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Quantitative analysis of opioids and cannabinoids in wastewater samples

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Abstract

Wastewater-based epidemiology is an innovative approach that uses the analysis of human excretion products in wastewater to obtain information about exposure to drugs in defined population groups. We developed and validated an analytical method for the detection and quantification of opioids (morphine, oxycodone, hydrocodone, oxymorphone and hydromorphone), and cannabinoids (Δ^9-tetrahydrocannabinol, 11-*nor*-9-carboxy- tetrahydrocannabinol (THCCOOH) and THCCOOH-glucuronide) in raw-influent wastewater samples by ultra-high performance liquid chromatography-tandem mass spectrometry. Method validation included linearity (5–1 000 ng/L for opioids, 10–1 000 ng/L for cannabinoids), imprecision (<21.2%), accuracy (83%–131%), matrix effect (from –35.1% to –14.7%) and extraction efficiency (25%–84%), limit of detection (1–5 ng/L) and quantification (5–10 ng/L) and auto-sampler stability (no loss detected). River, sewage overflow and wastewater samples were collected in triplicate from different locations in New York City and stored at -20 °C until analysis. River water samples were negative for all the compounds. Water from sewage overflow location tested positive for morphine (10.7 ng/L), oxycodone (4.2–23.5 ng/L), oxymorphone (4.8 ng/L) and hydromorphone (4.2 ng/L). Wastewater samples tested positive for morphine (133.0–258.3 ng/L), oxycodone (31.1– 63.6 ng/L), oxymorphone (16.0–56.8 ng/L), hydromorphone (6.8–18.0 ng/L), hydrocodone (4.0– 12.8 ng/L) and THCCOOH (168.2–772.0 ng/L). This method is sensitive and specific for opioids and marijuana determination in wastewater samples.

Keywords: liquid chromatography; mass spectrometry; solid phase extraction; wastewater; opioid; cannabis

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Introduction

Wastewater analysis is becoming the method of choice for determining what drug(s) are being used within geographical areas that wastewater treatment plants service (Castiglioni, Thomas, Kasprzyk-Hordern, Vandam, & Griffiths, 2014). By observing human biomarkers in sewage water, analysts can monitor the consumption of various drugs. These findings can then be compared to, and even supplement, traditional anonymous surveys. Wastewater epidemiology/toxicology is inexpensive, provides virtually real-time data, and is reliable for assessing the extent of drug use in a geographical region of interest. Its ability to rapidly determine drug use trends in an area can help with the development of targeted public health programs and policy initiatives in these specific communities. However, some disadvantages of wastewater analysis include uncertainties because of population flow variations (e.g. with tourists, peak travel holidays), sewage flow changes, rainfall, and varying inter-individual drug excretion rates (Daghir & Markuszewski, 2010; Daughton, 2011). Whereas wastewater analysis is a rapidly growing field in Europe (EMCDDA, 2016), data for the evaluation of wastewater in the United States (USA) are scarce (Daughton, 2011; Subedi & Kannan, 2015). This type of study has never been performed in New York City (NYC), which is the largest city in the USA.

Prescription opioids are used to treat chronic pain, and their use has increased dramatically in recent years. This has been strongly associated with increasing rates of nonmedical use of prescription opioids in the USA (Paulozzi, Mack, & Hockenberry, 2014). This situation has led to opioids being the most abused class of prescription drugs (*Nationwide Trends*, 2015). According to statistics from the New York City Health

Department, 59 opioid-related deaths occurred in 2000, and this increased to 220 opioid-related deaths in 2013 (http://www1. nyc.gov/site/doh/health/health-topics/alcohol-and-drug-use-data-tables.page). Between 2005 and 2014, the rate of deaths because of prescription opioids increased 250% (rate of increase per 100 000 general population). In 2005, prescription opioids contributed to 29% of the drug overdoses in New York, and this figure rose to 43% by 2014 (http://www.osc. state.ny.us/press/releases/june16/heroin_and_opioids.pdf). According to the 2014 National Survey on Drug Use and Health (*Center for Behavioral Health Statistics and Quality*, 2015), 4.3 million people aged 12 or older have reported current nonmedical use of prescription pain relievers.

In the USA, marijuana is the most commonly used illicit drug, with 22.2 million marijuana users aged 12 or older that have used the drug in the past month (past-month users) (*Center for Behavioral Health Statistics and Quality*, (2015). This is followed by stimulants (1.6 million past-month users), cocaine (1.5 million past-month users) and heroin (400,000 past-month users). Based on National Statistics, 44% of adolescents 12 years and older have used marijuana in their lifetime, which is about the same percentage as individuals aged 26 and older. Individuals aged 18 to 25 years old have the highest percentage of marijuana users (52%) (https://www.drugabuse.gov/national-survey-drug-use-health). On July 7 2014, New York became the 23rd state to legalize medical marijuana (https://www. health.ny.gov/regulations/medical_marijuana/), allowing medical facilities in eight cities to prescribe capsules, liquids, oils, or vaporizable forms of cannabis. The effect of marijuana legalization on prevalence of use is still unknown.

