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Screening for illicit drugs in pooled human urine and urinated soil samples and studies on the stability of urinary excretion products of cocaine, MDMA, and MDEA in wastewater by hyphenated mass spectrometry techniques

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Monitoring population drug use through wastewater-based epidemiology (WBE) is a useful method to quantitatively follow trends and estimate total drug consumption in communities. Concentrations of drug biomarkers might be low in wastewater due to dilution; and therefore analysis of pooled urine (PU) is useful to detect consumed drugs and identify targets of illicit drugs use. The aims of the study were (1) to screen PU and urinated soil (US) samples collected at festivals for illicit drug excretion products using hyphenated techniques; (2) to develop and validate a hydrophilic interaction liquid chromatography – mass spectrometry / mass spectrometry (HILIC-MS/MS) method of quantifying urinary targets of identified drugs in wastewater; and (3) to conduct a 24 h stability study, using PU and US to better reflect the chemical environment for targets in wastewater. Cocaine (COC) and ecstasy-like compounds were the most frequently detected illicit drugs; an analytical method was developed to quantify their excretion products. Hydroxymethoxymethamphetamine (HMMA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), HMMA sulfate (HMMA-S), benzoylecgonine (BE), and cocaethylene (CE) had 85–102% of initial concentration after 8 h of incubation, whereas COC and ecgonine methyl ester (EME) had 74 and 67% after 8 h, respectively. HMMA showed a net increase during 24 h of incubation (107% ± 27, n = 8), possibly due to the cleavage of HMMA conjugates, and biotransformation of MDMA. The results suggest HMMA as analytical target for MDMA consumption in WBE, due to its stability in wastewater and its excretion as the main phase I metabolite of MDMA. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: pooled urine analysis; illicit drugs; phase II metabolites; stability; wastewater-based epidemiology

Introduction

Analysis of wastewater for illicit drugs is a complementary approach to surveys for obtaining data on drug consumption trends in a community, since wastewater can be considered as a large, strongly diluted, pooled urine (PU) sample.[1–3] Wastewater-based epidemiology (WBE) estimates drug consumption at the community level by quantifying excretion products (drug and/or its metabolites) in wastewater and back calculating concentration of these targets to actual drug consumption. An important prerequisite for these back calculations is the knowledge about the best biomarkers to reflect human consumption and their stability in the sewer.[4,5]

As illicit drugs can be extensively metabolized in humans, knowledge of their metabolism and stability of metabolites is of great interest.[6] Obtaining authentic urine samples with excretion products of illicit drugs can be challenging for ethical and legislative reasons. Pooled urine analysis (PUA) can overcome some of these issues and reveal the nature and type of drugs consumed by an investigated population.[7–10] Furthermore, PUA offers realistic excretion patterns of illicit drugs and is easily accessible. Apart from PU, another way to collect urine is through urinated soil (US) samples. At events where people urinate in places other than lavatories and urinals, pools of urine and urinary metabolites can accumulate on the soil.

Stability data in wastewater are mainly available for drugs, but also for some metabolites.[4,5,11–13] Gomes *et al.* tested the stability

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of estrogen phase II metabolites in wastewater and concluded that sulfates should have a high stability (above 80%) after 8 h and glucuronides being below detection limit after 8 h of incubation.[14] So far, only one study has investigated the stability of phase II metabolites of illicit drugs in wastewater. Glucuronides of 3,4-methylenedioxypyrovalerone (MDPV) degraded in wastewater after 24 h, whereas sulfates mostly remain intact.[4] Currently used targets for cocaine (COC) and 3,4-methylenedioxymethamphetamine (MDMA) consumption in WBE are benzoylecgonine (BE) and MDMA itself, respectively.[15] However, MDMA is known to be highly metabolized before being excreted into urine and hydroxymethoxymethamphetamine (HMMA) sulfates were found to be the most abundant products.[16] Using MDMA itself as a target in WBE will not allow distinction between drug consumption and discharge.

Therefore, the aims of the present study were to collect PU and US samples from festivals and to screen these samples for excretion products of illicit drugs by mass spectrometry. Furthermore, an analytical method to quantify COC, MDMA 3,4-methylenedioxyethyleamphetamine (MDEA), and their main metabolites (Figure 1) in wastewater for stability studies was validated. Finally, PU and US were incubated in wastewater and the stability of the investigated compounds, including their conjugates, was assessed over time.

