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The Quantification of Oxycodone and Its Phase I and II Metabolites in Urine

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Abstract

The purpose of this research was to develop and validate an analytical method for the detection and quantification of noroxymorphone-3β-D-glucuronide (NOMG), oxymorphone-3β-D-glucuronide (NOMG), noroxymorphone (NOM), oxymorphone (OM), 6α-oxycodol (αOCL), 6β-oxycodol (βOCL), noroxycodone (NOC) and oxycodone (OC) in urine by liquid chromatography tandem mass spectrometry to be used in a human study. The method was validated according to the Academy Standards Board Standard Practices for Method Development in Forensic Toxicology. The method was then applied to a single-dose pilot study of a subject. Urine samples were collected from the subject after ingesting 10-mg OC as an immediate-release tablet. Additionally, urine specimens ($n = 15$) that had previously been confirmed positive for OC were analyzed using the validated method. The calibration range for NOMG and OMG was 0.05–10 μg/mL; for all other analytes, it was 0.015–10 μg/mL. Validation parameters such as bias, precision, carryover and dilution integrity, all met the validation criteria. After the method was validated, urine samples from the first subject in the controlled dose study were analyzed. It was observed that OC, NOC and OMG contained the highest concentrations and were present in either the 0.5 or 1 h void. NOC and OMG were detected until the 48 h collection, while OC was detectable till the 24 h collection. Time to reach maximum concentration (T_{max}) in the urine was achieved within 1.5 h for OC and within 3 h for NOC and OMG. Maximum concentration (C_{max}) in the urine for OC, NOC and OMG was 3.15, 2.0 and 1.56 μg/mg, respectively. OC concentrations in authentic urines ranged from 0.015 to 12 μg/mL. Ranges for NOMG and OMG were 0.054–9.7 μg/mL and 0.14–67 μg/mL, respectively. A comprehensive method for the quantification of NOMG, OMG, NOM, OM, αOCL, βOCL, NOC and OC in urine was optimized and met the validation criteria. The concentrations of NOMG and OMG presented in this study provide the details needed in the forensic community to better comprehend OC pharmacokinetics.

Introduction

Oxycodone (OC) is a semi-synthetic opioid that is commonly prescribed due to its analgesic properties. Prevalence of abuse of this opioid was observed in a study by Rookey, where there was 1,000% increase of drivers who tested positive for OC in 2016 compared with 2001. Additionally, there was a significant increase in prescription opioid overdose deaths between 2001 and 2015 (1).

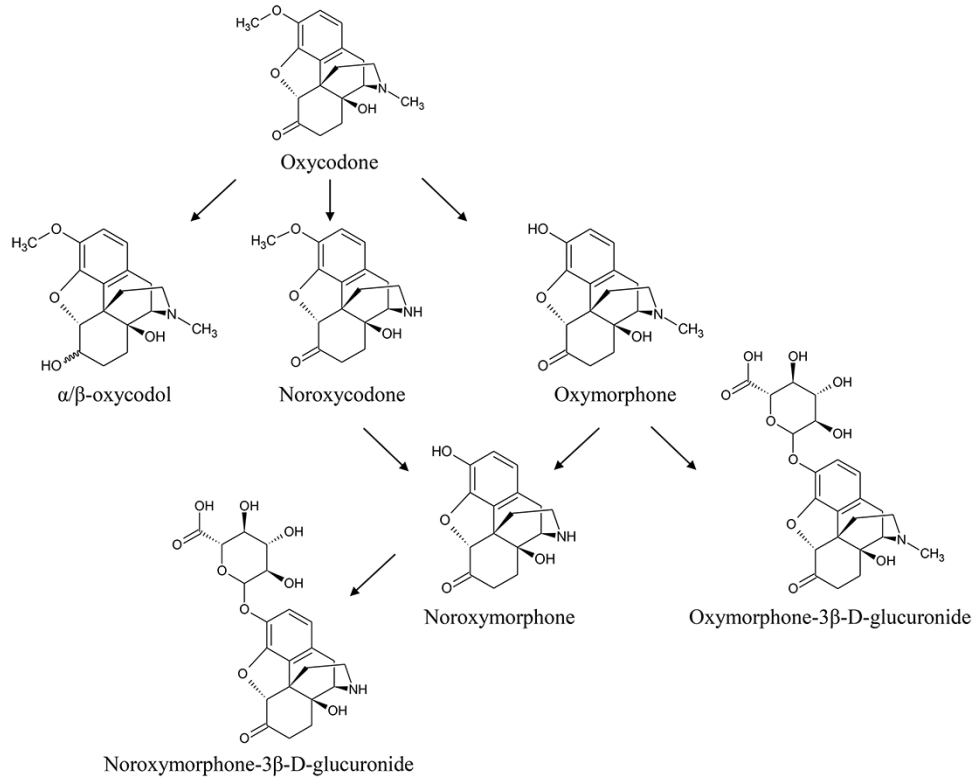

Figure 1. Metabolism of OC.

