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Analysis of 39 drugs and metabolites, including 8 glucuronide conjugates, in an upstream wastewater network via HPLC-MS/MS

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ABSTRACT

Pharmaceutical compounds ingested by humans are metabolized and excreted in urine and feces. These metabolites can be quantified in wastewater networks using wastewater-based epidemiology (WBE) methods. Standard WBE methods focus on samples collected at wastewater treatment plants (WWTPs). However, these methods do not capture more labile classes of metabolites such as glucuronide conjugates, products of the major phase II metabolic pathway for drug elimination. By shifting sample collection more upstream, these unambiguous markers of human exposure are captured before hydrolysis in the wastewater network. In this paper, we present an HPLC-MS/MS method that quantifies 8 glucuronide conjugates in addition to 31 parent and other metabolites of prescription and synthetic opioids, overdose treatment drugs, illicit drugs, and population markers. Calibration curves for all analytes are linear ($r^2 > 0.98$), except THC ($r^2 = 0.97$), and in the targeted range (0.1–1,000 ng mL^{-1}) with lower limits of quantification (S/N = 9) ranging from 0.098 to 48.75 ng mL^{-1}. This method is fast with an injection-to-injection time of 7.5 min. We demonstrate the application of the method to five wastewater samples collected from a manhole in a city in eastern Massachusetts. Collected wastewater samples were filtered and extracted via solid-phase extraction (SPE). The SPE cartridges are eluted and concentrated in the laboratory via nitrogen-drying. The method and case study presented here demonstrate the potential and application of expanding WBE to monitoring labile metabolites in upstream wastewater networks.

1. Introduction

The opioid epidemic is widespread in the US, causing nearly 450,000 overdose deaths since 1999 [1]. Each wave of the epidemic ushers in more fatal and non-fatal overdoses. Estimates of the intensity of each wave are based on first responder reports and hospital records. Unfortunately, compiling these types of data can take a matter of years. Moreover, individuals who experience non-fatal opioid overdoses may never actively seek medical care [2,3]. These hidden populations suffering from opioid use disorder may benefit from empirically proven

Abbreviations: ACT, Acetaminophen; ACT-Glu, Acetaminophen glucuronide; AMP, Amphetamine; BEG, Benzoylecgonine; BUP, Buprenorphine; CCN, Cocaine; CDN, Codeine; CDN-Glu, Codeine-6β-D-glucuronide; CFN, Caffeine; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; HCD, Hydrocodone; HCT, trans-3′-Hydroxycotinine; HMP, Hydromorphone; HMP-Glu, Hydromorphone-3-β-D-glucuronide; MAM, 6-monoacetylmorphine; MAMP, Methamphetamine; MDA, (±)-3,4-Methylenedioxyamphetamine; MDMA, (±)-3,4-Methylenedioxymethamphetamine; MPH, Morphine; MPH-Glu, Morphine-3-β-D-glucuronide; NBP-Glu, Norbuprenorphine glucuronide; NCD, Norcodeine; NHCD, Norhydrocodone; NLX, Naloxone; NLX-Glu, Naloxone-3ß-D-glucuronide; NMPH, Normorphine; NOCD, Noroxycodone HCl; NTRM, N-Desmethyl-*cis*-tramadol HCl; NTX, Naltrexone; OCD, Oxycodone; OMP, Oxymorphone; OMP-Glu, Oxymorphone-3-β-D-glucuronide; OTRM, O-Desmethyl-*cis*-tramadol HCl; PAG, Phenylacetylglutamine; THC, (−)-trans-Δ^9-tetrahydrocannabinol; THCCOOH, (±)-11-nor-9-Carboxy-Δ^9-THC; THCCOOH-Glu, (+)-11-nor-9-Carboxy-Δ^9- THC glucuronide; THCOH, (±)-11-Hydroxy-Δ^9-THC; TRM, *cis*-Tramadol HCl.

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interventions surrounding harm reduction and behavioral health. However, detection of these individuals continues to be challenging. Some tactics like syndromic surveillance using social media or development of prescription drug monitoring programs have been implemented by public health authorities to better understand the spread of the opioid epidemic, but these measures lack ground truth clinical sampling to confirm described trends. Therefore, a clear need exists for healthcare-independent, de-identified, noninvasive approaches for measuring opioid exposure.

