revisiting this ideology plausible. Additionally, high-throughput laboratories that succeed upon running several thousands of samples a day benefit from even seemingly miniscule minimizations of preparatory time. In that vein, this work demonstrates a unique sub-three-minute cycle time 'dilute-and-shoot' method with a sample preparation that includes little more than dilution of the sample and centrifugation. It carries out the analysis of eight opioid compounds with toxicologically acceptable limits of quantitation/limits of detection (LOQs/LODs) and robust instrument performance that saves both time and cost when analyzing this sample matrix (5–8). A comprehensive review of the literature herein is not possible and the reader is encouraged to consult several notable references on OF opioid testing (6, 9–13).

Materials and methods

Chemicals

All reference standards were purchased from Cerilliant (Round Rock, TX, USA). All solvents, including methanol (optima grade), acetonitrile (optima grade), isopropanol (optima grade) and formic acid (88%), were purchased from VWR (Radnor, PA, USA). Negative synthetic saliva was obtained from Immunalysis (Pomona, CA, USA).

Standard preparation

Reference standards were diluted to appropriate calibrator level concentrations (2.5, 5, 10, 100, 250 and 1,000 ng/mL) in negative synthetic saliva and were then diluted with an additional 10× by taking 100 μL of calibrator and adding it to 25 μL of internal standard (1,000 ng/mL, dissolved in deionized water) and 875 μL of a 20 : 80 methanol : deionized water diluent. Therefore, the

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standards are meant to mimic the already 4× diluted patient samples. Samples were then vortexed and centrifuged. No sample clean-up/extraction was performed.

Patient sample collection

A Quantisal™ sampling device from Immunalysis was used for patient sample collection. This device uses a collection pad to collect 1 mL (±10%) of sample, which is then submerged in 3 mL of buffer to stabilize the sample. Once at the testing facility, this sponge is wrung out to yield ca. 4 mL of total sample. Patient samples are diluted and prepared as per the standard protocol mentioned above. This 10× diluted sample was vortexed and spun down for 5 min at ∼8,000 *g*. No sample clean-up/extraction was performed. It is important to note that results on these samples must be multiplied by 4 in order to account for the upfront dilution in buffer and obtain the true 'in-mouth' concentration. For example, if a patient's saliva has 12 ng/mL of a drug present in the mouth, the specimen collector would take 1 mL of that saliva (in a sponge) and dilute it in 3 mL of buffer (yielding a 3-ng/mL sample). This 3 ng/mL sample would be sent to the testing facility where it would be diluted an additional 10× with internal standard (IS) and diluent (yielding an on-column concentration of 0.3 ng/mL) and injected into the LC–MS instrument.

Instrumentation

All analyses were conducted by LC–MS-MS on an AB SCIEX Triple Quad 4500 platform run in electrospray ionization mode using an Agilent 1290 chromatographic system (1290 Infinity Binary Pump, 1290 TCC, 1290 Sampler and 1290 Thermostat) with a Phenomenex phenyl guard column (AJ0-8774) followed by a Phenomenex Kinetex 2.6 μm phenyl-hexyl 100 Å, 50 × 4.6 mm (00B-4495-E0) analytical column. The run time for this method is 2.2 min with a cycle time of ∼2.9 min. Centrifugation took place on a Sorvall ST 40. No sample clean-up or extraction was performed beyond this.

This assay monitors two transitions for each of the following eight analytes: codeine, hydrocodone, hydromorphone, morphine, norhydrocodone, noroxycodone, oxycodone and oxymorphone; and five internal standards: codeine D_3, hydrocodone D_6, hydromorphone D_3, morphine D_3 and oxycodone D_3. Mass spectrometry method parameters are summarized in Table I. The chromatographic starting conditions are 95 : 5 0.1% formic acid (aqueous) :methanol (+0.1% formic acid) with a 0.7-mL/min flow throughout (Table II).

