Research article

Drug Testing and Analysis

Received: 20 December 2011 Revised: 9 January 2012 Accepted: 10 January 2012 Published online in Wiley Online Library: 28 February 2012

(www.drugtestinganalysis.com) DOI 10.1002/dta.1324

Disposition of opioids in oral fluid: Importance of chromatography and mass spectral transitions in LC-MS/MS

James Tuyay, Cynthia Coulter, Warren Rodrigues and Christine Moore*

The use of prescription pain relievers, specifically opioids, has been increasing over the last few years. Oral fluid is easier to collect than urine, is difficult to adulterate, and is a reflection of free drug in the body, so its analysis is becoming more widespread in the monitoring of opioids. The demethylated metabolites of oxycodone, hydrocodone, and codeine are present at higher concentrations in oral fluid than oxymorphone, hydromorphone, and morphine, respectively; therefore, their detection in saliva indicates ingestion of the medication rather than diversion, and should be included in the analysis of opioids in this matrix. Since the compounds have the same nominal molecular weights, the same $M+H^+$ precursor ions in positive electrospray mode, and potentially identical collisionally activated fragmentation patterns, the importance of chromatography to separate the various opioids as well as the selection of mass spectral transitions is critical for correct identification. A procedure for the simultaneous determination of 12 opioid related compounds in oral fluid using liquid chromatography with tandem mass spectrometry (LC-MS/MS) is presented. The recovery of opioids from the collection device was over 80% at 20 ng/ml; intra-day imprecision was less than 6.8%; inter-day imprecision less than 6.2%. In authentic specimens, the predominant metabolite of oxycodone was noroxycodone; for specimens containing codeine, no morphine was detected; and for hydrocodone positives, norhydrocodone was detected at significantly higher levels than hydromorphone. The importance of monitoring specific mass spectral transitions and chromatographic separation is demonstrated. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: liquid chromatography; tandem mass spectrometry; oral fluid; opioids

Introduction

In addition to the abuse of heroin, other opiates and semi-synthetic opioids are widely prescribed for the management of mild to moderate and chronic pain. The most widely used are codeine, dihydrocodeine (DHC, Parzone®), hydrocodone (Vicodin®), hydromorphone (Dilaudid®), morphine (MS-Contin®), oxycodone (OxyContin®), and oxymorphone (Opana®).

According to a recently released report from the Substance Abuse and Mental Health Service Administration (SAMHSA) entitled *Substance Abuse Treatment Admissions Involving Abuse of Pain Relievers: 1998 and 2008*, non-medical use of prescription pain relievers, specifically opioids, was the second, most prevalent type of illicit drug use, after marijuana use during that period.[1]

Oral fluid (saliva) is increasing in popularity as a drug testing matrix, due to its ease of collection, difficulty of adulteration, and improving sensitivity of analytical techniques. Since therapeutic drug monitoring is widespread using serum or plasma drug concentrations, it follows that an ultra-filtrate of the blood, oral fluid should be a better indicator of therapeutic concentrations than urine. In recent publications, several authors have concluded that oral fluid is a viable alternative to urine for use in compliance monitoring programs of chronic pain patients.[2,3]

The perceived drawbacks of oral fluid testing, such as unknown quantity collected, inadequate drug recovery from collection pads, and insufficient volume for multiple drug confirmations have been largely overcome by improved oral fluid collection devices and increased sensitivity of laboratory instrumentation, specifically mass spectrometers. The determination of codeine, morphine, their demethylated metabolites, and 6-acetylmorphine has been reported in oral fluid using liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS);[4] and a 2009 paper described the determination of several of these compounds in oral fluid collected with the Intercept® device, and using positive electrospray liquid chromatography-tandem mass spectrometry (LC-MS/MS).[5] The current report describes the disposition and simultaneous determination of several opioids in oral fluid collected with the Quantisal™ device, including mass spectral pathways and fragmentation patterns for drugs with similar chemical structures. The fully validated method highlights the importance of chromatographic separation and monitoring multiple specific mass spectral transitions.

Materials and methods

Collection devices, reagents, and reference standards

Oral fluid was collected using the Quantisal™ collection system (Immunalysis Corporation, Pomona, CA, USA). The device consists of a collection pad with a volume adequacy indicator, which turns blue when one milliliter of oral fluid (1 ml+/− 10%) has been collected. The pad is then placed into transport buffer (pH7; 3 ml), which provides drug stability during transport and storage, prevents bacterial growth, and produces a total specimen volume

* *Correspondence to: Christine Moore, Immunalysis Corporation, 829 Towne Center Drive, Pomona, CA 91767, USA. E-mail: cmoore@immunalysis.com*