One such approach is wastewater-based epidemiology (WBE), a technique of analyzing residential wastewater for human-excreted drug metabolites to estimate population-level exposures to pharmaceuticals and other drugs of abuse [4–9]. WBE permits noninvasive monitoring of large populations through existing public infrastructure. Thus, it enables population health assessments that are independent of disease indicators provided by healthcare facilities or based on expensive in-person survey protocols. WBE samples can be obtained as needed and wastewater analyte dynamics can be used to guide public health interventions and harm reduction policy development [10–11]. Several methods exist for quantifying licit and illicit drugs in wastewater utilizing a combination of solid-phase extraction and mass spectrometry, typically liquid chromatography coupled to triple quadrupole mass spectrometry [12–19]. Unfortunately, methods developed for this purpose often include only a subset of opioid metabolites or only those in their unchanged forms [8,20–24]. In the human body, opiates are primarily metabolized via phase II glucuronidation [25]. These glucuronide conjugates, unlike the unchanged parent forms, act as direct indicators of human exposure. Wastewater epidemiologists have recently called for the inclusion of glucuronide conjugates in back-calculations of human exposure [26]. Unfortunately, once introduced to the sewer system, the travel time of metabolites from the point of injection to the WWTP can be up to 24 h [27]. With some exceptions such as codeine-6-glucuronide, glucuronidated metabolites, once introduced to the wastewater system, undergo rapid degradation via enzymatic hydrolysis and may be absent or at such low concentrations that they evade the detection limit of analyzers at the WWTP level [24,28–31].

One approach to address this shortcoming is to move WBE sampling upstream in the sewer networks, thereby capturing glucuronide conjugates and other potentially time-sensitive metabolites prior to chemical, biological and physical transformations that occur in the sewer. Previous studies have sampled upstream to achieve more granular data of the contributing population such as in prisons or on college campuses [32–35]. This advance unlocks the potential for higher resolution community-level surveillance for opioid exposure and importantly expands the classes of target molecules to include direct metabolites of human exposure [36].

This study develops and validates a high performance liquid chromatography - tandem mass spectrometry method tailored to a panel of opioids including eight glucuronide conjugates: acetaminophen glucuronide, codeine-6-glucuronide, hydromorphone-3-glucuronide, morphine-3-glucuronide, norbuprenorphine glucuronide, naloxone-3-glucuronide, oxymorphone-3-glucuronide and (±)-11-nor-9-carboxy-delta9-THC glucuronide. We select three multiple reaction monitoring (MRM) transitions per target analyte to increase specificity between opioids which have similar structures and fragmentation patterns as opposed to existing methods which commonly utilize two per analyte. Finally, we pilot this technique in an upstream manhole catchment thereby demonstrating the feasibility of this analytical technique at critical access points in wastewater networks. To our knowledge, this is the first observation of naloxone-3-glucuronide in an upstream manhole catchment.

2. Materials

ACT, ACT-Glu, AMP, BEG, BUP, CCN, CDN, CDN-Glu, CFN, THCCOOH, EDDP, HCD, HCT, HMP, HMP-Glu, THCOH, MAM, MAMP, MDA, MDMA, MPH, MPH-Glu, NBP-Glu, NCD, NHCD, NLX, NLX-Glu, NMPH, NOCD, NTRM, NTX, OCD, OMP, OMP-Glu, OTRM, PAG, THC, THCCOOH-Glu and TRM reference standards and their deuterated analogues used for internal standards (ACT-D4, AMP-D8, BEG-D8, BUP-D4, CCN-D3, CDN-D6, CDN-Glu-D3, CFN-13C3, THCCOOH-D3, EDDP-D3, HCD-D3, HCT-D3, HMP-D3, THCOH-D3, MAM-D6, MAMP-D8, MDA-D5, MDMA-D5, MPH-D6, MPH-Glu-D3, NBP-Glu-D3, NCD-D3, NHCD-D3, NLX-D5, NLX-Glu-D5, NOCD-D3, NTRM-D3, NTX-D3, OCD-D6, OMP-D3, OMP-Glu-D3, OTRM-D6, PAG-D5, THC-D3, THCCOOH-Glu-D3 AND TRM-D3) were purchased from Cerilliant Corporation (Round Rock, TX, USA) at concentrations of either 1 mg mL^{-1}, 2 mg mL^{-1} or 100 μg mL^{-1}. ACT-Glu (5 mg, 99.9% grade purity) was purchased from Sigma Aldrich (St. Louis, MO, USA). LC/MS Optima Grade acetonitrile, methanol, water and formic acid used for mobile phases and elution solvents were obtained from ThermoFisher Scientific (Agawam, MA, USA). Ultra-high purity (UHP) Nitrogen gas for sample concentration and UHP Argon for the LC/MS collision gas were sourced from Airgas (Billerica, MA, USA). UHP Nitrogen for the LC/MS nebulizing gas was supplied by a nitrogen generator (Peak Scientific, Billerica, MA). Liquid chromatographic separation was performed on a Horizon Vanquish UHPLC system equipped with an autosampler compartment, a temperature-controlled column compartment, and a binary pump system coupled to a TSQ Altis triple stage quadrupole MS/MS system equipped with a heated electrospray ionization (H-ESI) source (ThermoFisher Scientific, San Jose, CA, USA). Analytical separations were performed using a reversed-phase AccucoreTM Biphenyl column (100 × 2.1 mm I.D., 2.6 μm). The column was set up in-line with a column pre-heater, pre-filter, and a DefenderGuard column (10 × 2.1 mm, AccucoreTM Biphenyl packing). TraceFinder 4.1 General Quantitation Software was used for data acquisition. The TSQ Altis Tune application software was used to select the MRM transitions and to optimize the MS ionization settings.