When compared to other Nordic countries (Denmark and Norway), Sweden has experienced a consistent increase in the prevalence of prescribed OC between 2006 and 2017 that was from 0.4 to 3.0% of the country's population (2).

In Figure 1, the extensive metabolism of OC is shown. OC is metabolized by cytochrome P450 (CYP)3A4 to noroxycodone (NOC) by *N*-demethylation, by CYP2D6 to oxymorphone (OM) by *O*-demethylation and by ketoreductase to 6α-oxycodol (αOCL)/6β-oxycodol (βOCL) by 6-keto reduction. OM and NOC are further metabolized by CYP2D6 and CYP3A4, respectively, to noroxymorphone (NOM), and then, OM and NOM are metabolized into phase II metabolites (oxymorphone-3β-D-glucuronide [OMG] and noroxymorphone-3β-D-glucuronide [NOMG]). In forensic casework, detecting these metabolites can help expand the window of detection for OC and increase the possibilities to determine the time passed from intake to sampling. In Heltsley et al., over 20,000 urine samples from chronic pain patients were analyzed for opiates and the corresponding metabolites. It was determined that the detection of NOC improved the identification of OC use by 6.1% (3). There have been previous studies that have investigated the pharmacokinetic profiles of OC and its metabolites in a controlled setting. In Cone et al., subjects ($n = 12$) were given a single dose of 20-mg controlled-release OC and urine was collected up to 52 h. The analytes that were monitored in that study were OC, NOC, OM and NOM (4). Metabolite ratios over time after administration were monitored, and it was determined that OC/NOC and OC/OM decreased, while OM/NOM increased. The study concluded that OM and NOC were detected longer than OC and that OM is primarily excreted as conjugate, and therefore, hydrolysis is required. The same analytes were studied by Pöyhia et al., except for NOM (5). The subjects ($n = 9$) were given OC intramuscularly once and twice orally. Then, urine and plasma were collected for up to 24 h. It was determined that OC was extensively excreted as NOC and that after 24 h, 8–14% of the OC dose is excreted. Although these studies do provide essential data on the pharmacokinetics of OC, neither of them have incorporated additional metabolites αOCL/βOCL or phase II metabolites.

In recent years, an abundance of methods were developed for the detection of OC and/or its metabolites in various matrices (6–18). However, methods that incorporate the conjugated metabolites are very limited. In Grabenauer et al., there was a method developed for the quantification of opiate conjugates in hair (19). Oxymorphone-3-glucuronide was one of the analytes of interest in this study and was detected in all six samples that contained concentrations of OM. The identification of this metabolite and parent in the hair matrix allows for the differentiation between external contamination and drug use. In a study performed by Dickerson et al., a method was developed for the detection of opioids and several glucuronides in urine (20). While the free opioids ($n = 14$) were quantitatively reported, the glucuronides were reported as present or not present at the established 50 ng/mL cutoff concentration. This method was then applied to urine samples ($n = 168$) collected from chronic pain patients. Of the 168 samples analyzed, 30% of the samples would have been misclassified as negative without the inclusion of OMG. This study further demonstrates the importance to incorporate phase II metabolites

into methods in order to avoid misclassifications of opiate positive cases.

The primary goal in this study was to develop and validate a quantitative method for the determination of OC and its phase I and phase II metabolites in urine samples from a controlled study. To evaluate suitability, the method was used to quantify opioids in urine samples from a pilot study, including one subject given a single dose of OC. Additionally, a limited number of samples were analyzed to investigate the concentration ranges in casework samples from suspected petty drug offenses.

Materials and Methods

Chemicals and reagents

NOMG, OMG, oxymorphone-3β-D-glucuronide-d_3 (OMG-d_3), NOM, OM, oxymorphone-d_3 (OM-d_3), NOC, noroxycodone-d_3 (NOC-d_3), OC and oxycodone-d_3 (OC-d_3) were purchased from Cerilliant (Round Rock, TX, USA). αOCL and βOCL were obtained from Cayman Chemical (Ann Arbor, MI, USA). Mobile phase purified water was acquired from a MilliQ® system (Millipore, Billerica, MA, USA). Gradient grade acetonitrile and formic acid (98–100%) were purchased from Merck (Darmstadt, Germany). Ammonium formate (BioUltra ≥99.0%) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Instrumentation