Immunalysis Corporation, Pomona, CA, USA

of 4 ml (3 ml buffer + 1 ml oral fluid) for laboratory analysis. This is specifically advantageous in cases where the specimen is positive for more than one drug, since the volume of specimen available for analysis can be an issue. In contrast to the Intercept® device, the collection pad itself is untreated so there are no stimulants to promote the secretion of oral fluid, other than that caused by the pad itself. When the production of saliva is assisted, more oral fluid is produced so measured drug concentrations are lower, which may give biased results. Deuterated internal standards as well as unlabelled drug reference standards were obtained from Cerilliant (Round Rock, TX, USA). Solid-phase extraction (SPE) columns (Clin II, 691-0353 T) were obtained from SPEWare (Baldwin Park, CA, USA). All solvents were high performance liquid chromatography (HPLC) grade or better, purchased from Spectrum Chemicals (Gardena, CA, USA) and all chemicals were ACS grade.

Calibrators and controls

The deuterated internal standards (codeine-d3, morphine-d3, 6-acetylmorphine-d3 and oxycodone-d6) stock solutions and the unlabelled drug standards for codeine (COD), norcodeine (nor-COD), morphine (MOR), hydrocodone (HYC), norhydrocodone (nor-HYC), hydromorphone (HYM), 6-acetylmorphine (6-AM), 6-acetylcodeine (6-AC), oxycodone (OXYC), noroxycodone (nor-OXYC), oxymorphone (OXYM) and dihydrocodeine (DHC) were prepared in methanol at a concentration of 100 μg/ml. The working solutions were diluted from the stock and were stored at −20 °C when not in use. Controls were prepared by fortifying drug free oral fluid with various concentrations of opioids. Drug-free negative specimens, positive controls at 5 and 50 ng/ml were included in every batch.

Sample preparation

For the chromatographic calibration standards, a working solution for the deuterated internal standards and unlabelled drugs were prepared in methanol at a concentration of 200 ng/ml and stored at −20 °C when not in use. For each batch, seven calibration standards at concentrations of 1, 4, 10, 20, 40, 80, and 160 ng/ml were prepared in synthetic oral fluid (1 ml) then transportation buffer from the Quantisal™ collection device was added (3 ml). An aliquot of saliva/buffer from the Quantisal™ collection device (1 ml), equivalent to 0.25 ml of neat oral fluid was removed and internal standard was added (100 μl). Solid-phase mixed mode extraction columns were conditioned with methanol (2 ml), and 0.1 M phosphate buffer (pH 6.0; 2 ml). The samples were allowed to flow through the columns, and then the columns were washed with deionized water (1 ml), 0.1 M acetate buffer (pH 4; 1 ml), methanol (1 ml) and ethyl acetate (1 ml). The columns were allowed to dry under nitrogen pressure (30 psi; 2 min). Opioids were eluted using freshly prepared ethyl acetate: ammonium hydroxide (98:2 v,v; 2 ml). The extracts were evaporated to dryness under nitrogen and reconstituted in methanol (50 μl).

Liquid chromatography-mass spectrometry conditions (LC-MS/MS)

A 1200 Series LC pump coupled to a 6430 triple quadrupole mass spectrometer, operating in positive electrospray ionization (ESI) mode was used for analysis (Agilent Technologies, Santa Clara, CA, USA). The column used was a Zorbax Eclipse XDB C18 (4.6 x 50 mm x 1.8 μm) and held at a temperature of 60 °C; injection volume was 5 μl. The mobile phase consisted of 20 mM ammonium formate pH 6.4 (Solvent A) and methanol (Solvent B). Initially, the mobile phase composition was 85% A at a flow rate of 0.7 ml/min over a period of 7 min, then the percentage of methanol was increased to 90%, then returned again to 85% A after 8 min.

The gas temperature was 350 °C, the gas flow was 10 l/min and the nebulizer pressure was maintained at 50 psi. Nitrogen was used as the collision gas and the capillary voltage was 4000 V.

Each compound was run in product scan mode to assess fragmentation of the precursor ($M+H^+$) ion. The nominal molecular weight of MOR, nor-COD, HYM and nor-HYC is *m/z* 285; the nominal molecular weight of DHC, OXYM and nor-OXYC is *m/z* 301; the nominal molecular weight of COD and HYC is *m/z* 299; therefore isobaric compounds with similar molecular structures (Figure 1) may generate identical product ions. For the first group (*m/z* 285), morphine and nor-hydrocodone were separated from the other analytes on the basis of retention time, but nor-COD and HYM co-eluted. The product ion full scan spectra obtained by collisions of the precursor ion $M+H^+$ are shown in Figure 2. Fragment ions of *m/z* 185 and 157 were monitored for HYM; 165 and 268 for nor-COD. The $286 > 268$ transition was due to water loss, which is generally avoided if possible, but in this case was shown to be stable, free from interference and consistent.

Method validation

The method was validated according to accepted scientific criteria including: selectivity, linearity, sensitivity, imprecision, accuracy, drug recovery, ion suppression or enhancement, drug stability, and extract stability.