3. Method development

A summary of the target analytes in the method with their respective drug classifications and parent compounds can be found in Table S1. The precursor and fragment ions for each target analyte and internal standard were determined by preparing individual 1,000 ng mL^{-1} solutions in 50:50 v/v methanol:water and directly infusing them to the mass spectrometer. Using a glass syringe and a syringe pump, the solutions were infused at a rate of 30 μL min^{-1} and teed into the LC flow until good spray stability was observed (<15% RSD). The CID gas was set to 1.5 mmTorr. Three MRMs for each target analyte and 2 MRMs for each internal standard were recorded along with their optimized collision energies, polarities and RF lens voltages (Table S2). A mixture of all reference materials was prepared at 10,000 ng mL^{-1} and infused to the H-ESI source, in-line with the LC flow at 50% B to optimize the ionization settings (Table S3). The optimized LC mobile phase solutions were 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) at a flow rate of 0.5 mL min-1. We compared both methanol and acetonitrile as the 'B' mobile phase and observed methanol to yield better retention and separation. The developed gradient elution program was: 3.50 min equilibration at 0% B, hold 0% B for 0.2 min, increase to 99% B over 2.3 min, hold constant for 1 min, decrease to 0% B and hold for 0.5 min. The autosampler compartment was set to 4 °C. The column oven was maintained at 40 °C in the 'Still Air' thermostatting mode. The injection volume was set to 1 μL, and the autosampler needle was rinsed before and after each injection for 15 s with a solution of 40:20:20 acetonitrile: isopropyl alcohol:acetone.

We investigated two chromatographic columns for this work: the Accucore™ Biphenyl and the Accucore™ Polar Premium reversed-phase columns from Thermo Fisher Scientific. We found the Accucore™ Biphenyl to retain and separate these target analytes better than the Accucore Polar Premium column.

During method development, we ran an experiment to determine the

minimum elution volume required to elute our compounds from the 15 g SPE cartridge. Milli-Q samples were spiked in triplicate with a mixture of target analytes for a concentration of 200 ng mL^{-1} in the final extract. A blank sample was also prepared. The cartridges (n = 4) were washed with 40 mL of a solution of 5% methanol in water v/v and eluted with 120 mL of 100% methanol with the wash and elution fractions collected at 20 mL intervals. Each fraction was spiked with a solution of internal standards for a final extract concentration of 50 ng mL^{-1}. The eluents were then dried, reconstructed in 1 mL of HPLC grade water and analyzed by the HPLC-MS/MS method.

Additionally, we performed a spiking recovery experiment to confirm the feasibility of the solid-phase extraction protocol in this matrix. For this experiment, we scaled down the loading volume and SPE cartridge resin to replicate field sampling while also preserving expensive reference standards. In the laboratory, we prepared triplicate samples (V = 140 mL) of Milli-Q water (spiked), wastewater (spiked) and grab wastewater (blank). The Milli-Q and wastewater samples were spiked with a mixture of target analytes at 5 ng L^{-1}. All solutions were loaded at a rate of 1 mL min^{-1} onto 6 cc, 500 mg Waters HLB SPE cartridges using a vacuum manifold. The cartridges were washed with 4 mL of 5% methanol in water v/v and eluted with 20 mL of 100% methanol. The eluents were spiked with a mixture of internal standards for a final extract concentration of 50 ng mL^{-1}, dried with UHP nitrogen, reconstructed in 1 mL of HPLC grade water and analyzed by the HPLC-MS/MS method described previously. The recovery in wastewater was corrected by the background concentration in the wastewater blank.