Several authors have published methods for the determination of licit and illicit drugs in wastewater (Baker & Kasprzyk-Hordern, 2011; Berset, Brenneisen, & Mathieu, 2010; Bijlsma, Sancho, Pitarch, Ibáñez, & Hernández, 2009; Bisceglia, Lynn Roberts, Schantz, & Lippa, 2010; Boleda, Galceran, & Ventura, 2007; Castiglioni et al., 2006; Chiaia, Banta-Green, & Field, 2008; Fedorova, Randak, Lindberg, & Grabic, 2013; González-Mariño, Quintana, Rodríguez, Gonzáez-Díez, & Cela, 2012; Gul, Stamper, Godfrey, Gul, & ElSohly, 2016; Heuett, Ramirez, Fernandez, & Gardinali, 2015; Hummel, Löffler, Fink, & Ternes, 2006; Mastroianni, Postigo, De Alda, & Barcelo, 2013; Postigo & Alda, 2008; Senta, Krizman, Ahel, & Terzic, 2013; Vazquez-Roig, Andreu, Blasco, & Picó, 2010). However, prescription opioid data are scarce, and wastewater samples have never been analyzed for the major cannabis metabolite in human urine, 11-*nor*-9-carboxy-tetrahydrocannabinol-glucuronide (THCCOOH-glucuronide). The objective of this study was to develop and validate an analytical method for the detection of morphine, common prescription opioids (oxycodone, oxymorphone, hydrocodone and hydromorphone) and cannabinoids (Δ^9-tetrahydrocannabinol (THC) and its metabolites THCCOOH and THCCOOH-glucuronide) in wastewater samples. Then, for proof of concept, this method was applied to river water, sewage overflow and wastewater samples collected from different locations within NYC.

Materials and methods

Reagents and materials

Morphine, oxycodone, hydrocodone, oxymorphone, hydromorphone, Δ^9-tetrahydrocannabinol (THC) and its metabolites 11-*nor*-9-carboxy-tetrahydrocannabinol

(THCCOOH) and THCCOOH-glucuronide were purchased from Cerilliant (Round Rock, TX, USA). The deuterated analogs THC-d_3, THCCOOH-d_3, THCCOOH-glucuronide-d_3, morphine-d_3, oxycodone-d_6, hydrocodone-d_6, oxymorphone-d_3 and hydromorphone-d_6 were also purchased from Cerilliant. Strata XC 33 mm polymeric strong cation exchange cartridges of 3 mL/60 mg for calibrators and 6 mL/200 mg for quality control (QC) and authentic wastewater samples were purchased from Phenomenex (Torrance, CA, USA). Hydrochloric acid (HCl), ultra-high performance liquid chromatography (UHPLC) grade methanol, dichloromethane and ammonium hydroxide was purchased from PharmcoAaper (Brookfield, CT, USA). Isopropanol, liquid chromatography mass spectrometry grade acetonitrile, and Whatman glass microfiber filters (outside diameter 4.7 cm, particle retention 1.6 mm, and thickness 0.26 mm) were from Thermo Fisher Scientific (Waltham, MA, USA).

Method Optimization

To ensure the best method would be implemented for the extraction and analysis of target compounds in wastewater samples, mass spectrometry (MS) optimization of each compound (precursor and product ions) was performed. After which, multiple extraction methods, solid phase extraction cartridges, liquid chromatography columns and separation programs/ reconstitution solutions were tested.

MS optimization of compounds involved injecting 1 or 2 μL of individual solutions of each compound at either 0.1 or 1 μg/mL. The precursor ion and all products produced from that precursor ion at various collision energies were manually reviewed. Product ions created in the most abundance and with consistency across energies were

chosen as quantifier or qualifier, and the respective voltages of the two quadrupoles and the collision energy cell was recorded.

To obtain the best separation among compounds in a short period of time, different columns and gradients were attempted. Two types of columns were tested (C-18 and EVO) to ascertain which would provide the best Gaussian peak shapes and great separation. The mobile phase was a mixture of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile).

A variety of solid phase extraction (SPE) cartridges were tested with different extraction procedures to ascertain which would give the best yield for all compounds of interest. SPE cartridges included Oasis HLB, Hypersep Verify Ax, Strata X-B Drug, and Strata XC cartridges. Extraction steps (conditioning, loading, washing, drying, and eluting) were optimized by using solvent mixtures on the same cartridge type to compare yields. Test samples were prepared in either diluted hydrochloric acid or diluted acetic acid for the load step. Depending on the cartridge type, washing steps varied from acidic to basic with the use of ammonium hydroxide, acetic acid or hydrochloric acid, along with methanol and water. The elution step varied as well depending on the cartridge type by utilizing a combination of solvents such as ethyl acetate/ isopropanol/ ammonium hydroxide, dichloromethane/ isopropanol, dichloromethane/ isopropanol/ ammonium hydroxide or methanol/ ammonium hydroxide in different percentages. Elution was also tested as a 1-step or 2-step process.