Experimental

Chemicals and reagents

COC, COC-d_3, cocaethylene (CE), CE-d_3, BE, BE-d_3, MDEA-d_5, and HMMA were obtained by LGC standards, ecgonine methyl ester (EME) and EME-d_3 were obtained from Cerilliant (Round Rock, TX, USA). HMMA-d_3, MDMA, MDMA-d_5, 3,4-methylenedioxymethamphetamine (MDA), and MDA-d_5 were obtained from Sigma-Aldrich (St Louis, MO, USA). HMMA sulfate (HMMA-S) was produced as described previously.[17] Hydroxymethoxyethylamphetamine (HMEA) was produced as described previously.[18] MDEA was obtained from seized materials. Dilutions and storage of COC and its metabolites were done in acetonitrile, for MDMA and metabolites in methanol. Isolute C18 (500 mg, 3 mL) and HCX cartridges (130 mg, 3 mL) were obtained from Biotage (Uppsala, Sweden). Mixture (100

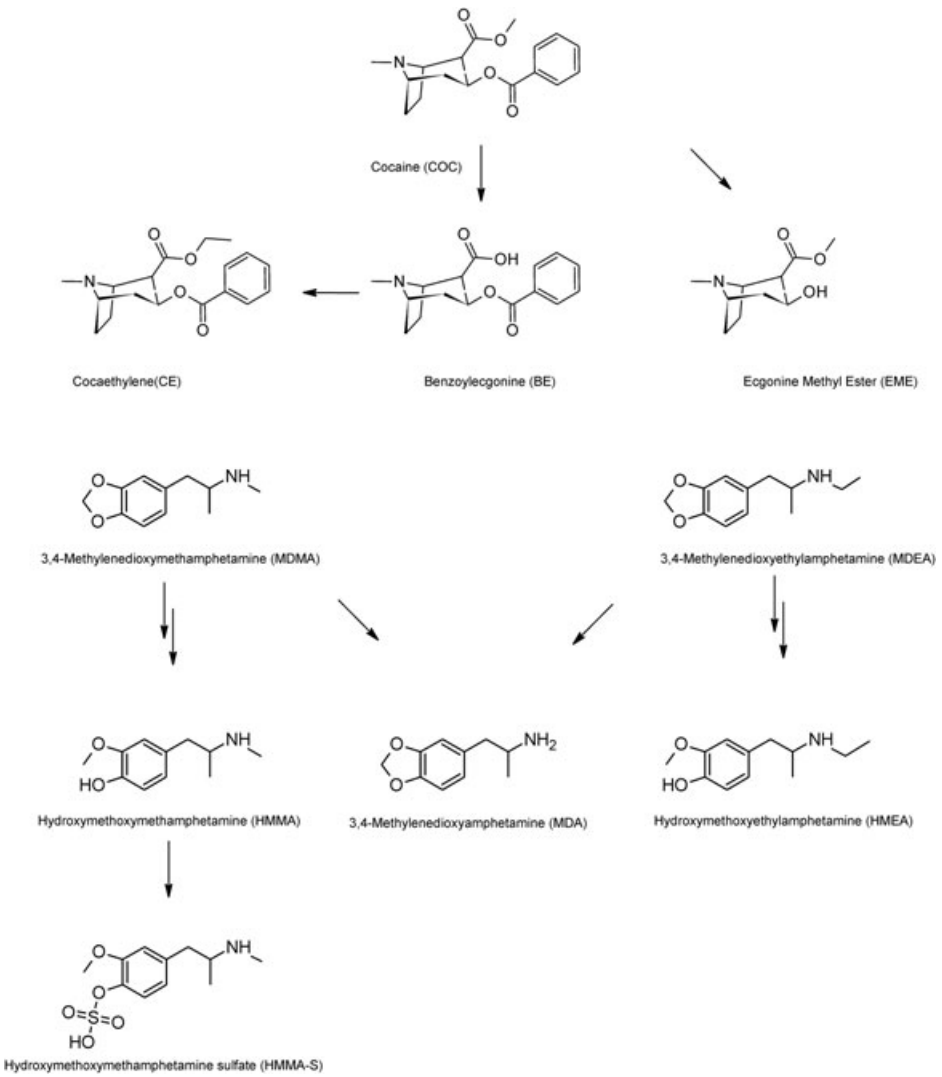

Figure 1. Chemical structures of the investigated excretion products. Arrows indicate metabolic pathways.