4. Method validation and quality control

The developed method was validated for specificity, linearity (r^2), accuracy, carryover, precision, range, instrumental quantification limit (IQL) and detection limit (IDL) in accordance with the FDA's *Analytical Procedures and Methods Validation for Drugs and Biologics Guidance for Industry* [37]. We prepared a series of dilutions from a 5,000 ng mL^{-1} working mixture of all target analytes into nine calibrators in a solution of 1% methanol in Milli-Q water: 0.1, 0.5, 1.0, 4.9, 10.0, 48.8, 97.5, 487.5, and 975 ng mL^{-1}. Each calibrator was spiked with the appropriate amount of internal standard (IS) mixture for a final amount of 50 ng mL^{-1}. Calibrators>20% different from the theoretical concentration were excluded from the calibration curve and a minimum of seven calibrators were required to validate a calibration curve. The calibration curves for all analytes were set to ignore the origin and had 1/X weighting. Area ratios of the target analytes to its respective internal standard were used to create the calibration curves. The IDL for each target analyte was determined by selecting the lowest concentration which had a signal-to-noise ratio (S/N) equal to 3, good chromatographic peak shape (6–10 MS scans per peak), the presence of at least one confirming ion, and fragment ion ratios within 20% of the expected fragmentation pattern pulled from the calibrators. The IQL for each target was determined by selecting the lowest concentration which satisfied the IDL requirements with both confirming ions present and S/N = 9 (see Table 1). An $r^2 > 0.98$ was considered suitable for linearity. The specificity of the method was ensured by using the internal standard mode of calibration. Any effects of the wastewater matrix such as retention time drift, chemical interferences, or ion suppression or enhancement are corrected by the internal standard. Carryover for all analytes was evaluated by injecting the highest calibrator in the series followed by an injection of milli-Q water solvent blank spiked with the appropriate amount of the internal standard working mixture and calculating the percent carryover between the injections.

The precision of the method was determined by repeatedly injecting a sample with 5 ng mL^{-1} of a solution of target analytes (n = 8) and calculating the relative standard deviation (%RSD). A %RSD < 15% was considered acceptable for validation. To ensure the quality of the method, we prepared a pooled sample consisting of 10 μL combined from each sample in the batch. The pooled sample was analyzed every five injections to check for instrument drift. A continuous calibration verification (CCV) sample equivalent to the fifth calibrator concentration is also injected periodically. Concentrations in the CCV<20% different than the theoretical amount are considered acceptable. A solvent blank spiked with internal standards is injected periodically to account for carryover.

Table 1
Summary of method validation parameters: instrumental detection limit (IDL), instrumental quantitation limit (IQL), linearity and precision.

Compound	IDL (S/N = 3), pg injected	IQL (S/N = 9), pg injected	Precision (% RSD)
ACT	9.8	9.8	9.9
ACT-Glu	4.9	4.9	9.1
AMP	4.9	4.9	20.1
BEG	0.5	0.5	36.9
BUP	0.5	0.5	6.5
CCN	1.0	0.1	3.6
CDN	1.0	4.9	13.8
CDN-Glu	0.5	1.0	11.9
CFN	1.0	4.9	6.2
EDDP	0.1	0.1	2.7
HCD	1.0	4.9	11.4
HCT	0.1	0.1	18.7
HMP	1.0	4.9	11.4
HMP-Glu	0.1	1.0	23.5
MAM	1.0	4.9	6.3
MAMP	0.1	0.5	2.9
MDA	0.5	0.5	2.8
MDMA	0.1	4.9	40.0
MPH	1.0	4.9	9.0
MPH-Glu	0.1	4.9	9.3
NBP-Glu	4.9	9.8	4.3
NCD	1.0	4.9	11.4
NHCD	4.9	4.9	6.0
NLX	0.5	1.0	8.3
NLX-Glu	0.5	1.0	8.1
NMPH	1.0	4.9	21.6
NOCD	0.5	1.0	10.2
NTRM	0.5	1.0	3.1
NTX	0.5	4.9	6.9
OCD	0.5	1.0	8.4
OMP	1.0	1.0	7.5
OMP-Glu	0.1	0.5	6.7
OTRM	0.1	1.0	9.4
PAG	0.5	4.9	34.5
THC	9.8	48.8	18.5
THCCOOH	1.0	4.9	8.6
THCCOOH-Glu	4.9	48.8	15.3
THCOH	1.0	4.9	2.8
TRM	0.1	4.9	3.4

5. Application to wastewater samples

We applied the developed method on wastewater samples (n = 5) collected from a manhole in a city in eastern Massachusetts. We used a custom-designed sampling device consisting of peristaltic pumps, a Durapore 0.22 μm filter, a custom-packed 15 g Oasis HLB solid-phase extraction (SPE) cartridge and a silicone rubber pickup tube equipped with a pond filter to prevent large debris from clogging the tubing (described further in Endo et al 2020). The sampling device was set to pump wastewater for 30 s every five minutes for 24 h. The effluent from the SPE cartridge was collected and the loading volume was measured for each sample. Upon completion of sample collection, the SPE cartridges were removed from the device and returned to the laboratory on ice. The samples were kept at −20 °C until analysis and analyzed within 72 h. Gonzalez-Marino et al. investigated the storage stability of similar analytes on HLB resin SPE cartridges and found minimal losses [38].