Two multiple reaction monitoring (MRM) transitions were identified, optimized, and integrated for each drug at dwell times of 50 ms; the retention times, transitions, optimal fragmentation voltages and collision energies for the opioids are shown in Table 1. The relative MRM transition intensity of the second transition (qualifier) was calculated with respect to the abundance of the primary MRM transition (quantifier). The ratio of the qualifier transition to the quantifier transition was determined from replicate analyses of a calibration standard at approximately the mid-point of the analytical range: 20 ng/ml. For a specimen to be considered positive, the ratio between the quantitation and qualification product transition was required to be within + −20% of that established using the calibration standard. Deuterated 6-AM was used as the internal standard for 6-AM, 6-AC; deuterated codeine was used as the internal standard for COD, nor-COD, DHC, HYC and nor-HYC; deuterated morphine was used as the internal standard for MOR and HYM; and deuterated oxycodone was used for OXYC, nor-OXYC and OXYM.

Selectivity

Five drug-free oral fluid specimens were collected using the Quantisal™ device. An aliquot of each was taken and subjected to extraction and analysis as described, in order to assess potential interferences associated with endogenous compounds or the transportation buffer. Commonly prescribed and illicit drugs were added to other oral fluid aliquots at concentrations of 2000 ng/ml. Tetrahydrocannabinol (THC), 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid, 11-hydroxy-THC, cannabinol, cannabidiol, cocaine, benzoylecgonine, norcocaine, cocaethylene, amphetamine,

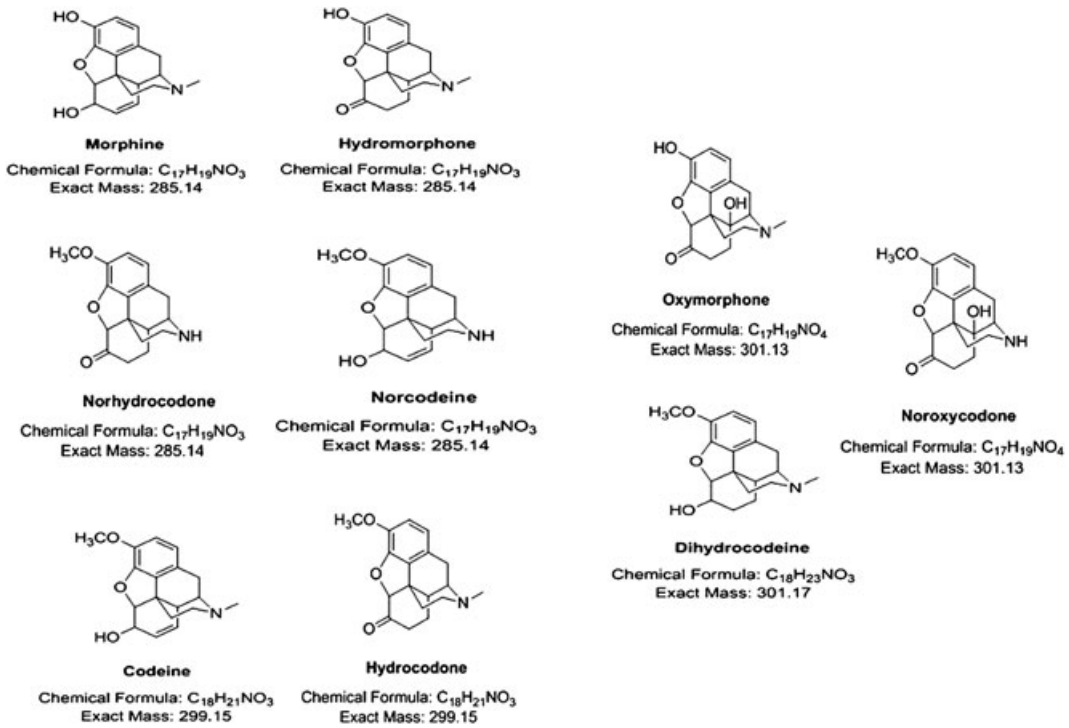

Figure 1. Molecular structures of opioids and their demethylated metabolites. Molecular weight *m/z* 285: morphine; hydromorphone; norhydrocodone; norcodeine. Molecular weight *m/z* 299: codeine; hydrocodone. Molecular weight *m/z* 301: oxymorphone; noroxycodone; dihydrocodeine.