Analysis was performed on an Acquity UPLC® I-class (Waters Corp, Milford, MA, USA) coupled to a Waters Xevo TQD. An Acquity HSS T3 column (1.7 μm, 2.1 × 100 mm) at 30°C was used to achieve chromatographic separation at 0.5 mL/min. Mobile phase A consisted of 0.001% formic acid in 10 mM ammonium formate (pH 5.2), and mobile phase B consisted of 0.001% formic acid in acetonitrile. The gradient started with 2% mobile phase B for 1.5 min and then increased to 25% over the next 4.7 min, followed by high organic wash (95% mobile phase B) for 1 min, then re-equilibration at 2% for 1 min and ending with a final run time of 7.1 min. The chromatographic separation of all analytes is shown in Figure 2. Waters MassLynx™ software (Waters, version 4.1 SCN 940) was used to acquire and analyze data. A minimum of two transitions for each analyte and one transition for each internal standard were obtained using electrospray ionization in positive mode

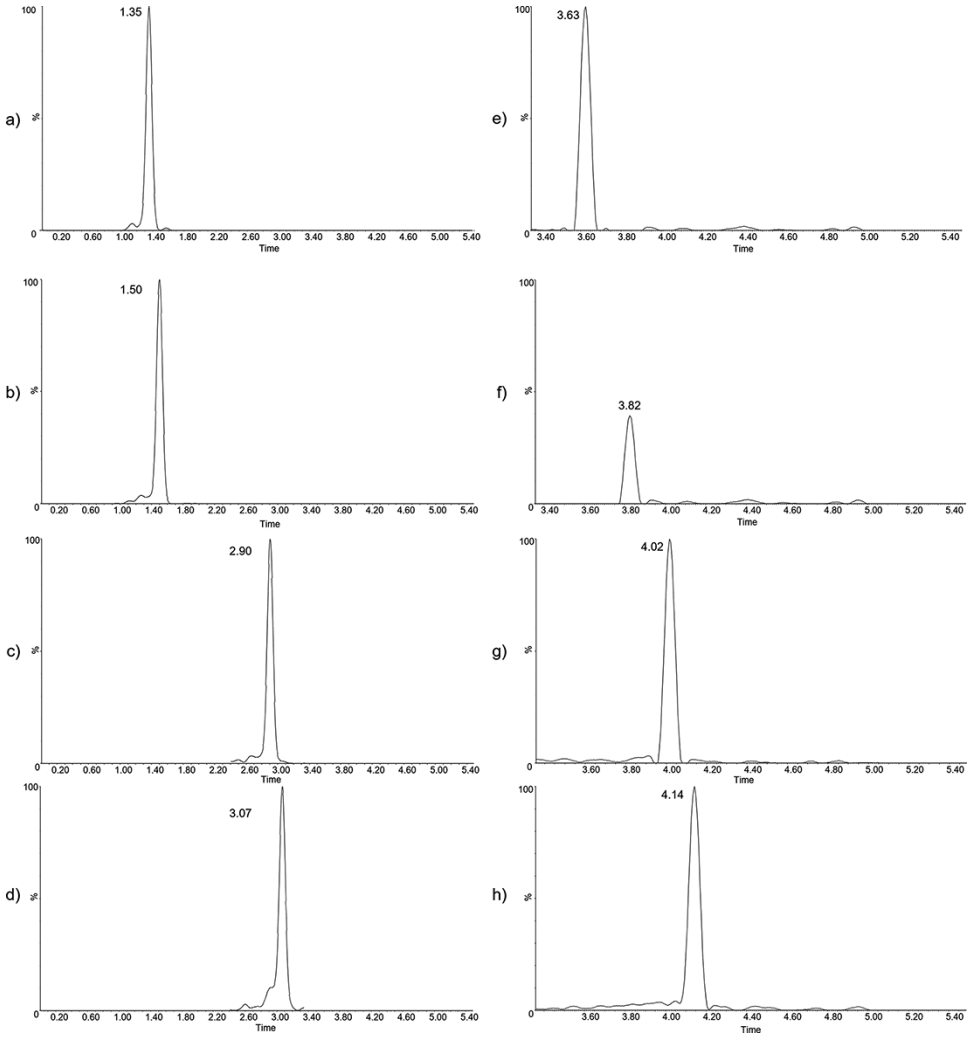

Figure 2. Chromatograms of analytes at low QC (0.03 μg/mL for NOM, OM, αOCL, βOCL, NOC and OC, 0.1 μg/mL for OMG and NOMG): (a) NOMG, (b) OMG, (c) NOM, (d) OM, (e) αOCL, (f) βOCL, (g) NOC and (h) OC.