The SPE cartridges were washed with 40 mL of a 5% v/v solution of methanol in water then eluted with 120 mL of 100% methanol at a rate of 1 mL min^{-1}. We observed that a minimum of 120 mL of 100%

methanol was required to elute some cannabinoids (THCCOOH and THC) which determined the elution volume used (Figure S1). The methanol fractions were collected in 4 × 40 mL glass vials and spiked with 50 ng of the internal standard mixture. The eluents were placed under a gentle stream of UHP nitrogen until the samples were at near dryness. Samples were reconstructed in 1 mL of 1% v/v methanol in water solution.

The wastewater concentration (C_w) for each analyte was calculated by summing the concentrations of all four vials, multiplying by the reconstruction volume (V_r), and dividing by the total SPE cartridge loading volume (V_l) (1). The vial concentrations described here are all blank-corrected.

$$Cw = \frac{[(C1 + C2 + C3 + C4)xVr]}{Vl} \quad (1)$$

6. Results and discussion

6.1. Method development and validation results

The developed HPLC-MS/MS method is fast with an injection-to-injection time of 7.5 min. The chromatographic column can retain and separate the opioid compounds despite their similar chemical structures. This instrumental method is suitable for high-throughput applications, especially in labs interested in monitoring opioid usage in the community. To maximize the efficiency of the instrument turnaround time, the sample preparation steps need to be further optimized to shorten the overall processing time.

The method is sensitive for all analytes with IDLs lower than 1 ng mL^{-1} except for ACT-Glu, AMP, NBP-Glu, NHCD, and THCCOOH-Glu which have IDLs below 5 ng mL^{-1}. ACT and THC have a slightly higher IDL of 10 ng mL^{-1}. Similarly, the instrumental limit of quantification (IQL) was as low as 0.1 ng mL^{-1} for some analytes. THC and THCCOOH-Glu have slightly higher IQL of 50 ng mL^{-1}. The upper limit of the calibration curve is 1,000 ng mL^{-1}. If the observed concentration in an unknown sample falls above the upper limit, the sample is diluted to an appropriate level, spiked again with internal standards and re-analyzed by the instrument.

The calibration curve linear equation parameters and r^2 values can be found in Table S4. The linearity for all analytes is excellent ($r^2 > 0.99$) except for BUP, EDDP, THCOH, and NMPH which have $r^2 > 0.98$. THC has a reasonable r^2 of 0.97 that fell below the threshold for validation. As a result, THC could only be qualitatively detected here. The precision of the instrument in measuring each target analyte in wastewater is below the threshold of 15% RSD for all compounds except for PAG, THC, BEG, AMP, HCT, NMPH, MDMA, HMP-Glu which ranged from 16 to 40% RSD. In each of the solvent blank samples, the concentration of all target analytes was not detected, so we were unable to calculate the percent carryover.

6.2. Results from case study

The manhole used here serves an area that is 84% residential with a population of approximately 5,300 people. The estimated maximum hydraulic retention time from the point of injection to the manhole selected is 45 min. The distance from the manhole to the wastewater treatment plant is approximately 10 miles. The five samples were collected daily from Friday, October 4th to Tuesday, October8th, 2019. There were no significant rain events during the sampling period. The sample volumes collected on days 1–5 were 1.270, 1.475, 1.510, 1.485, and 1.370 L, respectively.

The analytes with the highest average wastewater concentrations over the five-day period were benzoylecgonine, the major urinary metabolite of cocaine and the population biomarker class compounds: caffeine, acetaminophen, phenylacetylglutamine, and hydroxycotinine (Fig. 1, Table 2). Most analytes were detected in all samples, except for NBP-Glu, 6-MAM, NLX-Glu and THCCOOH-Glu which were detected in four samples and ACT-Glu and NCD which were detected in fewer than

Table 2
Observed wastewater concentrations (ng L^{-1}) for samples taken over five days from a manhole in eastern Massachusetts. n.d.: Not detected.