Instrumentation

The chromatographic separations were carried out on an UHPLC–tandem mass spectrometry (MS/MS) instrument from Shimadzu (Kyoto, Japan). The Nexera UHPLC

system consisted of a binary LC-20ADXR high-performance liquid chromatography pump, Nexera LC-30AD micro mixer, online degassing unit (DGU-20A3R) and cooled autosampler (SIL-20SCHT UFLC). The chromatographic column was a Kinetex C18 (2.1 mm x 100 mm, 1.7 mm particle size, 100 A pore size) and the guard column was a SecurityGuard ULTRA Cartridges C18 (2.1 mm, Phenomenex). Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The following gradient program was used for elution of cannabinoids: held at 40% B for 4 min, increased to 95% B and held for 1 min, decreased to 40% B in 0.5 min and held at 40% B for 1.5 min (Figure 1.). The total run time was 7 min and the mobile phase flow rate was 0.5 mL/min. The following gradient program was used for elution of opioids: held at 2% B for 1 min, increased to 30% B in 3 min, increased to 95% B in 2 min and held for 1 min, decreased to 2% B in 0.5 min and held for 2.5 min (Figure 2.). The total run time was 10 min and the mobile phase flow rate was 0.3 mL/min. The column oven was operated at 40 °C for both gradients. The injection volume was 50 µL for each set of compounds.

The mass spectrometer was a triple quadrupole LC- MS 8030 from Shimadzu equipped with a dual ionization source (atmospheric pressure chemical ionization and electrospray ionization). The nebulizing gas flow was set to 2 L/min, the desolvation line was at 250 °C, the heating block was at 400 °C and the drying gas flow was at 15 L/min. The dual ionization source corona needle voltage and interface voltage were both set to 4.5 kV. Two multiple reaction monitoring (MRM) transitions were monitored for each compound (Table 1), with one used as a quantifier and the other as a qualifier.

Sample preparation

An aliquot (100 mL) of each wastewater sample was measured using a graduated cylinder and placed in a beaker, spiked with 50 µL of internal standard mixture (0.1 µg/mL), and filtered through a glass microfiber filter. Then, 0.5 mL of HCl was added immediately to acidify the solution to maximize retention onto mixed- mode cartridges.

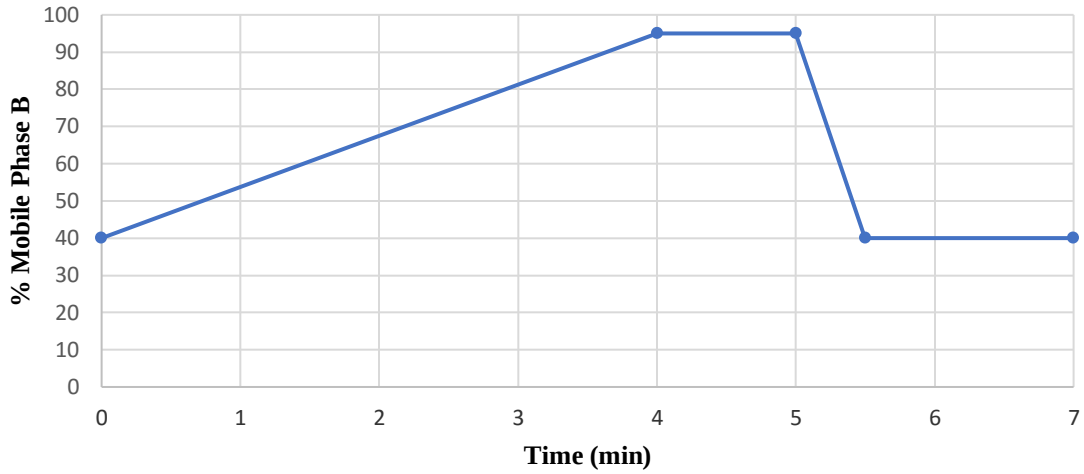

Figure 1. Liquid chromatography gradient for cannabinoids. Mobile phase A was 0.1% formic acid in water and B 0.1% formic acid in acetonitrile.

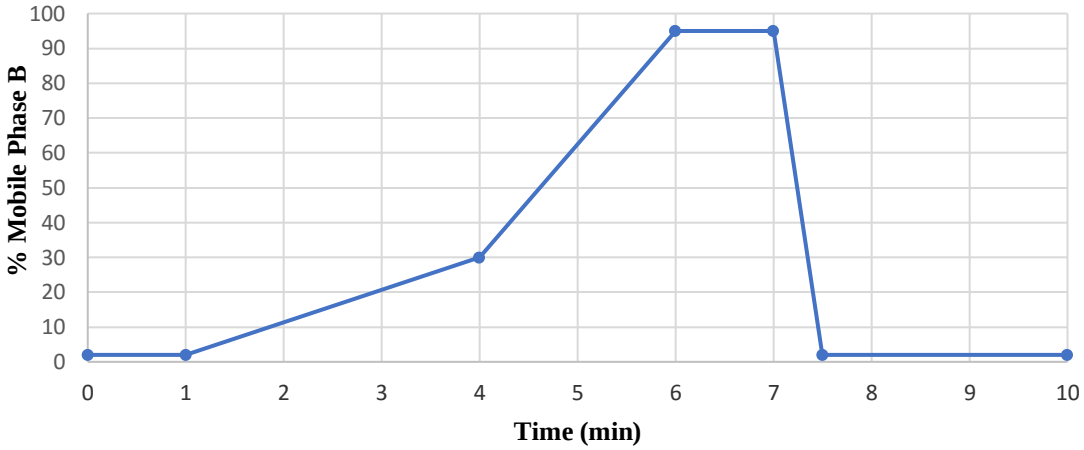

Figure 2. Liquid chromatography gradient for opioids. Mobile phase A was 0.1% formic acid in water and B 0.1% formic acid in acetonitrile.