Table I. Optimized Parameters for NOMG, OMG, NOM, OM, 6α-Oxycodol, 6β-Oxycodol, NOC, OC and Deuterated Internal Standards

Compound name	Parent (*m/z*)	Product (*m/z*)	Cone (V)	Collision (V)	Retention time (min)	Transition ratio	Internal standard
NOMG	*464.2*	*270.1*	40	26	1.36	1.99	OMG-d_3
	464.2	213.0	40	50			
OMG	*478.2*	*284.1*	46	32	1.51	2.80	OMG-d_3
	478.2	227.1	46	48			
NOM	*288.1*	*213.0*	32	28	2.92	2.25	OM-d_3
	288.1	184.1	32	46			
OM	*302.1*	*242.1*	40	28	3.09	0.37	OM-d_3
	302.1	227.0	40	30			
αOCL	*318.2*	*199.1*	34	34	3.68	1.75	NOC-d_3
	318.2	256.1	34	30			
βOCL	*318.2*	*199.1*	34	34	3.88	0.83	NOC-d_3
	318.2	256.1	34	30			
NOC	*302.1*	*187.0*	32	24	4.02	1.21	NOC-d_3
	302.1	227.0	32	28			
OC	*316.2*	*241.0*	34	32	4.15	1.73	OC-d_3
	316.2	256.1	34	30			
OMG-d_3	481.2	287.1	48	34	1.49		
OM-d_3	305.1	230.0	36	30	3.06		
NOC-d_3	305.1	190.0	36	24	3.99		
OC-d_3	319.1	244.0	40	32	4.12		

Quantifying transitions are indicated with italics.

(Table I). The following were the source parameters used: cone voltage at 41 V, capillary voltage at 0.39 kV, desolvation temperature at 550°C and gas flow for desolvation (1,000 L/h) and cone (50 L/h).

Sample preparation

Samples were prepared by diluting urine (100 µL) with internal standard mixture in MilliQ water (900 µL). The final concentration for OMG-d_3, OM-d_3, NOC-d_3 and OC-d_3 in sample was 0.1 µg/mL. After dilution, 2 µL of sample was injected onto the instrument for analysis.

Validation

The method was validated according to the Academy Standards Board Standard Practices for Method Development in Forensic Toxicology (21). The following parameters were assessed: bias and precision, calibration model, carryover, interferences, ionization enhancement/suppression, process efficiency, limit of detection (LOD), lower limit of quantitation (LLOQ), dilution integrity and processed sample stability. Bias and precision were determined by analyzing all analytes at three quality control (QC) levels (low, medium and high) in triplicate over 5 days. The three concentration levels were low (0.03 µg/mL for NOM, OM, αOCL, βOCL, NOC and OC, 0.1 µg/mL for NOMG and OMG), medium (4 µg/mL for all analytes) and high (8 µg/mL for all analytes). The combined interday and intraday precision were calculated using the one-way analysis of variance approach. Calibration model was evaluated by using five replicates of at least seven nonzero calibrators. Carryover was assessed by examining a blank sample after the highest calibrator (10 µg/mL). Interference studies involved evaluating matrix, internal standard and common analyte interferences. To examine matrix interferences, urine samples from petty drug offenses that reported negative ($n = 10$), were analyzed without internal standard. Internal standard interferences were assessed by spiking negative urine with only internal standard mix solution and by analyzing the highest calibrator without internal standard. Common drugs of abuse (benzodiazepines, opiates, stimulants, etc.) and isomers of the analytes of interest were analyzed for potential interferences. Ionization suppression or enhancement was determined qualitatively by postcolumn infusion of an analyte mixture at 1 µg/mL. Process efficiency was evaluated at low (0.2 µg/mL) and high (2 µg/mL) concentrations in 10 negative urine samples. Neat standards at each concentration were analyzed six times and used as reference for process efficiency calculations. LOD and LLOQ were determined by the lowest concentration of each analyte in QC samples that were within acceptable limits for accuracy and coefficient of variance (%CV). Dilution integrity was assessed by performing a 10× dilution on the highest calibrator in triplicate. Processed sample stability was evaluated at low and high concentrations at 24, 48 and 72 h in the autosampler at 10°C.