Compound	Wastewater Concentration (ng L^{-1})				
	Day 1	Day 2	Day 3	Day 4	Day 5
ACT	58619.6	70558.4	46603.7	75501.6	43012.6
ACTG	8.6	13.4	n.d.	n.d.	n.d.
APT	258.1	292.5	222.5	181.4	337.6
BEG	2510.7	1456	3894	3115	1060.6
BUP	13.3	17.6	33.7	56.1	36
CCN	267.8	471.3	283.7	226.3	73
CDN	26	16	16.4	21.3	20.6
CDNG	104.4	58.4	80.9	40.9	27.9
CFN	71660.4	74241.8	69271.4	69825.4	76495.9
CTHC	453.3	435.4	466.5	382.6	469.1
EDDP	1.1	4.8	0.8	0.9	0.5
HCD	6	2.6	3.5	4.4	4.3
HCT	1319.4	1280.1	1107.9	1060.9	1348.9
HMP	2.5	2.5	1.2	1	2.2
HMPG	138.2	90.1	185.2	27.7	26.7
HTHC	149.3	214.4	208.6	539.4	482.5
MAM	3.2	n.d.	6.5	8.8	4.5
MAMP	19.2	22.4	17	9.3	12.1
MDA	9.7	12.6	6.5	7.2	12.6
MDMA	13.1	5.6	27.2	10.2	4.6
MPH	95.9	85.1	84.2	68.5	118.8
MPHG	133	87.9	178.8	26.7	26.6
NBPG	5.7	4.7	3.2	3.1	n.d.
NCD	21.8	n.d.	n.d.	n.d.	n.d.
NHCD	5.4	4.4	5.1	5.5	3.5
NLX	3.3	5.4	7.9	6.2	6.5
NLXG	8	7.9	5.7	6.8	n.d.
NOCD	16.8	20.9	15.8	21.4	86.6
NTRM	25.6	16.3	19.4	25.2	33.3
NTX	n.d.	n.d.	n.d.	n.d.	n.d.
OCD	8.2	8.9	5.6	2.8	25.3
OMP	6	4.9	4.5	9.3	12.1
OMPG	3.6	6.1	4.3	3.5	25.7
OTRM	43.1	41.5	62.1	65.9	82.4
PAG	6659.9	5319.9	6501.5	5068.1	5012.3
THC	29.6	49.5	18	33.4	30.9
THCG	30.7	35.3	39.2	15.2	n.d.
TRM	51.4	44.9	77.1	146.9	88.2

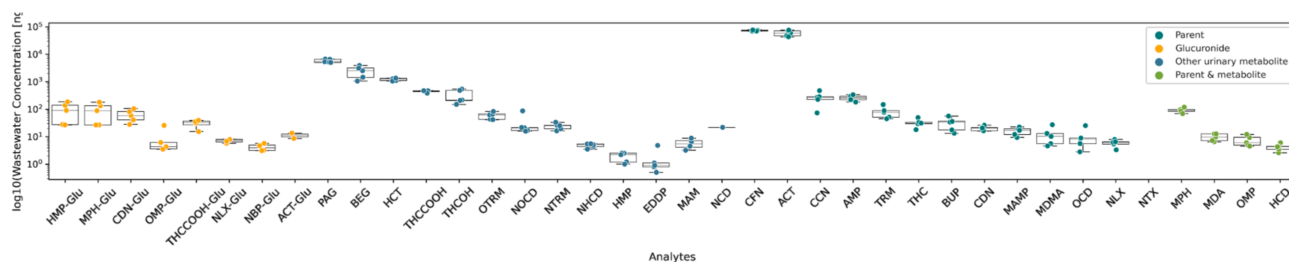

Fig. 1. Measured concentrations of all analytes in wastewater samples using the developed method.

two samples (Fig. 2). For some of the parent and glucuronide conjugate pairs such as THC and THCCOOH-Glu, NLX and NLX-Glu, HMP and HMP-Glu, MPH and MPH-Glu, CDN and CDN-Glu, the wastewater concentration of the glucuronide conjugate was higher or roughly equal to the amount of the parent compound (Fig. 2). This ratio of glucuronide conjugate to parent compound may be explained by the glucuronide being the primary urinary metabolite for these pairs [25,39–40]. Conversely, the average wastewater concentration of acetaminophen is four orders of magnitude higher than its glucuronide conjugate, acetaminophen glucuronide. This could potentially be explained by larger amounts of acetaminophen discarded down the drain. The relatively low detection rate of norcodeine may be because it is only a minor urinary metabolite of codeine [41]. Naltrexone, a treatment for alcohol and opioid abuse, was not detected in any of the samples. This could potentially be explained by fewer naltrexone users in our selected catchment area.

We found that THCCOOH is the metabolite reliably used to estimate human consumption of cannabis (THC) in a WBE context. Previous studies have shown difficulty measuring THC in aqueous wastewater without also including sludge due to its high lipophilicity [42] Furthermore, THCOH is only an intermediary metabolite which is ultimately excreted in urine as THCCOOH. It is also expected that some amount of THCCOOH-Glu transforms back to the carboxylic form in the wastewater environment. Although, Bijlsma et al. suggest acidifying the samples to pH 2 to reliably quantify THCCOOH in a wastewater matrix, adjusting the pH in an in-situ extraction device is challenging in this application and further work is needed to achieve pre-acidification.