000 U/mL) of glucuronidase (EC No. 3.2.1.31) and arylsulfatase (EC No. 3.1.6.1) from *H. Pomatia* and analytical grade ammonium acetate from Merck (Darmstadt, Germany). All other chemicals and reagents (analytical grade), and centrifugal filters were from VWR (Darmstadt, Germany). The P-Tree Urinal was from Aandeboom (Utrecht, the Netherlands).

Collection of pooled urine samples and urinated soil samples

All samples were collected at two European music festivals in 2014. PU samples were collected by two different methods:

A. Extracted from tulip urinals (individually collected PU samples, n = 11) using a plastic hose and plastic syringe, collected from three independent urinals at different time points over three days.
B. Collected from urinals (Pee tree urinals, n = 10) mounted on a wall, connected to a 30 L container located in a protected environment.

All PU samples were spiked on site with HMMA-d_3 to a final concentration of 100 ng/mL and stored in polystyrene containers and immediately put on ice until freezing (max 12 h).

US was collected from areas (fences, bushes, etc.) where pools of urine were visible on the surface. The characteristics (liquid content, colour, type of soil) differed from urine with small amounts of sand to humus with high organic content.

Sample preparation and screening analysis of urine samples and urinated soil samples

An overview of sample preparation of PU and US is presented in Figure 2. Sample preparation for PU was according to previously published standard urine sample protocols, to identify excretion products (parent drug and metabolites) of illicit and therapeutic drugs.[19] Briefly, Phase I metabolites were identified after enzymatic cleavage of conjugates in 1 mL PU added internal standard (1 μg/mL trimipramin-d_3); basic and neutral/acidic phase I metabolites were then separated by applying the cleaved PU onto HCX cartridges and dividing each fraction into two. One fraction was acetylated and injected onto gas chromatography-mass spectrometry (GC-MS) using a Hewlett Packard (HP, Waldbronn, Germany) 5890 Series II gas chromatograph system. The other part was analyzed by liquid chromatography-high resolution-tandem mass spectrometry (LC-HR-MS/MS) using a Thermo Fisher Q Exactive and LC-ion trap mass spectrometry (LC-MS^n) using a Thermo Fisher LXQ both coupled to Accela LC systems. Phase II metabolites were identified after extracting 1 mL PU added internal standard (1 μg/mL trimipramin-d_3) on C18 cartridges and after precipitation of 100 μL PU with 500 μL acetonitrile, then analyzed using LC-HR-MS/MS and LC-MS^n mentioned above (Figure 2).[20,21] Acquired LC-HR-MS/MS data were analyzed for common new psychoactive substances (NPS) and metabolites, by extracting ion chromatograms from full scan, as well as mining MS spectra in data-dependent mode.[20]

Sample preparation for US was as follows: 0.5 g of US was mixed with 0.5 mL acetonitrile with internal standard (100 ng/mL HMMA-d_3). This mixture was thoroughly vortexed and centrifuged for 5 min at 18,407 *g* and 10 μL were injected onto the LC-HR-MS/MS and LC-MS^n systems as used for the PU samples, with instrument settings as previously reported for standard human urine samples.[19–21]

Collection of wastewater samples

Wastewater was collected for the stability assessment of urinary targets on a dry day as a 24 h time-proportional composite sample from a wastewater treatment plant in Wulpen, Belgium.

Instrumentation for wastewater incubations

The LC system consisted of an Agilent 1200 series High Pressure LC fitted with a degasser, a binary high-pressure gradient pump, a heated column compartment and an autosampler module without cooling. Chromatographic separation was achieved using a Phenomenex LUNA HILIC 200 Å (150 x 3 mm, 5 μm) column, with a mobile phase composed of A) 5 mM ammonium acetate in ultrapure water and B) acetonitrile, at a flow rate of 0.4 mL/min. The gradient was as follows: 0–0.5 min 95% B, 0.5–6.5: 60% B, 6.5–9 min: 52% B, 9–9.5 min: 95% B. The total runtime, including column