Table I
The Mass Spectrometric Ion Fragmentation Transitions for this OF Opioid Method

Analyte	Transition	Declustering potential (DP)	Collision energy (CE, V)	Cell exit potential (CXP)	IS compound
Codeine	300.0 → 151.9	86	85	10	Codeine D_3
	300.0 → 165.0	86	55	10	
Hydrocodone	300.0 → 198.9	111	41	14	Hydrocodone D_6
	300.0 → 127.9	111	75	10	
Hydromorphone	286.0 → 184.9	101	41	8	Hydromorphone D_3
	286.0 → 156.9	101	51	12	
Morphine	285.9 → 164.8	76	55	12	Morphine D_3
	285.9 → 128.0	76	77	12	
Norhydrocodone	285.9 → 198.9	56	39	16	Hydrocodone D_6
	285.9 → 127.9	56	73	12	
Noroxycodone	302.0 → 227.0	51	41	12	Oxycodone D_3
	302.0 → 186.9	51	33	12	
Oxycodone	316.0 → 241.1	61	39	10	Oxycodone D_3
	316.0 → 256.0	61	35	12	
Oxymorphone	302.0 → 226.9	81	39	16	Oxycodone D_3
	302.0 → 197.8	81	57	14	
IS	Transition	DP	CE (V)	CXP	IS compound
Codeine D_3	302.9 → 151.8	91	83	10	–
	302.9 → 164.9	91	57	12	
Hydrocodone D_6	306.0 → 202.0	101	41	8	–
	306.0 → 174.0	101	53	12	
Hydromorphone D_3	289.0 → 185.0	116	41	12	–
	289.0 → 157.0	116	55	10	
Morphine D_3	289.0 → 152.0	106	85	10	–
	289.0 → 164.9	106	53	12	
Oxycodone D_3	319.0 → 243.8	76	41	18	–
	319.0 → 215.1	76	44	15	

Only morphine and norhydrocodone share an ion transition, m/z 286 → m/z 128.

Table II
The LC Gradient Parameters for the Present OF Opioids Method

Step	Time (min)	%A (H_2O with 0.1% formic acid)	%B (MeOH with 0.1% formic acid)
0	Initial	95	5
1	1.2	60	40
2	1.5	50	50
3	1.6	2	98
4	2.0	2	98
5	2.1	95	5
6	2.2	95	5

Method validation

This method was validated according to the Clinical Laboratory Improvement Amendments (CLIA) guidelines and a few other notable sources (14–19). Using these guidelines and recommended protocols, this method was assessed for limits/sensitivity (the lower and upper concentration limits at which the method could accurately identify and quantify the analyte), linearity (the reproducible regression or fit of the calibration curve), carryover (the highest concentration of analyte present which does not produce a concentration level above the lower limit in a blank injection proceeding it), precision and accuracy (the capability of the method to produce the reproducible accurate results over a period of multiple days at concentrations spanning the concentration range of interest), interference/selectivity (the ability of the method to be unaffected by the presence of other medications/compounds) and matrix effects (the suppression or enhancement of analyte signals of interest due to the presence of matrix—in this case saliva). The last validation study performed was the patient comparison portion which seeks to confirm that the new method accurately quantitates actual patient samples when compared with the currently in-use and accepted method for this particular class of compounds. Because this method did not require an extraction, recovery and extraction efficiency assessments have been omitted.

To achieve a passing result, the standard must return a quantitative value within a specified percentage of the target value. For limits, linearity and precision and accuracy, this value is 25%. These quantitation values are based on a calibration curve which must be prepared and run fresh on the same day that the particular validation study was conducted, passing without dropping any calibration points. This calibration curve must have an R^2 greater than 0.99 with a passing quality control

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(QC; at a mid-range concentration) passing with a threshold of 30%. Visual inspection of both chromatograms and the calibration curves is completed to assure the absence of outliers. Additionally, a blank run after the highest calibrator point must show no signs of carryover. For this reason, the highest calibrator and the carryover limit level will often be the same value. The internal standard quantifier ion transition for unknown samples must have an area within 75% of that established by this calibration curve.