In order to collect and observe the more labile analytes of interest in this study, we utilized an in-situ wastewater sampling device. Unlike most WBE studies which obtain a composite sample from a WWTP before bringing it to the laboratory for processing, this device filters and loads the wastewater sample to the SPE cartridge instantaneously, thereby preserving the glucuronide conjugates and other sensitive compounds. However, one significant and notable disadvantage of this sampling method is that the internal standards are only introduced to the wastewater sample after the cartridge is returned and eluted in the lab. Therefore, the extraction efficiency is not considered, making further WBE calculations of population consumption challenging.

While the sample collection procedure described here is outside the scope of the HPLC-MS/MS method validation, this sampling strategy is valuable for the future of wastewater surveillance at the neighborhood level. To demonstrate the effectiveness of the SPE cartridges in the field, we performed a spiking recovery experiment in the laboratory to investigate the recoveries of the SPE protocol in both Milli-Q and wastewater matrices. The recoveries from the experiment are summarized in Table S5. Overall, the opiates had reasonable recoveries in Milli-Q water (>30%) with HMP-Glu and MPH-Glu having lower average percent recoveries of 22.8 and 27%, respectively. Many compounds had better percent recoveries in wastewater even after being wastewater blank corrected with average percent recoveries >70% for all compounds selected except for BUP which had a recovery in wastewater of 17.7%.

6.3. Implications and future work

The ability to assay wastewater samples at both upstream and

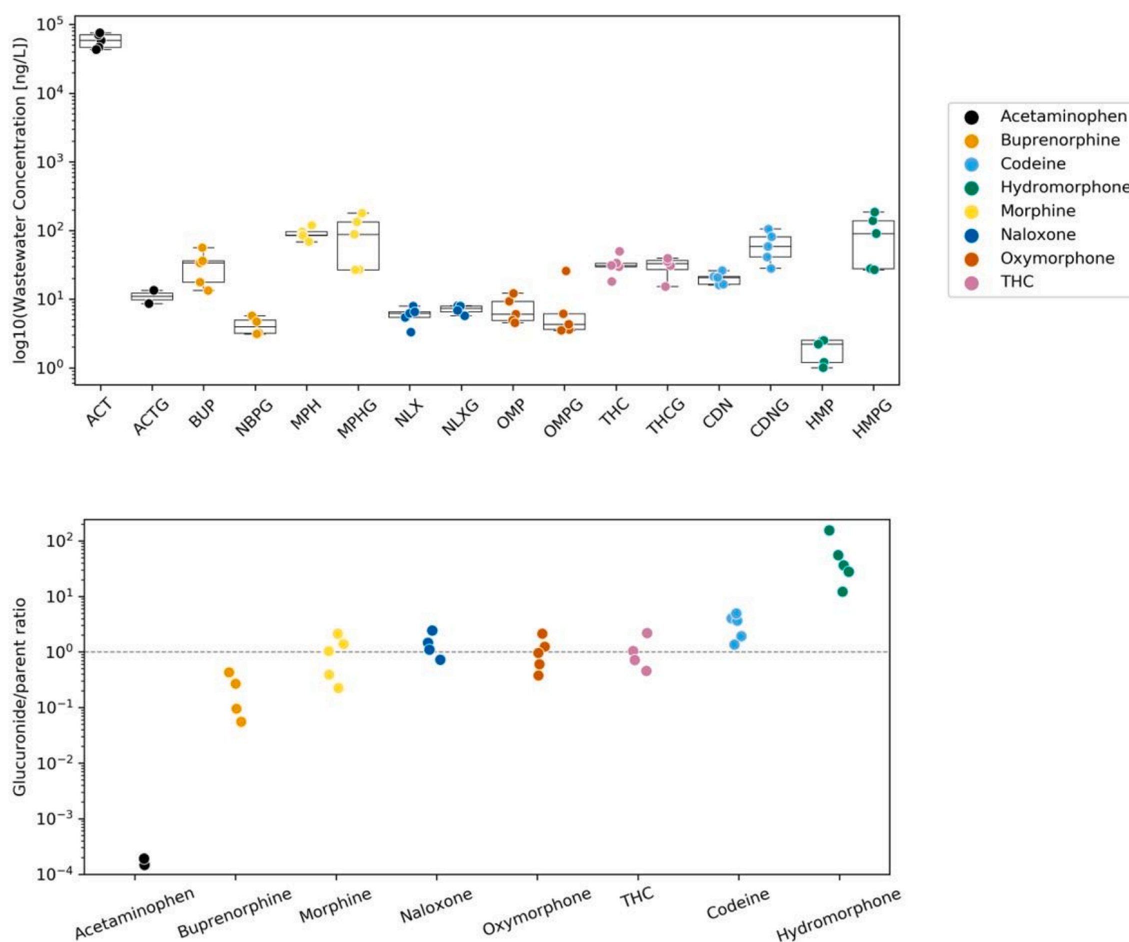

Fig. 2. (Top) Comparison of the wastewater concentrations for the glucuronide conjugates and their respective parent compound. (Bottom) The ratio of the wastewater concentration for the glucuronide conjugate to its parent.

downstream sources for a large panel of both prescription and illicit opioids significantly advances the ability of researchers and public officials to understand exposure to opioids among key populations. Importantly, the incorporation of glucuronide conjugates draws the distinction between human exposure to opioids compared to opioids that may be discarded via the wastewater system. This distinction allows public health officials to recognize patterns of opioid use that are a result of actual human exposure compared to a composite analysis of both human exposure plus potential discarded opioids.