Single-dose pilot study

As proof of concept, urine samples were collected from a male subject after ingesting a single immediate-release 10-mg tablet of OC Actavis. The time of collections was 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 9, 10, 12, 14, 24, 48, 72 and 96 h. Samples were immediately refrigerated and analyzed within 24 h after collection. This study was approved by the Swedish Ethical Review Authority Dnr 2020-00102, and written consent was obtained from the subject. Creatinine concentrations were determined using an AU680 (Beckman Coulter, Indianapolis, IN, USA). Graph Pad Prism V 8.3.0 (San Diego, CA, USA) was used for data analyses. Using the 5 h sample, stability was assessed for NOMG, OMG, NOM, αOCL/βOCL, NOC and OC in the following conditions: 48 h at room temperature, 1, 2 and 3 weeks at 5 ± 4°C and 1 month at -20 ± 10°C. The 5 h sample was analyzed 6 times to establish the concentrations at T_0 and again in 6 replicates at each time point.

Authentic samples

Samples were obtained under the approval of the regional ethics committee in Linköping (Dnr: 2018-186/31). A total of 15

antemortem urine samples were collected that had reported OC concentrations from a routine method at the National Board of Forensic Medicine in Linköping, Sweden. The samples were stored in a refrigerated room (5 ± 4°C) 4 to 8 weeks prior to analysis.

Results and Discussion

Validation

The main aim of this study was to develop and validate a comprehensive method for the quantitation of NOMG, OMG, NOM, OM, αOCL/βOCL, NOC and OC in urine that would later be used for human dosing experiments. In Table II, results for validation parameters such as precision and bias, dilution integrity, process efficiency, LLOQ and upper limit of quantitation (ULOQ) are shown. The bias at all three levels evaluated was between −12.2 and 2.4%, within the ±20% criteria and therefore was deemed acceptable. The interday precision ranged from 1.8–10.6%CV, and the intraday precision was determined to be between 1.8–13%CV. Both ranges for precision were within the allotted ±20% and deemed acceptable. After performing a 10× dilution on the 10-µg/mL calibrator, all analytes quantified within an acceptable limit (98–111%) of the target concentration. The process efficiency of all analytes was within ±25% with the exception of NOMG (71%) at the high concentration. The matched deuterated internal standards had comparable process efficiencies to the corresponding analytes. NOMG used OMG-d_3 as its internal standard due to a lack of matched deuterated internal standard. Although NOMG was not within the set criteria, it was deemed acceptable for this method as it did not compromise precision, bias and LLOQ. Additionally, the low concentration that was evaluated was within acceptable limits, and therefore, the 71% process efficiency of NOMG at the high level was considered acceptable for this study. The LLOQ and LOD were determined to be 0.015 µg/mL for OC, NOC, αOCL/βOCL, OM and NOM and 0.050 µg/mL for OMG and NOMG. The acceptable validation criteria for these concentrations was accuracy between 75–125%, %CV within ±25% and signal to noise >10.

Calibration ranges were 0.05–10 µg/mL for NOMG and OMG and 0.015–10 µg/mL for all other analytes. Calibration function was determined using residuals plots of curves over 5 days and deemed acceptable if $R^2 \geq 0.99$. All analytes had R^2 values ≥0.99 and a linear range with a 1/x weighting except for NOM, αOCL and βOCL, which were quadratic with a 1/x weighting. There were no detectable peaks in the blank that was analyzed after the highest calibrator when evaluating carryover. After evaluating internal standard interference, it was determined that there were no significant (<LOD) interferences present when analyzing internal standard without analyte and vice versa. There were no interferences detected after injecting blank matrix ($n = 10$) from 10 different sources. These sources of blank matrix were used to evaluate ion enhancement/suppression using postcolumn infusion, and it was determined that there was no significant (<25%) matrix effect from the blank matrices. When evaluating potential interferences from common drugs of abuse, there was a peak detected for NOM after injecting morphine standard. The transition ratio and retention time could not differentiate this peak from NOM. After further investigation, it was determined that this peak observed was due to morphine possessing M + 2 peak in the spectra. Although this would cause interpretation issues when analyzing casework with very high free morphine concentrations, for the purpose of a planned controlled

Table II. Validation Parameters for NOMG, OMG, NOM, OM, 6α-Oxycodol, 6β-Oxycodol, NOC and OC