The analyte signal must also display reliable chromatography. This means that the retention time must be within 25% of the retention time established for that particular analyte in the calibration curve. For a two-minute method, for example, this would give a tolerance of ± 30 s. Additionally, the chromatographic peak must be roughly Gaussian in shape and not contain any shoulders or any indication of splitting.

For each analyte, two mass transitions are selected. The first (typically the most intense) transition serves as the quantifier ion transition. The quantifier transitions for the internal standard and analyte are used to establish the relative response of the ion. By tracking the relative response at multiple concentrations, a quantitative calibration curve is generated. Relative responses of unknown samples are then applied to this curve to calculate a concentration for the unknown sample. For each analyte and internal standard, a second ion transition is also selected (often this is the second most intense transition for this ion, or at least the next most stable transition that is free of interferences). The peak area for this second ion transition is compared with the peak area of the quantifier ion transition within each analyte or internal standard to generate an ion ratio. For the analytes, the ion ratio of all calibrators (curve points) is averaged to arrive at the calibrated ion ratio. The same is done for the internal standards. Unknowns must then be within a certain tolerance of this ion ratio in order to be 'passing'. If the ion ratio (qualifier peak area/quantifier peak area) is greater than 50%, the tolerance is 20%. For average ion ratios greater than 20% but less than or equal to 50%, the tolerance is 25%. For average ion ratios greater than 10% but less than or equal to 20%, the tolerance is 30%. For average ion ratios equal to 10% and less, the tolerance is 50%.

Limits

In this particular aspect of validation, the lowest concentration at which the method is able to generate reproducible acceptable results for all analytes is determined. The LOQ is defined as the concentration level at which the analyte signal demonstrates reproducible quantitation and ion ratio stability. The LOD is defined as the concentration level at which the analyte signal demonstrates reproducible ion ratio stability. In the case of LOD, the quantitation may be incorrect, but the stability of the ion ratio establishes that it is indeed the analyte in question that is being observed; hence it is detectable, but not quantifiable. To establish this limit, four of five replicates must pass for the necessary criteria when compared with a passing curve (as described above).

Linearity

Linearity consists of running all calibration curve points five times, with four passing for quantitation ($\pm 25\%$), ion ratio and chromatographic requirements for all analytes at each level. Additionally, the percent coefficient of variation (CV) of each set of replicates must be $<15\%$.

Carryover

Carryover is investigated to determine how high a potentially unknown sample can be before it begins to affect the quantitation of the sample following it in the sequence. This test consists in running five replicates of the potential carryover limit concentration, each followed by a blank injection. Four out of five of these blank injections following carryover sample must not quantitate higher than the established LOQ concentration. In regular use, if a sample exhibits a concentration higher than the carryover limit all proceeding samples in the sequence that had the potential for carryover must be rerun.

Precision and accuracy

Precision and accuracy is used to validate the stability of the method at various concentrations over multiple days' time. Three concentrations (which do not coincide with the calibration points) in the analytical range of interest are chosen and run 10 times each on 3 separate days. These injections must each be prepared fresh and processed against a curve also prepared and run fresh on that same day. Of these 10 injections, 9 must pass within 30% of the target concentration.

Interference

Interference studies are meant to determine the contribution of compounds (both in and not in the method) to the signal of each analyte in the method. Interference would be considered confirmed if false signal met LOQ requirements (i.e., was higher than the LOQ and had a passing ion ratio) in greater than one of the five replicates that must be run for each individual test. Matrix run without IS and without analyte is injected to be sure the matrix does not contribute to any monitored ion transitions. Additionally, the following compounds were tested for contributing interference to the method: dihydrocodeine (at 10,000 ng/mL with IS), norcodeine (at 10,000 ng/mL with IS), 6-AM (at 1,000 ng/mL with IS), codeine (at 1,000 ng/mL without IS), hydrocodone (at 1,000 ng/mL without IS), hydromorphone (at 1,000 ng/mL without IS), morphine (at 1,000 ng/mL without IS), norhydrocodone (at 1,000 ng/mL without IS), oxycodone (at 1,000 ng/mL without IS), noroxycodone (at 1,000 ng/mL without IS) and oxymorphone (at 1,000 ng/mL without IS). Other compounds were not considered due to the lack of known interferences and the lack of isobaric possibilities for other commonly prescribed medications.