Solid phase extraction

Strata XC 6 mL/200 mg cartridges were conditioned with 6 mL of methanol, 6 mL of ultra-high purity (UHP) water, and 6 mL of 0.1% HCl. Then the 100 mL of acidified

wastewater was manually loaded 6 mL at a time (17 times) onto a cartridge with a small vacuum (<34 473 Pa). The cartridges were washed with 4 mL of UHP water and 4 mL of 0.1% HCl, and then dried under vacuum for 15 min. Finally, 8 mL of elution solvent ($V_{dichloromethane}$:$V_{isopropanol}$: $V_{ammonium\ hydroxide}$ = 78:20:2) was added. A vacuum was applied to retrieve all the solvent, and the eluate was split in half. The opioid samples were labelled set 1, and the cannabinoid samples were labelled set 2. Each set was evaporated to dryness under a steady stream of N_2 in a Biotage TurboVap (Uppsala, Sweden) at 40 °C. The opioid samples (set 1) were reconstituted in 200 μL of UHP water, and the cannabinoid samples (set 2) were reconstituted in 200 μL of a mixture (V_A:V_B = 60:40) of mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile).

Calibrators, quality controls and internal standards

An internal standard working solution was prepared by diluting each ampoule with pure methanol and combining all analogues to a final concentration of 0.1 μg/mL in methanol. Stock solutions of each compound were prepared in pure methanol (at either 10 or 100 μg/mL) and combined to a stock concentration of 1 μg/mL. This solution was then serially diluted to concentrations of 0.001, 0.01, 0.1, and 1 μg/mL and used to prepare quality control and calibration curve samples at concentrations of 0.5, 1, 3, 5, 10, 50, 100, 500 and 1 000 ng/L.

To reduce the cost and time of this process, calibrators were prepared in 3 mL of UHP water and spiked with the corresponding calibration working solution to match concentrations of 5 to 1 000 ng/L in a 100 mL sample. For the calibration curve, clean test tubes were prepared by adding 3 mL of UHP water, 50 μL of internal standard mixture (0.1 μg/mL), and the following volumes of the respective calibrator working

solution: 50 and 100 µL of the 0.01 µg/mL solution for 5 and 10 ng/L calibrators, 50 and 100 µL of the 0.1 µg/mL solution for 50 and 100 ng/L calibrators, and 50 and 100 µL of the 1 µg/mL solution for 500 and 1 000 ng/L calibrators. Lastly, 15 µL of HCl (0.5%) was added before vortex mixing and SPE. Strata XC 3 mL/60 mg cartridges were conditioned with 3 mL of methanol, followed by 3 mL of UHP water and 3 mL of 0.1% HCl. The acidified calibrator was loaded onto the mixed-mode cartridge. Cartridges were washed with 2 mL of UHP water and 2 mL of 0.1% HCl, and then dried under vacuum for 15 min. Sample elution was performed with 4 mL of a dichloromethane/isopropanol/ammonium hydroxide mixture ($V_{dichloromethane}$:$V_{isopropanol}$: $V_{ammonium\ hydroxide}$ = 78:20:2).

Table 1. MRM transitions, retention time (RT), and precursor ion for each analyte of interest.

Compound	RT (min)	Precursor ion (m/z)	Quantifier product ion (m/z)	Collision energy[1] (eV)	Qualifier product ion (m/z)	Collision energy[2] (eV)
Morphine	3.14	286	165	-40	181	-33
Morphine-d_3	3.13	289	164	-44	153	-43
Hydromorphone	3.44	286	184	-33	157	-43
Hydromorphone-d_6	3.42	292	185	-33	157	-49
Oxymorphone	3.29	302	226	-32	242	-28
Oxymorphone-d_3	3.29	304	201	-45	230	-31
Oxycodone	3.98	316	256	-26	212	-46
Oxycodone-d_6	3.97	322	247	-31	262	-29
Hydrocodone	4.10	300	199	-31	170	-40
Hydrocodone-d_6	4.08	306	202	-36	174	-44
THC	4.01	315	193	-23	122	-38
THC-d_3	4.00	318	195	-27	122	-39
THCCOOH	2.87	345	299	-21	192	-28
THCCOOH-d_3	2.86	348	330	-17	302	-23

THCCOOH-Glucuronide	1.98	521	345	-15	326	-18
THCCOOH-Glucunoride-d_3	1.94	524	348	-15	330	-21

[1]Collision energy for Quantifier production
[2]Collision energy for Qualifier production

The eluate was split in half. The samples were labelled as set 1 for opioids and set 2 for cannabinoids. Each set was evaporated to dryness under a steady stream of N_2 in a Biotage TurboVap at 40 °C. Opioid samples (set 1) were reconstituted in 200 μL of UHP water, and cannabinoids samples (set 2) were reconstituted in 200 μL of a mixture (V_A:V_B = 60:40) of mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile).