			Interday precision (%CV)			Intraday precision (%CV)			Bias (%)				Process efficiency (%) Analyte (d_3)	
Analyte	LLOQ (µg/mL)	ULOQ (µg/mL)	Low[a]	Mid[b]	High[c]	Low[a]	Mid[b]	High[c]	Low[a]	Mid[b]	High[c]	Dilution integrity (%)	Low[d]	High[e]
NOMG	0.05	10	10.5	3.6	3.8	12.9	4.3	4.4	−2.1	−5.1	−2.0	111	81	71
OMG	0.05	10	5.5	2.9	3.4	6.7	3.6	3.9	2.4	−10.2	−3.7	100	98 (105)	91 (92)
NOM	0.015	10	8.5	2.8	4.6	10.4	3.4	5.2	−0.4	−1.3	−5.5	101	86	105
OM	0.015	10	10.6	3.0	3.5	13.0	3.6	4.0	−10.9	−2.0	−6.2	98	93 (91)	96 (94)
αOCL	0.015	10	6.2	2.1	2.6	5.0	1.8	2.9	−4.4	−3.2	−9.8	101	109	114
βOCL	0.015	10	8.8	1.8	2.8	10.8	2.2	3.2	−5.3	−3.4	−4.6	98	102	109
NOC	0.015	10	5.5	2.0	2.9	6.8	2.4	3.3	−10.2	−5.5	−11.8	100	113 (119)	118 (115)
OC	0.015	10	7.4	2.2	2.2	9.0	2.7	2.6	−10.0	−7.3	−12.2	103	118 (118)	116 (114)

[a]Low concentration: 0.03 µg/mL for NOM, OM, αOCL, βOCL, NOC and OC, 0.1 µg/mL for NOMG and OMG.
[b]Middle concentration: 4 µg/mL for all analytes.
[c]High concentration: 8 µg/mL for all analytes.
[d]Low concentration was 0.2 µg/mL for all analytes.
[e]High concentration was 2 µg/mL for all analytes.

Table III. Concentrations for NOMG, OMG, NOM, 6α-Oxycodol, 6β-Oxycodol, NOC and OC after a Single Dose of OC

Time (h)	Creatinine (mg/mL)	NOMG (μg/mL)	OMG (μg/mL)	NOM (μg/mL)	αOCL (μg/mL)	βOCL (μg/mL)	NOC (μg/mL)	OC (μg/mL)	NOMG (μg/mg) Creatinine	OMG (μg/mg) Creatinine	NOM (μg/mg) Creatinine	αOCL (μg/mg) Creatinine	βOCL (μg/mg) Creatinine	NOC (μg/mg) Creatinine	OC (μg/mg) Creatinine
0	1.5														
0.5	0.95						0.058	0.091						0.06	0.096
1	0.34		0.18	0.045	0.024	0.031	0.46	0.85		0.53	0.13	0.071	0.091	1.4	2.5
1.5	0.31		0.33	0.055	0.030	0.038	0.51	0.98		1.1	0.18	0.097	0.12	1.6	3.2
2	0.26		0.39	0.050	0.024	0.034	0.47	0.69		1.5	0.19	0.092	0.13	1.8	2.7
3	0.25		0.39	0.057	0.023	0.041	0.50	0.61		1.6	0.23	0.092	0.16	2.0	2.4
4	0.27		0.32	0.054	0.020	0.041	0.46	0.47		1.2	0.20	0.074	0.15	1.7	1.7
5	0.83	0.097	0.89	0.14	0.053	0.11	1.4	1.1	0.12	1.1	0.17	0.064	0.13	1.7	1.3
6	0.65	0.059	0.57	0.10	0.030	0.062	0.96	0.57	0.090	0.88	0.15	0.046	0.10	1.5	0.88
8	0.26		0.20	0.050		0.029	0.34	0.19		0.77	0.19		0.11	1.3	0.73
9	0.33		0.18	0.055		0.038	0.35	0.18		0.55	0.17		0.12	1.1	0.55
10	0.45		0.23	0.064	0.016	0.040	0.49	0.21		0.51	0.14	0.036	0.089	1.1	0.47
12	0.28		0.17	0.036		0.018	0.25	0.059		0.61	0.13		0.064	0.89	0.21
14	1.3	0.11	0.91	0.075	0.023	0.06	0.95	0.20	0.085	0.70	0.058	0.018	0.046	0.73	0.15
24	2.5	0.14	1.1	0.11	0.018	0.065	1.1	0.18	0.056	0.44	0.044	0.0072	0.026	0.44	0.072
48	2.6		0.13				0.087			0.050				0.033	
72	2.0														
96	2.0														

study, it will not pose a problem due to all other medications being prohibited. All other common drugs of abuse did not have peaks detected for analytes of interest. All analytes were determined to be stable (±20% of initial concentration) at 24, 48 and 72 h in the autosampler.