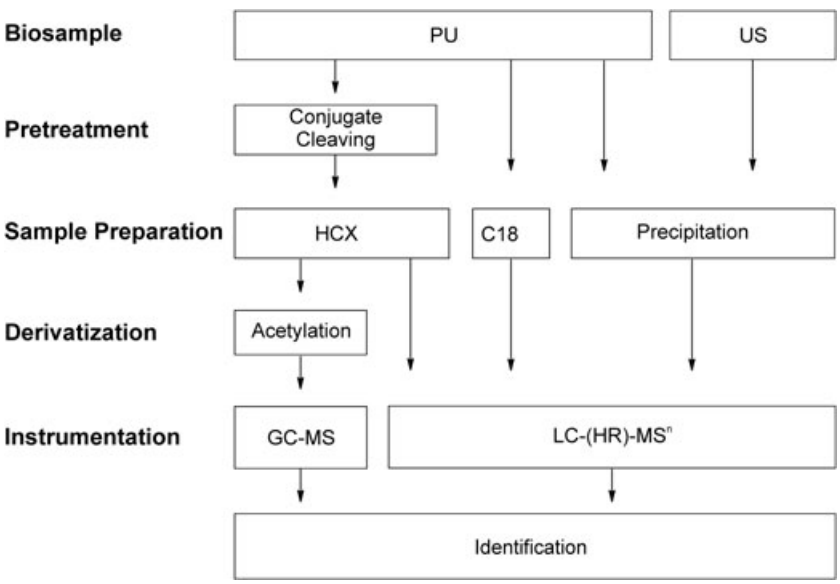

Figure 2. Overview of instrumentation and sample preparation methods for screening pooled urine and urinated soil samples for illicit drugs and therapeutic drugs.

equilibration, was 15.5 min. All compounds were eluted within 8 min. The injection volume was set to 2 μL. The LC system was coupled to an Agilent 6410 triple quadrupole mass spectrometer with an electrospray interface (ESI) for detection and quantification of the compounds. Source parameters were as follows: gas temperature of 350 °C, gas flow 12 L/min, nebulizer 40 psi, capillary voltage +4000 V and delta EMV 450, time frame was set to 0.15 min. The mass spectrometer compound dependent parameters were optimized for each compound individually (Table 1) by direct injection (without analytical column) of standards using MassHunter Optimizer (v. B 03.01) Source parameters (gas temperature, gas flow, nebulizer pressure, and capillary voltage) were optimized to acquire an intense protonated molecular species $[M+H]^+$ for each compound. Mass spectrometer compound dependent parameters, fragmentor voltage, and collision energy were optimized to acquire two multiple reaction monitoring (MRM) transitions (qualifier and quantifier) for each compound, including internal standards. The most abundant transition was chosen as quantifier (Q) and the second most abundant transition as qualifier (q). The Q/q ratio was monitored for variation (RSD 20%) to provide an additional identification criterion besides the retention time. For LC optimization, a mix of standards with all compounds was injected on three different HILIC columns for evaluation of separation power. In addition, different injection volumes (1, 2, 3, and 5 μL) were tested, and optimized based on effect on peak shape.

Method validation for analysis of wastewater incubations

Method validation was based on the EMA guidelines on bioanalytical method validation with some exceptions.[22] Precision, accuracy, linearity, selectivity, calibration range, lower limit of quantification (LLOQ) and sensitivity were tested. Multi-component calibration curves were established in wastewater with a minimum of six calibration points, by spiking stock solution into previously collected grab samples wastewater, then mixing 50 μL spiked wastewater with 50 μL internal standard in acetonitrile, filtering samples through 0.45 μm nylon membrane filter units and centrifuging them for 5 min at 18 000 *g*. Signals from each analyte were corrected for their corresponding deuterated internal standard (IS), with the exceptions of HMMA and HMMA-S, where MDMA-d_5 was used as internal standard. IS was prepared by diluting stock solutions in acetonitrile to a final concentration in prepared sample of 84 ng/mL for COC-d_3, CE-d_3, and MDMA-d_5, 40 ng/mL for EME-d_3 and BE-d_3, and 140 ng/mL for MDA-d_5 and MDEA-d_5.