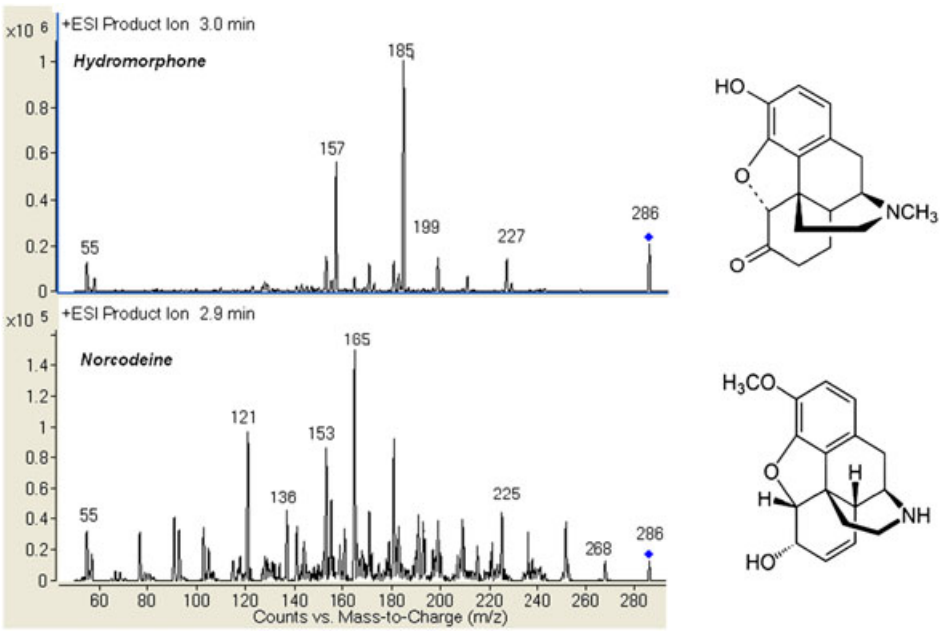

Figure 2. Product ion full scan obtained by collisions of $M+H^+$ ions of hydromorphone and nor-codeine.

methamphetamine, methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), methylenedioxyethylamphetamine (MDEA), phentermine, ketamine, zolpidem, naltrexone, fentanyl, phencyclidine, citalopram, venlafaxine, amitriptyline, cyclobenzaprine, imipramine, dothiepin, doxepin, fluoxetine, sertraline, trimipramine, protriptyline, chlorpromazine, clomipramine, nortriptyline, paroxetine, desipramine, bromazepam, alprazolam, clonazepam, lorazepam, oxazepam, diazepam, midazolam, flurazepam, flunitrazepam, nordiazepam, triazolam, temazepam, nitrazepam, chlordiazepoxide, secobarbital, phenobarbital, pentobarbital, butalbital, and butabarbital as well as other pain medications: carisoprodol, meprobamate, buprenorphine, dextromethorphan, meperidine, propoxyphene, tapentadol, tramadol, and methadone were all added to drug-free oral fluid, extracted and analyzed as described.

Linearity and sensitivity

Calibration using deuterated internal standards was calculated using linear regression analysis over the concentration range.

Table 1. Multiple reaction monitoring (MRM) transitions, retention times and optimized fragmentation voltages for opioids in oral fluid

Compound	*Transition*	*Retention time (min)*	*Fragment voltage (V)*	*Collision energy (eV)*
Oxymorphone	**302 ≥ 227**	1.6	140	30
	302 > 198		120	45
D3-morphine	*289 ≥ 165*	*2.0*	*130*	*40*
Morphine	**286 ≥ 165**	2.1	170	35
	286 > 153		150	45
Hydromorphone	286 ≥ 185	2.5	160	35
	286 > 157		160	50
Norcodeine	**286 ≥ 165**	2.6	170	35
	286 > 268		180	35
Noroxycodone	302 ≥ 284	2.6	140	10
	302 > 187		140	20
Norhydrocodone	286 ≥ 199	3.0	180	25
	286 > 171		180	35
D6-oxycodone	*322 ≥ 247*	*3.0*	*160*	*25*
Dihydrocodeine	302 ≥ 199	3.0	180	35
	302 > 227		140	30
Oxycodone	316 ≥ 298	3.1	160	15
	316 > 241		160	30
D3-codeine	*303 ≥ 165*	*3.3*	*140*	*40*
Codeine	**300 ≥ 165**	3.4	140	45
	300 > 215		140	25
D3-6-acetylmorphine	*331 ≥ 165*	*3.5*	*160*	*35*
6-acetylmorphine	328 ≥ 165	3.6	160	40
	328 > 211		160	40
Hydrocodone	300 ≥ 199	3.8	160	35
	300 > 165		140	45
6-acetylcodeine	342 ≥ 225	5.0	160	30
	342 > 165		160	40

Underlined transition used as quantifiers.
Bold transitions are the same for multiple compounds.