Single-dose pilot study

The results for the concentrations obtained from urine collections are shown in Table III. In this table, concentrations for creatinine and the analytes of interest are shown. Additionally, the creatinine corrected concentrations of the analytes were also determined. OC and the two major metabolites observed in this study (NOC and OMG) are shown in Figure 3a. OC and NOC were present in the first void (0.5 h), while OMG first appeared in the 1 h void. OC was detected until 24 h collection, while NOC and OMG were detected until the 48 h collection. The longer detection of these metabolites compared with the parent compound was also observed in Cone et al., where OM and NOC were detectable until the endpoint of the study (36 h) (4). The current study shows that NOC and OMG are still able to be detected up to 48 h after the administration of a single 10-mg tablet of OC. T_{max} for OC, NOC and OMG were 1.5, 3 and 3 h, respectively. The T_{max} values in the current study were comparable with those determined by Pöyhia et al. (1 h for OC and 4 h for NOC) and lower than those from Cone et al. (free OC and NOC was 6 and 7.2 h, respectively) (4, 5). The difference can be attributed to the dose (10 vs. 20 mg) and release type of the tablet (immediate vs. controlled). C_{max} for OC, NOC and OMG were 3.15, 2.0 and 1.56 μg/mg, respectively.

NOMG, NOM, αOCL and βOCL time–concentration profiles are shown in Figure 3b. NOMG was first detected in the 5 h urine sample, while NOM, αOCL and βOCL were all first detected in the 1 h sample. NOMG, NOM, αOCL and βOCL were all detected until the 24 h collection, like OC. NOM and OC were also determined to have similar detection times in Cone et al. (4). There were no concentrations of unconjugated OM determined using this method after the single dose of OC (10 mg). This aligns with the Cone et al. study that established the need for hydrolysis to detect OM in urinary excretions after OC administration (4).

Stability for NOMG, OMG, NOM, αOCL/βOCL, NOC and OC was determined using the 5 h sample shown in Table IV. All

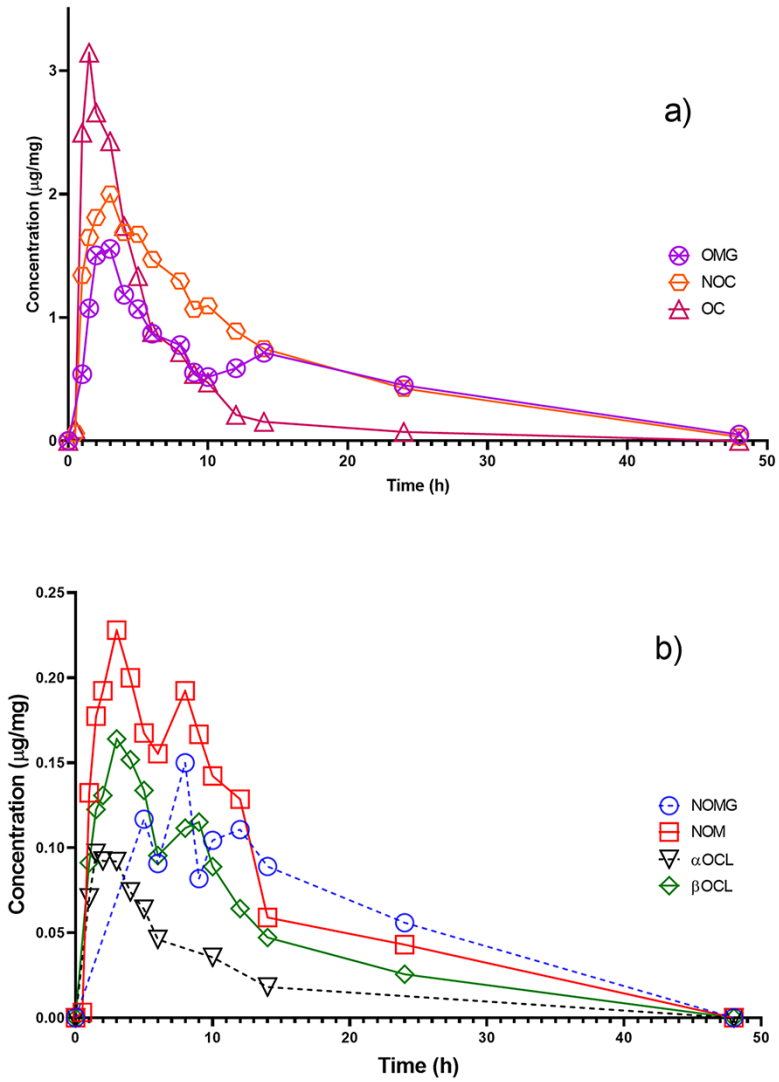

Figure 3. Time–concentration profiles after a single dose of 10-mg OC immediate release: (a) OMG, NOC and OC, (b) NOMG, NOM, αOCL and βOCL.