A blank sample (wastewater sample with acetonitrile) and a zero sample (wastewater sample with IS) were run together with the calibration curves. The medium quality control (QC_{med}) was prepared for the method validation, and additionally two quality controls, one low (QC_{low}) and one high (QC_{high}) were added for the analytical runs. QCs were prepared in the same way as calibration points and used to calculate accuracy and precision. Calibration curves for quantification were constructed in Agilent MassHunter Quantitative Browser by plotting peak areas of the analytes divided by the corresponding internal standard, against the spiked concentration and weighted by 1/x. Carryover was tested by injecting the blank after the highest calibrator and repeated three times.

Intra- and inter-day accuracy and precision were based on calculating concentrations of quality control samples from established calibration curves, using QC_{med} ($n = 6$) for intra-day and all three QCs ($n = 6$-13) for inter-day accuracy and precision. The results were assessed with an acceptance criterion within 85–115% (mean) accuracy and $< 15\%$ precision, as confidence value (CV). LLOQ was based on visual inspection, and complied with EMA guidelines on ± 20 % back-calculated concentrations.

Stability assessment of urinary targets in wastewater

The workflow for the multi-step preparation of the incubations is presented in Figure 3. Screening analysis provided estimations on which PU and US samples had high and low concentrations of COC, MDMA, and MDEA excretion products, respectively. These estimations were used to design wastewater incubations with high, low, or medium concentrations of COC, MDMA, and MDEA excretion products. A blank incubation was run to correct for possible

Table 1. Optimized MRM transitions and retention times for selected compounds and the deuterated internal standards.

Compound Name	Retention time, min	Precursor ion, *m/z*	Fragmentor voltage, V	Collision energy, V	Quantifier Product ion, *m/z*	Qualifier Product ion, *m/z*
Benzoylecgonine	5.3	290.1	124	14	168.1	105.1
Benzoylecgonine-d_3	5.3	293.2	122	13	171.1	105.0
Cocaethylene	5.8	318.2	134	14	196.1	82.1
Cocaethylene-d_3	5.9	321.2	132	16	199.1	85.1
Cocaine	5.4	304.2	124	14	182.1	82.1
Cocaine-d_3	5.6	307.1	124	14	185.1	85.1
Ecgonine Methyl Ester	7.5	200.2	124	14	182.1	82.1
Ecgonine Methyl Ester-d_3	7.6	203.2	118	14	185.1	85.1
HMEA	7.4	210.1	89	4	165.1	137.1
HMMA-S	4.1	276.0	105	12	165.1	137.1
HMMA	7.7	196.1	88	8	165.1	137.1
MDA	7.4	180.2	80	4	163.1	105.1
MDA-d_5	7.5	185.2	80	4	168.1	110.1
MDEA	7.1	208.2	88	9	163.0	105.1
MDEA-d_5	7.1	213.1	92	8	163.1	105.1
MDMA	7.3	194.1	92	9	163.0	105.1
MDMA-d_5	7.3	199.1	92	21	163.1	107.1

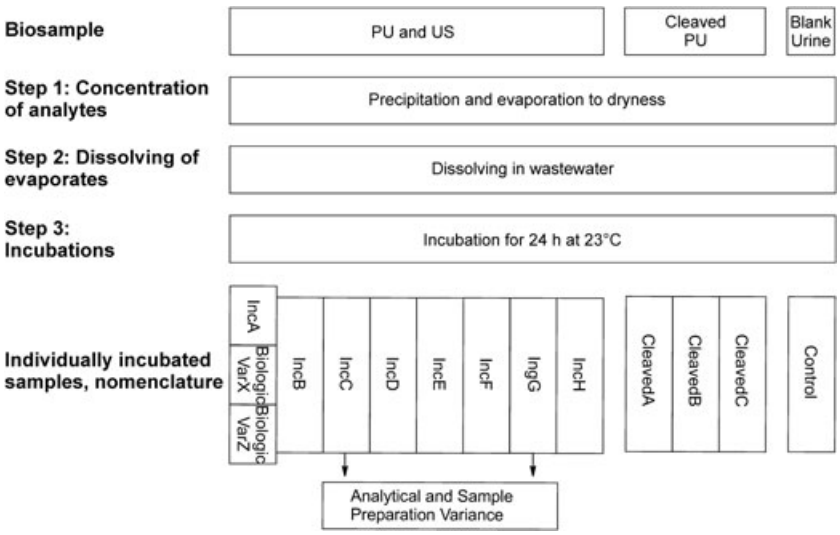

Figure 3. Overview of incubation workflow, which samples are used to assess biological, analytic and sample preparation variance, and nomenclature for samples. Biosample: PU, US, cleaved PU, or blank urine