QC samples were prepared at 10 and 100 ng/L by spiking 100 mL of UHP water with the required amount of the working solution and 50 μL of the internal standard mixture. These samples were then filtered, and 0.5 mL of HCl was added before SPE.

Authentic sample collection

For proof of concept, 33 samples were collected from river water (22 samples), sewage overflow (6 samples) and raw influent from wastewater treatment plants (5 samples) in NYC. River samples were collected from the Hudson and East Rivers in the Bronx, Manhattan, Queens and Roosevelt Island. Sewage overflow samples were collected from Newtown Creek (Brooklyn), and wastewater samples were collected from the Tallman and Jamaica wastewater treatment plants in Queens. Samples were collected for 1–3 days before and after national holidays (Independence Day, July 4 2015; Labor Day, September 7 2015; New Year's Day, January 1 2016) and on March 25th and 30th 2016. The samples were collected at one time point on each of these days (between 7 and 11 am) in 200 mL Nalgene™ Certified Wide-Mouth Amber high-density polyurethane

bottles (Thermo Fisher Scientific). To prevent degradation of the target drugs, the samples were stored in a freezer at –20 °C until required for analysis.

Validation parameters

The method was validated using various procedures outlined by the Scientific Working Group for Forensic Toxicology guidelines ("Scientific working group for forensic toxicology (SWGTOX) standard practices for method validation in forensic toxicology," 2013) for the linearity, limit of detection (LOD), limit of quantification (LOQ), interferences (specificity), autosampler stability, imprecision, accuracy, carryover, extraction efficiency, process efficiency and matrix effect.

Linearity was determined over five different days by least-squares regression and different weighting factors (none, 1/x and $1/x^2$) were evaluated. The linearity was acceptable if the coefficient of determination (R^2) was ≥ 0.99 and the residuals were within ±20%. The LOD and the LOQ were evaluated with decreasing analyte concentrations in spiked samples from three different sources. The LOD was the lowest concentration with acceptable chromatography, a signal-to-noise ratio > 3, the presence of all product ions, the correct ion ratio (within ±20% of the average of the calibrators) and a suitable retention time (within ±0.2 min of the retention time of the calibrators). The LOQ satisfied the LOD criteria and was quantified within ±20% imprecision and 80%–120% accuracy.

Interferences from matrix components were evaluated by analyzing river (n =22) and wastewater (n = 4) samples negative for the compounds of interest, after spiking with the internal standard solution. Interferences were considered insignificant if the analytes of interest were not detected in these samples. Method specificity was demonstrated by

analyzing high concentrations (1,000 ng/L) of potentially interfering drugs. The following compounds and their metabolites were examined: opioids (morphine-3-glucuronide, morphine-6-glucuronide, hydromorphone-3-glucuronide, oxymorphone-3-glucuronide, oxymorphone-6-glucuronide and 6-acetylmorphine), cannabinoids (11-hydroxy-THC, cannabinol, and cannabidiol) and common drugs of abuse (cocaine, benzoylecgonine, amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine, 3,4-methylenedioxymethamphetamine and methadone). Sufficient specificity was achieved if the analytes of interest were below the LOD.

To determine carryover, blank samples spiked with the internal standard (negative calibrator) were injected immediately after samples spiked at 2,000 ng/L (twice the highest calibrator concentration). The carryover was considered negligible if the measured concentration was less than the LOD. Before SPE, the 2,000 ng/L samples were prepared using 3 mL of UHP water and spiking it with 50 μL of internal standard, 200 μL of the 1 μg/mL calibrator solution and 15 μL of HCl.

Inter- and intra-day QC samples at 10 ng/L and 100 ng/L were prepared with 100 mL of UHP water spiked with 100 μL of the 0.01 μg/mL solution (for 10 ng/L concentration) or the 0.1 μg/mL solution (for 100 ng/L concentration). The imprecision and accuracy were determined at these two concentrations with four repeat analyses in one day (intra-day n = 4) and over five days (inter-day n = 5). The imprecision was determined using the coefficient of variation of the measured values and expected to be less than 20%. The intra- and inter-day imprecision were calculated as the standard deviation of the QC concentrations x 100/ mean QC concentrations. The accuracy was calculated as a percentage of the target concentration, and was required to be within

80%–120%. The intra- and inter- day accuracy was calculated as the mean QC concentrations x 100/QC target concentration.

Autosampler stability was evaluated by reinjecting four QC samples after 24 h in the autosampler at 10 °C. The QC samples were prepared at 10 ng/L using 100 mL of UHP water and 100 μL of 0.01 μg/mL calibrator working solution. The concentrations within ±20% of the initial concentration were considered acceptable.

To evaluate the matrix effect, extraction efficiency and process efficiency, three sets of samples were prepared in duplicate at the same concentration (10 ng/L). Set 1 contained neat samples prepared by adding 2 mL of elution solvent, 50 μL of internal standard, and 100 μL of the 0.01 μg/mL solution to a clean test tube. This sample was then split, evaporated to dryness and reconstituted in the appropriate opioid or cannabis mobile phase for LC-MSMS separation and analysis. Set 2 contained QC samples spiked at 10 ng/L with the internal standard and submitted to the same sample preparation and extraction steps as normal samples. Set 3 contained QC samples spiked at 10 ng/L and with the internal standard post-extraction. The samples were from four different sources, one set prepared with UHP water and three sets using authentic wastewater samples that tested negative for the target drugs. The peak areas for Set 1 and 3 were compared to determine if there were any matrix effects. The peak areas for the Set 2 and 3 samples were compared to assess the extraction efficiency, and those for Set 1 and Set 2 were used to assess the process efficiency.