Peak area ratios of the target analyte and the internal standard were calculated using Mass Hunter software (Agilent). The data were fit to a linear least-squares regression curve with a weighting factor of 1/x and not forced through the origin. The linearity of the assays was established with seven calibration points, excluding the drug free matrix. The limit of quantitation (LOQ) of the method was determined using serial dilutions to the lowest point where the acceptable criteria for the quantitation of a compound were met, i.e. the chromatographic peak shape, retention time (within 2% of calibration standard) and qualifier transition ratio (+ − 20%) compared to the 20 ng/ml calibration standard were acceptable. The quantitative value of the LOQ had to be within + −20% of the target concentration and replicate analyses were required to have low variation in response (n = 5; CV,15%). The highest variation was nor-HYC (CV = 9.1%).

Matrix effects

Oral fluid specimens were obtained from drug-free volunteers, extracted and analyzed according to the described procedures in order to assess matrix effects according to the protocol of Matuszewski.[6] Briefly, a non-extracted drug standard at a concentration of 20 ng/ml was prepared as well as drug-free matrix extracts and negative controls (extracts containing only internal standard). The recovery of the opioids from oral fluid was determined by assessing the response of the extracted samples (n = 5) {R_{ES}}. Next, oral fluid was extracted and drug (20 ng/ml) was added post-extraction (n = 5) {R_{PES}}. The percentage recovery was then calculated from the equation (R_{ES} / R_{PES}) x 100. The percentage reduction or improvement in response due to matrix effects (ion suppression / ion enhancement) was determined by assessing the peak area response of a non-extracted neat drug standard (n = 5) at a concentration of 20 ng/ml {R_{NES}}. The non-extracted solution was analyzed in the same reconstitution solvent as the extracted specimens. The matrix effect was then calculated using the equation (R_{PES} / R_{NES}) -1 x 100. A negative result indicated ion suppression and a positive result indicated ion enhancement of the signal. The overall efficiency of the process was calculated as (R_{ES} / R_{NES}) x 100. Reduction of matrix effects is best achieved by utility of deuterated internal standards where possible, extensive matrix clean-up before injection and optimal chromatographic and mass spectral conditions.

Accuracy and imprecision

The accuracy of the procedure was determined over six replicates at 5 and 50 ng/ml. Accuracy was calculated as mean measured concentration divided by the fortified concentration x 100%.

Imprecision was evaluated at two concentrations. Specimens were fortified with the opioids simultaneously at concentrations of 5 and 50 ng/ml and six aliquots were analyzed according to the described procedure. Intra-day data were obtained from six

analyses performed on one day (n = 6); inter-day data were obtained by analyzing six specimens each day for five days (n =30).

Drug recovery from the collection device

The efficiency of extraction from the collection pad and transportation buffer was determined. Synthetic oral fluid was fortified with drugs at a concentration of 20 ng/ml. A collection pad was placed into the fluid until the volume adequacy indicator turned blue showing that 1 ml (+ − 10%) of oral fluid had been absorbed. The pads were then placed into the Quantisal™ buffer (3 ml), capped, and allowed to remain at room temperature overnight, to simulate transportation to the laboratory. The following day, the pads were removed after separation from the stem, and an aliquot (1 ml) of the specimen was analyzed. The procedure was repeated five times.

Stability of the opioids in oral fluid and as extracts

The stability of opioids in oral fluid at a concentration of 20 ng/ml was determined over seven days at both room temperature and at 4 °C. One set of specimens remained at room temperature; one set were refrigerated at 4 °C for seven days. On days 1 and 7 an aliquot was removed and analyzed as described.

The stability of the extracts was also investigated. Auto-sampler vials, after analysis, were re-capped and stored at 7 °C overnight, before being re-analyzed. The concentration change between the days was noted.

Authentic samples

Specimens obtained from various research projects were subjected to the described procedure.

Table 2. Average linearity; correlation coefficient for calibration curve (r^2); established range for product ion transition ratio at 20 ng/mL (n = 5); ion suppression or enhancement, recovery, matrix effect and process efficiency for opioid drugs in oral fluid

Analyte	*Equation*	*Correlation (r^2)*	*Transition ratio (+ − 20%)*	*Matrix effects Ion suppression or enhancement*		
				Recovery (%) (R_{ES} / R_{PES}) x 100	*Matrix effect (%)* (R_{PES} / R_{NES}) -1 x 100	*Process efficiency (%)* (R_{ES} / R_{NES}) x 100
Oxymorphone	*y = 0.046x −0.004*	*0.998*	22.4 – 33.6	102	−19	82.6
Morphine	*y = 0.026-0.006*	*0.998*	56.4 – 84.7	94.2	−16	79.1
Hydromorphone	*y = 0.06 +0.004*	*0.998*	72.9 – 109	103	−21	81.7
Norcodeine	*y = 0.014-0.01*	*0.996*	42.8 – 64.3	97.7	−11	86.4
Noroxycodone	*y = 0.036-0.029*	*0.995*	22.1 – 33.1	98.3	−11	86.6
Norhydrocodone	*y = 0.018-0.004*	*0.999*	29.9 – 44.9	101	−13	87.3
Dihydrocodeine	*y = 0.028-0.002*	*0.997*	69.4 – 104	97.5	−9.9	87.9
Oxycodone	*y = 0.063-0.003*	*0.999*	20.2 – 30.4	101	−12	88.8
Codeine	*y = 0.03-0.003*	*0.999*	38.7 – 58.2	100	−9.8	90.7
6-acetylmorphine	*y = 0.014-0.001*	*0.998*	12.3 – 18.5	102	−9.4	93.3
Hydrocodone	*y = 0.046 +0.004*	*0.999*	16.8 – 25.2	102	−18	83.5
6-acetylcodeine	*y = 0.023-0.001*	*0.998*	28.6 – 43.0	103	−11	91.6