Matrix effects

The effect of matrix on the method was evaluated by running the QC concentration of 40 ng/mL both in matrix (in this case, synthetic negative saliva from Immunalysis) and in the LC starting conditions [95/5 water (with 0.1% formic acid)/methanol (with 0.1% formic acid)]. It should be noted that SWGTOX validations require a matrix comparison with 10 negative patient saliva samples from different sources (20). While this was not completed *per se*, examination of the patient positive samples speaks to the concentration of matrix interference (see below).

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Patient comparison

For patient comparison studies, this method was evaluated by comparing the results it achieved with those achieved using the current validated, accepted method in the laboratory. This accepted method, which uses an Agilent 6490 and Agilent 1290 chromatographic stack, was previously validated and had been in regular daily use for over a year. To make this comparison, all patient positive OF samples (not yet expired) for the analytes in this method were prepared using the new procedure and compared with the previous method. An agreement of $\pm 30\%$ is required for this study to be considered passing.

Results

Throughout validation and while in regular use, this method has produced calibration data which exhibit a reproducible quadratic correlation with a weighting of $1/x^2$. Consistent peak shapes and ion ratios are achieved for codeine, hydrocodone, hydromorphone, morphine, norhydrocodone, noroxycodone, oxycodone and oxymorphone. Daily six-point (2.5, 5, 10, 100, 250 and 1,000 ng/mL) calibration curves routinely demonstrate $100 \pm 25\%$ accuracy for each point and excellently fit to the quadratic correlation ($R^2 > 0.99$). A typical chromatogram is shown in Figure 1 for the LOQ concentration of 2.5 ng/mL, demonstrating the separation achieved for this 2.2 min method. The chromatograms for the internal standards have been omitted since they reached maximum intensities of ca. 2E5. These traces have had no smoothing applied. Importantly, morphine and norhydrocodone (which share an ion fragmentation transition, m/z 286 → m/z 128) are baseline separated in the chromatogram.

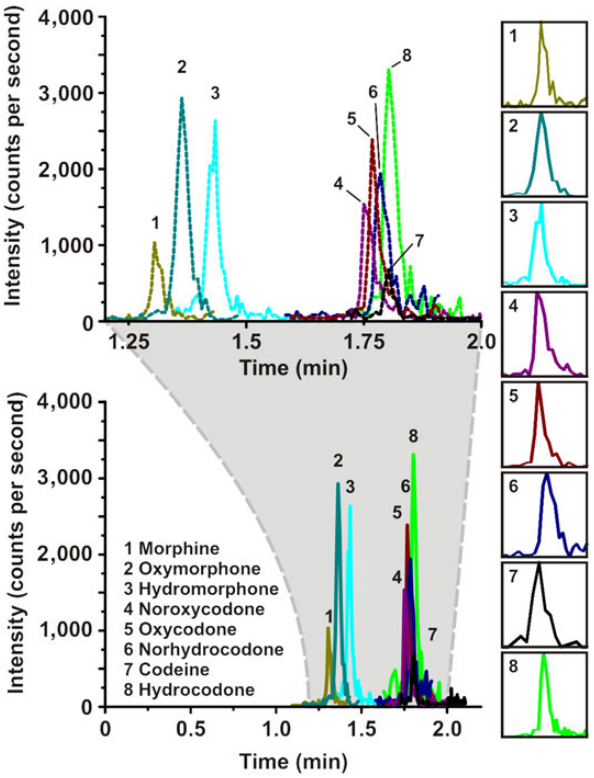

Figure 1. The chromatogram at the bottom displays an injection of a sample at the LOQ concentration of 2.5 ng/mL (internal standards have been omitted). The chromatogram at the top is extracted from the region of interest highlighting the separation achieved using this method. The individual analyte peaks have been extracted and displayed along the right hand side of the figure, numbered accordingly. These data have not had any smoothing applied.