Identification criteria

The identification criteria included retention time within ±0.2 min of the calibrators retention time, the presence of two product ions (quantitative and qualitative) and an ion

ratio within ±20% of the average of the calibrators.

Table 2. Inter- and intra-day imprecision and accuracy for quality controls at 10 and 100 ng/L.

Compound	**Imprecision (%)**				**Accuracy (%)**			
	Inter-day (n=5)		*Intra-day (n=4)*		*Inter-day (n=5)*		*Intra-day (n=4)*	
	10 (ng/L)	*100 (ng/L)*	*10 (ng/L)*	*100 (ng/L)*	*10 (ng/L)*	*100 (ng/L)*	*10 (ng/L)*	*100 (ng/L)*
Morphine	11.4	6.2	5.6	3.4	93.3	94.7	116.0	110.9
Oxymorphone	10.8	3.9	4.9	3.3	106.5	106.6	126.5	109.1
Hydromorphone	14.1	3.3	12.9	4.4	98.3	103.3	119.5	121.1
Oxycodone	8.9	7.6	10.9	5.0	98.3	101.1	110.0	102.0
Hydrocodone	10.0	8.8	6.1	4.8	107.5	103.7	131.0	127.2
THC	9.7	9.0	21.2	7.9	102.8	98.8	110.3	106.6
THCCOOH	8.0	6.1	5.0	7.3	97.3	105.6	119.3	103.4
THCCOOH-Glucuronide	6.0	10.3	4.1	5.5	95.0	98.9	83.0	102.3

Results

Method validation

The LOQ and LOD for all opioids were 5 and 1 ng/L, respectively, and the linear range was 5–1,000 ng/L. For the cannabinoids, the LOQ, LOD and linear range were 10, 5 and 10–1,000 ng/L, respectively. Acceptable linearity for opioids and cannabinoids ($R^2 \geq 0.99$ and residuals within ±20%) were achieved with $1/x^2$ weighting. No endogenous or exogenous interferences were detected.

For opioids, the intra- and inter-day imprecision were 3.3% to 14.1%, respectively, and the accuracy was 93.3%–131.0%. For cannabinoids, the intra and inter-day imprecision were 4.1% to 21.2%, respectively, and the accuracy was 83.0% to 119.3%. For opioids, the extraction efficiency range was 75.0%–84.0%, and the process efficiency range from 63.1% to 73.3%. For cannabinoids, the extraction efficiency range was 25.4%– 66.5% and the process efficiency range was 22.7%– 62.7%. For opioids, the

matrix effects range was –35.1% to –7.6% (ion suppression), with a coefficient of variation of 28.3% (n = 4). For cannabinoids, the matrix effects range was –14.7% to –5.8%, with a coefficient of variation of 13.9% (n = 4). These results are summarized in Tables 2 and 3.

Carryover was assessed by injecting a blank after injection of a sample prepared at 2,000 ng/L (twice the concentration of the highest calibrator). The results for all target compounds for the blank were below LOD. Autosampler stability was assessed by injecting the same samples fresh and after 24 h in the autosampler at 10 °C. The mean concentrations from these injections were compared to determine the percentage difference. The concentrations of all target compounds were within the accepted ±20%, except for oxymorphone (–21.3%).

Table 3. Extraction efficiency, process efficiency, matrix efficient (n=4).

Compound	Extraction Efficiency (%)	Process Efficiency (%)	Matrix Effect (%)	Matrix Effect CV
Morphine	79.0	73.3	-7.6	9.9
Oxymorphone	84.0	63.1	-24.5	8.7
Hydromorphone	75.0	48.8	-35.1	28.3
Oxycodone	82.0	63.5	-23.1	21.7
Hydrocodone	84.0	72.6	-13.8	16.1
THC	25.4	22.7	-10.6	13.9
THCCOOH	66.5	62.7	-5.8	11.6
THCCOOH-Glucuronide	53.1	45.3	-14.7	9.5

Application to authentic samples

Samples from the East and Hudson rivers tested negative for morphine, prescription opioids and cannabis. Samples from sewage overflows (Newtown Creek, Brooklyn) tested positive for morphine (10.7 ng/L), oxycodone (4.2–23.5 ng/L), oxymorphone (4.8 ng/L) and hydromorphone (4.2 ng/L). Wastewater samples from the Tallman and Jamaica

plants in Queens tested positive for morphine (133.0–258.3 ng/L), oxycodone (31.1–63.6 ng/L), oxymorphone (16.0–56.8 ng/L), hydromorphone (6.8–18.0 ng/L), hydrocodone (4.0– 12.8 ng/L) and THCCOOH (168.2–772.0 ng/L) (Table 4). Figure 3 shows a chromatogram of an authentic wastewater sample that tested positive for opioids and cannabinoids.