R_{ES} = response of extracted specimens (n = 5); R_{PES} = response of post-extracted specimens (n = 5); R_{NES} = response of non-extracted drug standards (n = 5).

Table 3. Method validation: Extraction efficiency, accuracy, intra- and inter-day imprecision at two concentrations

Drug	*Extraction efficiency, accuracy and imprecision*						
	Pad recovery (%) (n = 6)	*Accuracy (%)*		*Imprecision (5 ng/ml)*		*Imprecision (50 ng/ml)*	
		5 ng/ml	*50 ng/ml*	*Intra-day (n = 6)*	*Inter-day (n = 30)*	*Intra-day (n = 6)*	*Inter-day (n = 30)*
Oxymorphone	92.1	107	101	2.4	5.4	2.3	3.8
Morphine	85.1	100	104	4.0	3.6	2.1	4.0
Hydromorphone	83.8	93.3	98.7	4.2	5.2	4.1	4.4
Norcodeine	86.9	94.1	96.7	6.8	5.5	3.3	4.8
Noroxycodone	90.5	94.2	102	5.9	6.0	2.8	4.3
Norhydrocodone	87.3	92.3	97.7	6.6	6.2	3.0	4.1
Dihydrocodeine	83.9	98.1	101	2.5	3.7	4.8	3.5
Oxycodone	90.0	102	101	1.5	4.9	1.0	3.1
Codeine	85.8	101	92.7	3.8	3.9	3.7	4.1
6-acetylmorphine	83.9	100	101	5.0	4.6	2.3	3.2
Hydrocodone	84.5	94.6	97.0	5.7	3.8	3.3	3.7
6-acetylcodeine	80.9	100	100	4.9	5.3	4.7	5.0

Table 4. Concentrations of oxycodone and noroxycodone in authentic oral fluid specimens

Specimen	Oxycodone (ng/ml)	Nor-oxycodone (ng/ml)	Percentage of nor-metabolite
1	49	0	n/a
2	296	90	30.4
3	675	96	14.2
4	844	126	14.9
5	1040	252	24.3
6	1815	674	37.1
7	1879	919	48.9
		Average % noroxycodone = 28%	

Results and discussion

The method was predominantly validated using drug free oral fluid specimens diluted in 0.1% bovine serum albumin (BSA) buffer, fortified with various concentrations of the analytes.

Selectivity

No endogenous interference was noted from drug-free extracts; or for exogenous interference from any of the commonly encountered drugs, which were analyzed at high concentration.

Linearity and sensitivity

Good linearity was obtained for all the analytes over the range 1–160 ng/ml. The LOQ was determined to be 1 ng/ml for all analytes; the limit of detection (LOD) was not determined since all specimens were quantitated. The average linearity of the calibration standards (n = 5), correlation coefficients and acceptable ratios for transitions produced by the various opioids (n = 5) are shown in Table 2.

Matrix effects

The matrix effects associated with LC-MS/MS systems are minimized when deuterated internal standards and efficient extraction procedures are incorporated into the assay. As a result, the only opioid with ion suppression greater than 20% was hydromorphone (21%). The process efficiency for all drugs was over 80% showing good optimization of the entire assay (Table 2).

Accuracy and imprecision

The results for the accuracy and imprecision of the assay are shown in Table 3. Overall the method was consistent with accuracy at both concentrations no less than 92%; intra-day imprecision values for all drugs less than 6.6% and 4.8% at 5 and 50 ng/ml, respectively (n = 6); inter-day imprecision maximum values were 6.0% and 5.0% for 5 and 50 ng/ml, respectively (n = 30).

Drug recovery from the collection device

The recovery of the opioids from the collection pad was determined and the results are shown in Table 3. All the drugs were well recovered from the collection pad with 6-AC showing the lowest percentage recovery at 80.9%; OXYM showed the highest recovery at 92.1% (n = 6).