Validation of this method followed the guidelines mentioned above and accounted for goodness of fit for the concentration ratio vs. area ratio of the analytes over the determined calibration range; absence of carryover of the analytes into a negative sample following a high concentration injection; the precision and accuracy of the method over 3 days; the effect of the matrix on the response of the analytes and any interferences which may be a source of type I error (false positives) (21). Compiled results are displayed in Table III.

The LOD/LOQ for all analytes was determined to be 2.5 ng/mL. This concentration reflects the buffered OF sample prior to the final 10× dilution, which is then injected onto the column. Thus, the 'in-mouth' LOD/LOQ would be four times this value (due to the in-clinic buffer dilution) or 10 ng/mL. At this concentration of 2.5 ng/mL, it was possible to achieve passing results for accuracy and ion ratio tolerance. Additionally, no appreciable carryover was observed following the highest curve point of 1,000 ng/mL, with the highest carryover value owing to oxymorphone at 1.51 ng/mL.

The precision and accuracy over a 3-day span was evaluated by running 10 replicates of standards at 7.5, 25 and 500 ng/mL on 3 separate days. Excellent accuracy, precision and ion ratio tolerance were observed for all analytes with the worst percent deviation belonging to norhydrocodone at 88.1% (at 7.5 ng/mL) and the highest percent CV belonging to oxymorphone at 7.5%.

All analytes, except morphine, showed an increase in signal response when dissolved in negative synthetic saliva compared with a 'neat' preparation in mobile phase initial conditions (94.9% water, 5% MeOH and 0.1% formic acid). Norhydrocodone, noroxycodone and oxymorphone all showed a greater than 10% increase in peak area, whereas morphine saw a 3.1% decrease in response.

Some interferences were observed. Notably, norcodeine, when run at 10 μg/mL, resulted in a codeine signal that quantitated above the LOQ. The same was observed for dihydrocodeine, which, when run at 10 μg/mL, also produced a peak that quantitated above the LOQ for hydrocodone. Both of these signals were below the LOQ when the interfering compound concentration was at 5 μg/mL. The interference concentrations of norcodeine and dihydrocodeine were at or in excess of 10,000 ng/mL. Samples with these concentrations of norcodeine and dihydrocodeine are very rare if present at all in OF. Hence, the impact on codeine concentration (which is also increasingly rare in all samples) is minimal if present at all.

Discussion

OF, as a biological matrix, is made up of various components supplied by numerous salivary glands. These salivary glands draw upon the blood supply and therefore allow 'rapid transference' of various drugs in the blood to the oral cavity. This drug transference is highly dependent on a number of factors including the ionization state of the drug. Typically, only unionized drugs can migrate across epithelial membranes. Since basic drugs may be

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Table III
Compiled Validation Data from This OF Opioid Method

	Linearity[a]			Carryover	Precision and accuracy						Matrix	Interference	
	LOQ/LOD	ULOL	R^2	Average concentration (ng/mL) ($N = 5$)[b]	Average % target ($N = 90$)			Average % CV ($N = 90$)			% Matrix effect	Interfering compounds	Concentrations (μg/mL)
					7.5 ng/mL	25 ng/mL	500 ng/mL	7.5 ng/mL	25 ng/mL	500 ng/mL			
Codeine	10	4,000	0.9986	0.93	99.3	103.5	96.4	7.2	4.3	3.4	+7.1	Norcodeine	>5
Hydrocodone	10	4,000	0.9975	1.28	97.5	101.8	96.3	5.8	3.7	3.1	+3.0	Dihydrocodeine	>5
Hydromorphone	10	4,000	0.9987	1.30	92.2	98.9	98.3	7.0	3.8	3.3	+1.0	None	–
Morphine	10	4,000	0.9979	1.03	97.0	106.2	98.0	5.8	4.2	2.4	−3.1	None	–
Norhydrocodone	10	4,000	0.9978	1.29	88.1	93.7	95.7	5.8	2.7	2.9	+17.2	None	–
Noroxycodone	10	4,000	0.9975	1.48	96.1	98.2	97.0	7.1	5.8	4.3	+14.8	None	–
Oxycodone	10	4,000	0.9956	1.28	97.8	101.5	98.5	6.9	7.1	4.8	+2.1	None	–
Oxymorphone	10	4,000	0.9946	1.51	103.3	105.5	95.3	7.5	4.7	4.1	+10.6	None	–