Table 4. Results from wastewater plants (Tallman and Jamaica, New York City, NY) collected at one time point 1-3 days before and after national holidays (Independence Day, July 4, 2015; Labor Day, September 7, 2015; New Year's Day, January 1, 2016) and March 2016

Analyte	Concentration range (ng/L)	N cases
Morphine	133.0 – 258.0	5
Hydrocodone	4.0 – 12.8	4
Oxycodone	31.1 – 63.6	5
Oxymorphone	16.0 – 56.8	5
Hydromorphone	6.8 – 10.0	5
THCCOOH	168.2 – 641.4	5

Discussion

We developed and validated a method for the detection of morphine, oxymorphone, oxycodone, hydromorphone, hydrocodone, THC and its metabolites THCCOOH and THCOOH-glucuronide in wastewater samples. Numerous methods for the analysis of licit and illicit drugs in wastewater samples have been published (Baker & Kasprzyk-Hordern, 2011; Berset et al., 2010; Bijlsma et al., 2009; Bisceglia et al., 2010; Boleda et al., 2007; Castiglioni et al., 2006; Chiaia et al., 2008; Fedorova et al., 2013; González-Mariño et al., 2012; Gul et al., 2016; Heuett et al., 2015; Hummel et al., 2006; Mastroianni et al., 2013; Postigo & Alda, 2008; Senta et al., 2013; Vazquez-Roig et al., 2010). These analytical methods allow for the quantification of opiates and prescription opioids (Baker & Kasprzyk-Hordern, 2011; Bisceglia et al., 2010; Chiaia et al., 2008; Gul et al., 2016; Hummel et al., 2006), cannabis (Bijlsma et al., 2009; Fedorova et al., 2013)

or both classes of compounds, opiates and cannabis (Baker & Kasprzyk-Hordern, 2011; Berset et al., 2010; Castiglioni et al., 2006; Fedorova et al., 2013; González-Mariño et al., 2012; Heuett et al., 2015; Mastroianni et al., 2013; Postigo & Alda, 2008; Senta et al., 2013). In the case of opiates, most of the methods can only detect morphine (Baker & Kasprzyk-Hordern, 2011; Berset et al., 2010; Bisceglia et al., 2010; Boleda et al., 2007; Castiglioni et al., 2006; González-Mariño et al., 2012; Gul et al., 2016; Heuett et al., 2015; Hummel et al., 2006; Mastroianni et al., 2013; Postigo & Alda, 2008; Senta et al., 2013; Vazquez-Roig et al., 2010), although some methods are suitable for prescription opioids such as oxycodone, oxymorphone, hydrocodone and hydromorphone (Baker & Kasprzyk-Hordern, 2011; Bisceglia et al., 2010; Chiaia et al., 2008; Fedorova et al., 2013; Gul et al., 2016; Heuett et al., 2015; Hummel et al., 2006). With regard to cannabis, most methods have been developed for THCCOOH (Berset et al., 2010; Bijlsma et al., 2009; Castiglioni et al., 2006; Fedorova et al., 2013; Senta et al., 2013) or for THC or THC and THCCOOH (Boleda et al., 2007; González-Mariño et al., 2012; Heuett et al., 2015; Mastroianni et al., 2013; Postigo & Alda, 2008; Vazquez-Roig et al., 2010). There is no data available for THCCOOH-glucuronide in wastewater samples, even though this compound is the predominant THC metabolite in human urine (Desrosiers et al., 2014). This may be because glucuronides are normally hydrolyzed in wastewater (Ternes, 1998), resulting in higher concentrations of the free compound. However, recent publications have reported high concentrations of glucuronides in wastewater samples (Wang & Gardinali, 2014; Zonja, Perez, & Barcelo, 2016). These results highlight the need for a method for detection of glucuronides in wastewater.

Currently, the most commonly used instrument for wastewater analysis is LC-MSMS. However, gas chromatography-mass spectrometry has been used as well (González-Mariño, Quintana, Rodríguez, & Cela, 2010). In the present method, all compounds were ionized in positive mode using dual ionization sources (atmospheric pressure chemical/electrospray ionization), despite other authors finding better sensitivity for cannabinoids in negative ionization mode (electrospray ionization) (Castiglioni et al., 2006; González-Mariño et al., 2012; Postigo & Alda, 2008; Senta et al., 2013). The sensitivity of our method (LOD 1–5 ng/L and LOQ 5–10 ng/L in 100 mL of wastewater) was within the range of methods in previous publications (Berset et al., 2010; Bisceglia et al., 2010; Fedorova et al., 2013; Mastroianni et al., 2013). Earlier studies have reported LOQs for the compounds of interest as low as 0.48 ng/L (Zuccato et al., 2008) and as high as 100 ng/L (Berset et al., 2010) for wastewater volumes between 15 mL (Gul et al., 2016) and 250 mL (Boleda et al., 2007). Sample preparation usually involves filtration and SPE with a reversed-phase (Oasis HLB, Waters Corp., Milford, MA, USA) or cation exchange (Oasis MCX, Waters Corp.) cartridges.