Stability of the opioids in oral fluid and as extracts

The stability of the opioids at room temperature and refrigerated (4 °C) over a period of seven days was studied. Overall, the demethylated metabolites were the most stable over seven days, losing less than 5% whether at room temperature or refrigerated. MOR and OXYM appeared to be less stable refrigerated than at room temperature, but all the other drugs lost less than 20% under both sets of conditions. Only 6-AC showed more than 20% degradation at room temperature (21.6%); 11.6% loss when

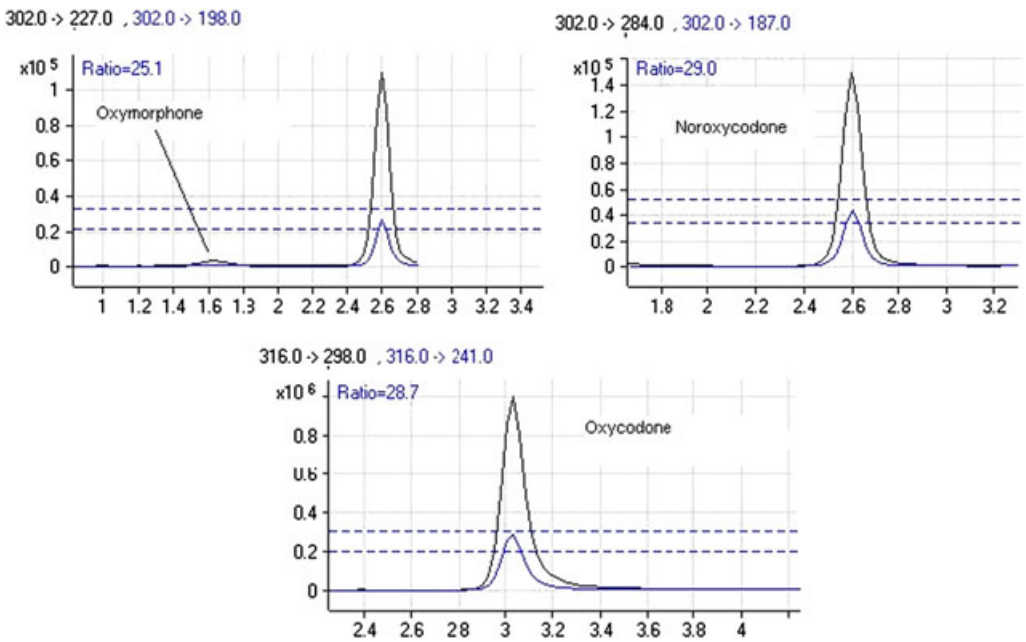

Figure 3. MRM transitions from Specimen #5, an authentic oral fluid specimen, showing the presence of oxycodone and noroxycodone; absence of oxymorphone.

refrigerated. Collected specimens are stable for at least seven days at room temperature; thereafter they should be refrigerated for extended storage in the Quantisal buffer.

The extracted specimens were stable over 48 h, with no measurable loss for any drug with the exception of MOR which showed a loss of 24% at both 5 and 50 ng/ml concentrations over 48 h while stored at 7 °C.

Authentic specimens

The procedure was applied to specimens received during various research projects. In urine, monitored metabolites of COD, HYC and OXYC tend to be MOR, HYM, and OXYM, respectively, even though the demethylated metabolites are also present, and recent papers have suggested they be included in drug-monitoring programmes. In oral fluid specimens analyzed using this procedure, while the parent drugs themselves were present in measurable concentration, the predominant metabolites detected were nor-COD, nor-HYC and nor-OXYC respectively, and not MOR, HYM and OXYM. Morphine was not detected at all in codeine positive specimens; this is in agreement with controlled dosage studies reported for codeine.[7] In oral fluid specimens positive for hydrocodone, the same pattern was observed; nor-HYC was the main metabolite detected. The results from seven specimens positive for oxycodone are shown in Table 4. With the exception of one low level oxycodone (49 ng/ml), nor-OXYC was detected in all specimens containing parent oxycodone in concentrations ranging from 14% to 48% of the parent drug (mean 28%; median 27%). Oxymorphone was not detected in any of the specimens.

The TIC from specimen # 5 is shown in Figure 3. The dotted lines represent the acceptable ion ratio for the qualifying transition. While the transitions for OXYM are visible at a retention time of 1.6 min, the concentrations was well below the LOQ and the intensity of nor-OXYC (retention time 2.6 min) is significantly greater.

In 2010, Cone *et al*. recommended the inclusion of the demethylated metabolites in urine test profiles for pain medications since their presence indicated drug ingestion as opposed to diversion, and reduced the incidence of false negatives. They reported that 8.6%, 7.8% and 9.4% of urine specimens contained nor-COD, nor-HYC and nor-OXYC respectively with no measurable parent drug.[8] When pain patients are monitored using oral fluid, it is often necessary to determine ingestion of the drug rather than diversion. Monitoring of metabolites allows this to be assessed, but mistakenly identifying oxymorphone as a metabolite of oxycodone; and/or hydromorphone as a metabolite of hydrocodone in oral fluid is a distinct possibility. Correct identification must be carried out on the basis of retention time and ratio of at least two mass spectral transitions. To date no oral fluid specimens with demethylated metabolites only have been reported. Presence of morphine, oxymorphone or hydromorphone in oral fluid likely indicates the ingestion of those specific drugs, and not a metabolic pathway.