The linearity results are compiled for all six curve points, 2.5, 5, 10, 100, 250 and 1,000 ng/mL, each run five times. Carryover was tested by running a matrix blank immediately following the highest curve point of 1,000 ng/mL. Precision and accuracy statistics were arrived at by compiling data from three separate concentration standards (7.5, 25 and 500 ng/mL), 10 replicates each, prepared and run on 3 separate days. Matrix data were calculated by dissolving the standards in negative synthetic saliva compared with a 'neat' preparation in chromatographic starting conditions (5% MeOH and 0.1% formic acid in water). An extensive list of drug compounds related to pain medication monitoring was tested to check for any interfering signals that may contribute to false negatives for this assay.
[a]In mouth concentrations. Actual method LOQ/LOD values are 2.5 ng/mL and ULOL values are 1,000 ng/mL.
[b]Average concentration after a 1,000-ng/mL injection.

unionized in the blood supply, they can transfer into the oral cavity and become ionized in the more acidic conditions there. This means that these drugs are unable to be rapidly exchanged back to the blood supply, and instead become trapped, effectively increasing their concentrations in OF relative to blood (7, 22, 23). The pKa of the opioids tested for here is all generally in the range 8–10 and therefore are found in greater quantities in the OF when compared with the blood as a result of this phenomenon.

Despite the common view that buffered OF samples cause serious instrument wear and failure, no appreciable effort was spent performing extra maintenance or on replacement of consumables (over the course of more than a year and 10,000+ actual patient sample injections). This low-maintenance convenience may be credited to the source or mass analyzer itself since similar preparations for OF matrices have proved largely unsuccessful on other manufacturer's models. The Phenomenex phenyl-hexyl column showed stable performance through greater than 2,000 injections and the curtain plate on the AB SCIEX 4500 was cleaned only roughly every week despite consistent use with this type of matrix. Using this 2.2 min method, sufficient separation was achieved and because of the unused time portion prior to the first analyte, multiplexing (or overlapping) injections is a reasonable option to greatly increase throughput (Figure 1).

Centrifugation steps are in place in this protocol to help prevent any debris in sample from being injected through small-bore tubing and analytical columns. The addition of a flow diversion step is prudent to minimize build-up of any salts or debris in the source and/or tubing that is inevitable no matter how great the dilution or clean-up. For this method, flow diversion is selected for the first minute. At the 1-min mark, the diversion valve switches and directs the flow to the mass spectrometer. The flow is diverted back to waste at Minute 2.2 to ensure flow is going to waste between all samples and in the event of a time-out or error.

This method was originally validated on an Agilent 1290 chromatography system (connected to the same SCIEX 4500) using an 80 : 20 MeOH : H_2O diluent; a remnant of a comprehensive multianalyte assay from which this opioid method was extracted and then optimized. This original system was plumbed with 0.17 mm ID steel tubing (i.e., Agilent's 'green' tubing) connecting the autosampler to the guard column. Later, this method was reverified on a different, yet identical system, except for this tubing which runs from the autosampler to the guard column. On the second setup, this tubing had an ID of 0.12 mm (i.e., Agilent's 'red' tubing), which in turn caused a dramatic broadening of the morphine and oxymorphone peaks due to the high organic solvent content of the injected bolus due to the sample diluent. It is theorized that with the larger ID tubing, which was used initially, this bolus had adequate time to effectively mix prior to reaching the analytical column. With the smaller ID tubing, however, it did not mix until reaching the analytical column. As the analytes adsorbed to the column, they were immediately partially desorbed (by the high organic content of the solvent around them) and then re-adsorbed as the high organic solvent sufficiently mixed with surrounding aqueous solvent, thus causing a broad 'smeared' peak (Figure 2) of the more hydrophilic compounds. Switching to a 20 : 80 MeOH : H_2O sample diluent promptly solved this issue. This example underlines the importance of sample diluent choice in LC–MS methods as well as attention to compatible tubing diameters and plumbing conditions.