Table IV. Matrix Stability (% Difference) for NOMG, OMG, NOM, 6α-Oxycodol, 6β-Oxycodol, NOC and OC in Urine

Analyte	48 h Room temperature (% difference, $n = 6$)	1 week $5 \pm 4°C$ (% difference, $n = 6$)	2 weeks $5 \pm 4°C$ (% difference, $n = 6$)	3 weeks $5 \pm 4°C$ (% difference, $n = 6$)	1 month $-20 \pm 10°C$ (% difference, $n = 6$)
NOMG	−11%	−1%	10%	10%	18%
OMG	−8%	7%	4%	2%	5%
NOM	−4%	2%	11%	23%	−4%
αOCL	−3%	4%	6%	1%	3%
βOCL	−4%	2%	3%	1%	3%
NOC	−5%	8%	14%	−4%	3%
OC	−5%	−1%	−4%	−5%	−3%

Concentrations for OM were below limit of quantitation.

Table V. Concentrations of NOMG, OMG, NOM, OM, 6α-Oxycodol, 6β-Oxycodol, NOC and OC in Authentic Urine Samples A-O

Sample	Creatinine (mg/mL)	NOMG (μg/mL)	OMG (μg/mL)	NOM (μg/mL)	OM (μg/mL)	αOCL (μg/mL)	βOCL (μg/mL)	NOC (μg/mL)	OC (μg/mL)
A	2.3	0.16	0.20	-	-	-	0.017	0.68	0.015
B	1.5	0.16	2.4	0.042	-	0.048	0.24	4.9	0.14
C	1.8	-	1.6	0.096	0.061	0.12	0.25	6.0	2.8
D[a]	1.9	9.7	61	6.1	0.60	0.75	3.7	53	6.9
E	1	-	0.18	0.030	-	-	-	0.16	0.031
F	0.47	-	0.098	0.020	-	-	0.41	0.95	0.10
G	0.21	0.12	0.21	0.038	-	-	0.040	0.63	0.14
H	1.8	0.054	1.5	0.11	0.052	0.19	0.48	8.1	2.8
I	1.4	0.35	3.7	0.28	0.079	0.27	1.0	6.9	6.2
J[a]	1.3	1.2	21	1.3	0.43	0.56	2.3	27	8.0
K	1.3	-	0.14	-	-	-	0.044	0.86	0.102
L[a]	2.4	4.2	67	5.4	0.79	1.1	2.1	32	30
M[a]	2.5	1.9	22	1.8	0.49	0.85	2.0	7.2	5.7
N	0.68	-	0.16	0.025	-	-	-	0.36	0.028
O[a]	0.91	5.6	55	3.9	0.47	0.87	3.2	52	12
	Median (Range)	1.2 (0.054–9.7)	2.0 (0.098–67)	0.11 (0.02–6.1)	0.45 (0.052–0.79)	0.56 (0.048–1.1)	0.74 (0.04–3.7)	6.5 (0.16–53)	2.8 (0.028–30)

[a]Ten-fold dilution.

analytes were stable at 48 h at room temperature, 1 and 2 weeks at $5 \pm 4°C$ and 1 month at $-20 \pm 10°C$. NOM (23%) exceeded acceptable limits of ±20% of T_0 at 3 weeks at $5 \pm 4°C$.

Authentic samples

Concentrations from the analyzed urine specimens are shown in Table V. There were samples ($n = 5$) that required a 10-fold dilution due to high metabolite concentrations. Of the 15 samples analyzed, all contained detectable OMG and 10 were positive for NOMG. Concentrations for NOMG and OMG were 0.054–9.7 μg/mL and 0.098–67 μg/mL, respectively. OC and NOC concentrations were also present in all analyzed samples and were 0.015–30 μg/mL and 0.16–52 μg/mL, respectively. The ranges and median of the other metabolites are shown in Table V. The median for OC concentration was 2.8 μg/mL, which is similar to the median established in Heltsley et al. (2.0 μg/mL), after analyzing 5,046 OC-positive specimens from chronic pain patients. Higher OC concentrations did not always correlate with higher concentrations of the glucuronide metabolites as seen in samples D and L. This further illustrates the need for studies on metabolite ratios in a controlled setting in order to correlate ratios with time of administration.

Conclusion

A comprehensive method for the quantification of NOMG, OMG, NOM, OM, αOCL, βOCL, NOC and OC in urine was optimized and met the validation criteria. This method has demonstrated its ability when applied to a pilot human study and therefore will be beneficial in future human studies with OC administration. Including NOC and phase II metabolites of OC benefited analyses by extending the window of detection (up to 48 h). The concentrations of NOMG and especially OMG presented in this study provide details needed in the forensic community to better comprehend OC pharmacokinetics.

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