In order to avoid misidentification of compounds, the necessity of including more than one MRM transition in MS/MS methods has been demonstrated by numerous researchers. It is recommended that at least two MRM product ions be present at specific abundances, as well as reproducible retention times and, where available, the inclusion of a stable isotopic internal standard. Fox *et al*. published an excellent paper on the criteria for opiate identification using liquid chromatography linked to tandem mass spectrometry and problems in routine practice.[9] They pointed out the similar transitions associated with dihydrocodeine and noroxycodone as well as the importance of retention time and good chromatography. Coles *et al*., while pointing out the advantages of LC-MS/MS, also discussed the importance of monitoring and using secondary transitions having observed increases in concentration of opioids due to the presence of other members of the same class.[10] The monitoring of three MS/MS transitions producing two ratios is recommended for urine assays where drug levels are relatively high, but for matrices where the concentrations are low, it is often not possible to achieve two qualifying transitions and maintain adequate sensitivity. However, it is essential to monitor at least two specific transitions (producing one ratio) in order to minimize the potential for misidentification of compounds with similar fragmentation patterns and retention time.

Summary

We report a sensitive, specific method for the simultaneous detection of opioids in oral fluid collected with the volume adequacy indicating Quantisal™ device. The procedure is applicable to the analysis of various opioids and their demethylated metabolites in oral fluid. Reproducible retention time, good chromatography, and monitoring of multiple mass spectral transitions are essential for the correct identification of opioids and their metabolites in oral fluid.

References

[1] Available at: http://oas.samhsa.gov/2k10/230/230PainRelvr2k10.cfm [15 July 2010].
[2] R. Heltsley, A. DePriest, D. L. Black, T. Robert, L. Marshall, V. M. Meaders, *et al*. Oral fluid drug testing of chronic pain patients. I. Positive prevalence rates of licit and illicit drugs. *J. Anal. Toxicol.* **2011**, *35*, 529.
[3] V. Vindenes, B. Yttredal, E. L. Oiestad, H. Waal, J. P. Bernard, J. G. Morland, *et al*. Oral fluid is a viable alternative for monitoring drug abuse: detection of drugs in oral fluid by liquid chromatography-tandem mass spectrometry and comparison to the results from urine samples from patients treated with methadone or buprenorphine. *J. Anal. Toxicol.* **2011**, *35*, 32.
[4] R. Dams, C. M. Murphy, R. E. Choo, W. E. Lambert, A. P. De Leenheer, M. A. Huestis. LC-atmospheric pressure chemical ionization-MS/MS analysis of multiple illicit drugs, methadone and their metabolites in oral fluid following protein precipitation. *Anal. Chem.* **2003**, *75*, 798.
[5] D. Fritch, K. Blum, S. Nonnemacher, B. J. Haggerty, M. P. Sullivan, E. J. Cone. Identification and quantitation of amphetamines, cocaine, opiates, and phencyclidine in oral fluid by liquid chromatography-tandem mass spectrometry. *J. Anal. Toxicol.* **2009**, *33*, 569.
[6] B. K. Matuszewski, M. L. Constanzer, C. M. Chavez-Eng. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal. Chem.* **2003**, *75*, 3019.
[7] I. Kim, A. J. Barnes, J. M. Oyler, R. Schepers, R. E. Joseph, E. J. Cone, *et al*. Plasma and oral fluid pharmacokinetics and pharmacodynamics after oral codeine administration. *Clin. Chem.* **2002**, *48*, 1486.
[8] E. J. Cone, A. Zichterman, R. Heltsley, D. L. Black, B. Cawthon, T. Robert, *et al*. Urine testing for norcodeine, norhydrocodone and noroxycodone facilitates interpretation and reduces false negatives. *Forensic Sci. Int.* **2010**, *198*, 58.
[9] E. J. Fox, S. Twigger, K. R. Allen. Criteria for opiate identification using liquid chromatography linked to tandem mass spectrometry: problems in routine practice. *Ann. Clin. Biochem.* **2009**, *46*, 50.
[10] R. Coles, M. M. Kushnir, G. J. Nelson, G. A. McMillin, F. M. Urry. Simultaneous determination of codeine, morphine, hydrocodone, hydromorphone, oxycodone, and 6-acetylmorphine in urine, serum, plasma, whole blood, and meconium by LC-MS-MS. *J. Anal. Toxicol.* **2007**, *31*, 1.