Numerous pharmacokinetic studies have been performed on the human excretion rates of the target analytes in this study which are further used in WBE back-calculations of human exposure of the parent compounds [44–54]. Adding glucuronide conjugates to these back-calculations expands the suite of human-specific markers of consumption, especially for analytes such as naloxone which are primarily excreted as the glucuronide conjugate [48].

The method described here is subject to some limitations. Regarding the sample collection, one major issue is the inability to introduce the internal standards to the sample prior to the solid-phase extraction loading step. Further experiments and hardware revisions should be explored to investigate if introducing the internal standards sooner is possible, perhaps during the in-situ sample collection or as a dose to the cartridge upon arrival to the lab. Another limitation of this method is the large elution volume required to elute each SPE cartridge. This results in long drying times and potential handling errors when splitting the sample into vial fractions. In future applications, the relatively large total elution volume could be collected and dried in a single container using a rotary evaporator rather than drying individual fractions with nitrogen gas.

Notably, some of the glucuronide conjugates were not observed on all five days of the study. There are many possibilities for why these conjugates were absent. One possible explanation could be the different usage dynamics of the parent drugs across the days of the week [43]. Their absence could also potentially be explained by partial degradation prior to sample collection or abnormal dumping of bleach cleaning products to the sewage network that day. We know that dilution can affect the signal of these compounds in wastewater, however we did not observe significant rain events during the sampling period. Another explanation could be changes in the bacterial concentrations in the wastewater across the different days. Future work is needed to better constrain the in-sewer stability of glucuronide conjugates at different injection points in the upstream wastewater network. Furthermore, the method itself can be expanded and revalidated to include emerging opioids or drugs of abuse (i.e. synthetic drugs or fentanyl analogs). This technique may enable higher resolution understanding of substance use and the impact of treatment programs at the community and neighborhood level.

7. Conclusions

We developed a sensitive HPLC-MS/MS method for the quantification of 31 opioids, illicit drugs, and population biomarkers as well as 8 glucuronide conjugates in wastewater. Chromatographic separations were performed using an Accucore™ Biphenyl column (100 × 2.1 mm I.D., 2.6 µm), with a 7.5-minute runtime between injections. The instrumental method was validated for selectivity, linearity, IQL, precision, and accuracy. The calibration curves for all analytes are linear ($r^2 > 0.98$) in the targeted range (0.1–1,000 ng mL^{-1}), except for THC with $r^2 = 0.97$, and instrumental limits of quantification (S/N = 9) for all analytes ranged from 0.1 to 48.8 ng mL^{-1}. We applied the method to five wastewater samples collected from a manhole using an in-situ wastewater sampling device. Results from a recovery experiment found reasonable recoveries utilizing an in-situ sampling device in a manhole. These results demonstrate the ability to capture glucuronide conjugates indicative of direct human exposure to various licit and illicit opioids as well as naloxone, the overdose reversal drug. This allows for high resolution detection of changes in population exposure to opioids as well as potential overdoses which may be reversed in the field and never present for medical care. By understanding the distribution of opioids and naloxone usage, public health officials can use these data to better allocate opioid abuse services and interventions in their communities [10]. Lastly, to more accurately estimate population consumption using an upstream sampling method, further investigation of internal standard spiking inside of an in-situ robotic sampling device is required.

CRediT authorship contribution statement

Katelyn S. Foppe: Conceptualization, Investigation, Methodology, Validation, Writing - original draft, Writing - review & editing. **Elizabeth B. Kujawinski:** Supervision, Writing - review & editing. **Claire Duvallet:** Data curation, Writing - review & editing, Visualization. **Noriko Endo:** Writing - review & editing, Project administration. **Timothy B. Erickson:** Writing - review & editing, Funding acquisition. **Peter R. Chai:** Writing - review & editing, Funding acquisition. **Mariana Matus:** Resources, Funding acquisition.

Declaration of Competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [MM is CEO and co-founder of Biobot Analytics; KF, NE, CD and MM are employed by Biobot Analytics; EK is a scientific advisor to Biobot Analytics. All of these authors hold shares in the company].

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2021.122747.

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