Traditional opinion on liquid chromatography typically dictates that all analytes be baseline separated. This may be founded on practices and techniques arrived at when UV detection followed LC separation or with the goal of separating out all interfering compounds from complex mixtures. When using a mass selective detector in conjunction with LC, it is counterproductive to require all analytes to be chromatographically baseline separated since the differences in parent and fragment masses are often enough to distinguish the analytes. This method does not baseline separate all analytes present. Although there are two sets of isobaric compounds in this group of analytes, these analytes do no exhibit appreciable interference on one another. For example hydromorphone, morphine and norhydrocodone, all with a parent mass of ~286 Da, are baseline separated in the chromatogram. This chromatographic separation is required as morphine and norhydrocodone share a mass transition (the qualifier ion for both compounds is the same). Codeine and

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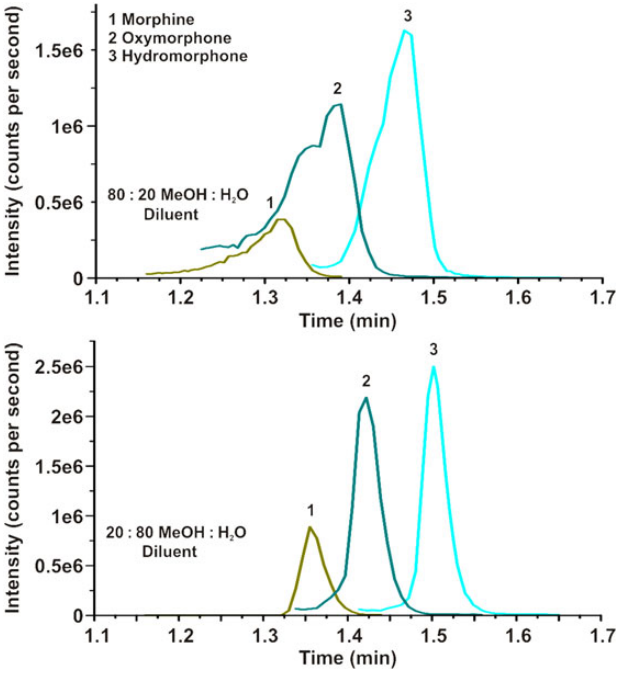

Figure 2. Two chromatograms demonstrating the effect of sample diluent on the final peak shape of morphine, oxymorphone and hydromorphone. The top chromatogram is the theorized result of slight de-adsorption of analyte while loading due to the highly organic composition of the sample diluent (80% methanol). The bottom chromatogram exhibits more favorable binding conditions as a result of lowering the organic composition of the sample diluent from 80 to 20%.

hydrocodone, on the other hand, have the same parent mass of ~300 Da, and are not baseline separated, but do not share any transitions. High-throughput methods of this variety, which do not emphasize pristine chromatographic separation, must be extensively tested for matrix and other interferences which may contribute unfavorably to an analyte's signal.

When compared with a previously validated extraction-based LC method, this method showed excellent agreement among actual patient samples. For example, hydrocodone showed an average relative change of 12.4% ($N = 62$), and morphine yielded 0.02% ($N = 17$). Because the occurrence of some analytes/metabolites is very rare and replacement and newly validated methods can only be run simultaneously for so long, some analytes were not confirmed often enough to produce statistically relevant comparison values. These changes are consistent with the matrix test results for negative saliva samples and point to a rather minor matrix effect in either positive or negative (e.g., morphine) direction.

Conclusion

This work demonstrates the analysis of opioids using a simple dilute-and-shoot procedure, a fast separation and sensitive mass spectral detection. Key features of the method include centrifugation and a 10× dilution with internal standard and diluent (20 : 80 MeOH : water). The results from this method compare well with the same samples run with an LC–MS-MS method that uses a SPE clean-up and sample concentration (10×), a different analytical column, but the same ion fragmentation transitions.

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