During method validation, matrix effect experiments were carried out using four different matrices instead of six ("Scientific working group for forensic toxicology (SWGTOX) standard practices for method validation in forensic toxicology," 2013). This limitation was because it was difficult to obtain wastewater samples that were negative for the compounds of interest. Another limitation of the present method was the intra-day accuracy above the established criteria for three opioids. Although oxymorphone's low QC, hydromorphone's high QC and hydrocodone's low and high QC gave an intra-day accuracy >120% (121.1% to 131%), the rest of the validation parameters were within the

established range ("Scientific working group for forensic toxicology (SWGTOX) standard practices for method validation in forensic toxicology," 2013).

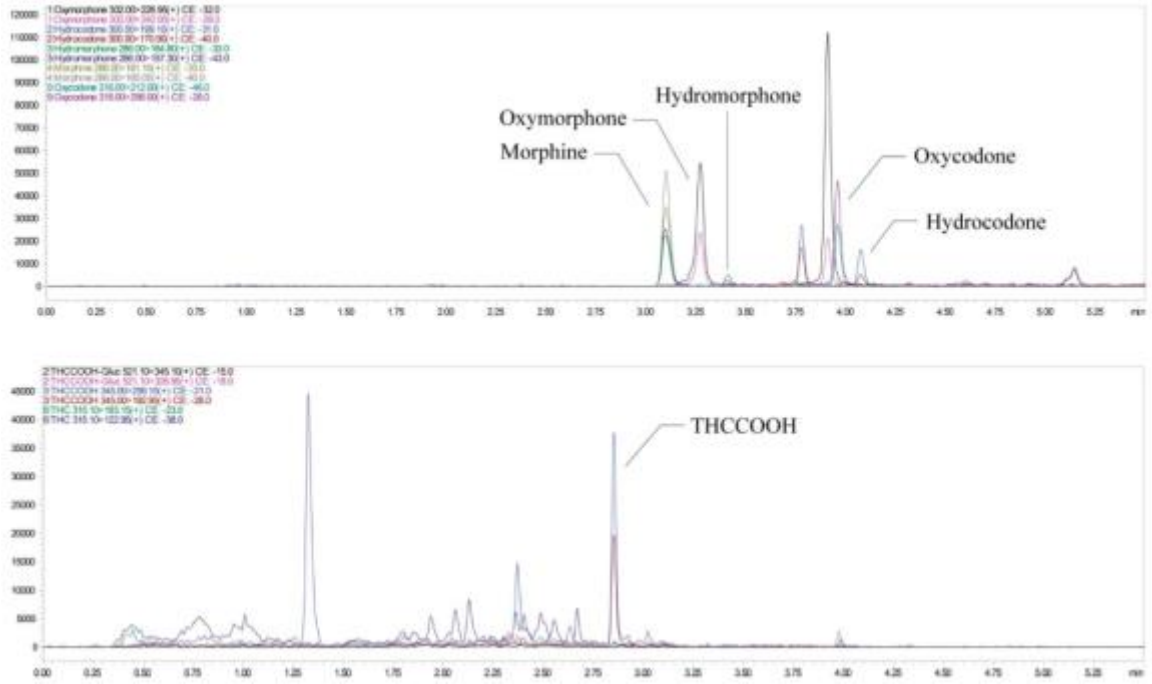

Figure 3. Multiple reaction monitoring chromatograms of an authentic wastewater sample from Tallman Island Wastewater Treatment Plant (Queens, NYC) showing positive results for THCCOOH (184.1 ng/L), morphine (181.9 ng/L), hydromorphone (10.0 ng/L), oxymorphone (56.8 ng/L), oxycodone (63.3 ng/L) and hydrocodone (10.3 ng/L).

As a proof of concept, we were able to detect THCCOOH, morphine and prescription opiates in water samples from sewage overflow locations and wastewater treatment plants. The concentrations of morphine (10.7– 258.3 ng/L), oxycodone (4.2–63.6 ng/L), oxymorphone (4.8–56.8 ng/L), hydromorphone (4.2–18.0 ng/L), hydrocodone (4.0–12.8 ng/L) and THCCOOH (168.2–772.0 ng/L) were similar to those found in previous studies (Baker & Kasprzyk-Hordern, 2011; Fedorova et al., 2013; Gul et al., 2016; "Scientific working group for forensic toxicology (SWGTOX) standard practices for method validation in forensic toxicology," 2013; Wang & Gardinali, 2014). THC and THCCOOH-glucuronide were not detected in any of the analyzed samples. Previously, THC was detected in wastewater samples (Boleda et al., 2007), but there are no reports of

the detection of THCCOOH-glucuronide. Continued research is required to investigate the importance of monitoring THCCOOH-glucuronide's in these types of samples, and to back calculate the drug exposure in communities based on wastewater drug concentrations.

Conclusion

We developed and validated an analytical method for the extraction, detection, and quantification of morphine, common prescription opioids (oxycodone, oxymorphone, hydrocodone and hydromorphone), and THC, THCCOOH and THCCOOH-glucuronide in wastewater samples. This technique was sensitive (LOD 1–5 ng/L and LOQ 5–10 ng/L in 100 mL of sample) and specific. This is the first report of testing for THCCOOH-glucuronide in wastewater samples. As a proof of concept, we were able to detect THCCOOH, morphine, and prescription opioids in samples from sewage overflow locations and wastewater plants throughout NYC.

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