back-ground excretion products present in wastewater. Only PU and US with COC, MDMA, and/or MDEA excretion products were used for stability assessment of urinary targets in wastewater. To investigate stability of phase I metabolites and parent drug with minimal interference from phase II metabolites, three wastewater incubations were prepared with conjugate cleaved PU (Figure 3), from PU samples tested positive for COC and MDMA excretion products in the screening analysis. Three PU samples were enzymatically cleaved by adjusting them to pH to 5.2 ± 0.1 with 0.1 M HCl and 0.1 M NaOH, then adding 50 μL glucuronidase/arylsulfatase, and incubating the mix for 30 min at 70 °C in a GC oven.

Step 1: Concentration of analytes

All samples were concentrated by precipitation with methanol and evaporation to dryness. Five mL PU, US, cleaved urine, or blank urine were mixed with ice cold methanol (50:50 *v/v* %) and centrifuged for 10 min at 2000 *g*. The supernatants were evaporated to dryness at 40 °C under a gentle stream of nitrogen.

Step 2: Dissolving in wastewater

The concentrated PU, US, cleaved urine, or blank urine was diluted in wastewater to fit the calibrated range. A total volume of 4 mL of each incubation was transferred to 50 mL falcon tubes.

Step 3: Incubations

All samples were incubated at 23 °C in a temperate water bath for 24 h. Sampling of each incubation was made after 0, 0.5, 1, 2, 3, 5, 8, 12, and 24 h.

One of the incubations (IncA) was divided into three separate containers to assess biological variance (IncA, BioVarX, BioVarZ), i.e., the variance introduced by different transformation from the biological communities in wastewater. Incubation pH was monitored with indicator paper.

Sample preparation of wastewater incubations

An aliquot of 50 μL from the wastewater incubations was mixed with 50 μL internal standard in acetonitrile and samples were filtered through 0.45 μm nylon membrane filter units that were centrifuged for 5 min at 18 000 *g*.

Evaluation of analytical data for stability studies

Variance introduced post-injection (analytical variance), from the sample preparation (sample preparation variance) and from variance introduced by different transformation from the biological communities in wastewater (biological variances) were assessed for MDMA and COC excretion products in real samples. Biological variance was assessed after 3, 12, and 24 h. Analytical and sample preparation variances were tested using IncC and IncG from a single time point, respectively. Both samples were prepared five times (sample preparation variance) and one of these was injected five times (analytical variance). F-tests were calculated using Microsoft Excel 2007 add-in program, Analysis ToolPak F-test.

Compound stability compared to initial concentration was classified using slightly modified definitions presented by McCall *et al.*[23] A compound had a high stability when more than 80 % of the original concentration remained after defined time period, medium stability when 40-80 % remained, and a low stability when less than 40% remained. Stability was assessed after 8 and 24 h.

Results and discussion

Screening of pooled urine samples and urinated soil samples

Two different analytical systems (GC and LC) have been used to cover a wide range of analytes. The methods used are expected to be comprehensive to each other and were therefore used to screen for therapeutic drugs as well as for drugs of abuse originating from different drug classes. COC and MDMA were the most frequently detected illicit drugs in the investigated PU and US samples (Table 2). Other identified illicit drugs were MDEA, MDA, methamphetamine, amphetamine, ketamine, and sildenafil. All compounds except amphetamine were also identified by metabolites. It should be noted, that the latter two are also therapeutic drugs, but have potential for non-prescription abuse. MDA is the main excretion product after MDA use, but is also an excretion product of MDMA and MDEA.[18,24,25] Signal intensities of MDA in the PU and US

Table 2. Frequency of detected illicit drugs by GC-MS and LC-(HR)-MS^n. M: metabolites.

	Tulip Urinals	PeeTree Urinals	Urinated Soil Samples
	n = 11	n = 10	n = 12
Cocaine + M	10	6	8
MDMA + M	9	1	8
Ketamine + M			1
Sildenafil + M			1
Amphetamine	3	1	1
MDEA + M	2		

samples were lower than MDMA and MDEA signal intensities, it is thus assumed to be present as a metabolite of the latter two. The US samples were found to be a useful addition to the PU samples. No standard urine screening approaches for illicit drug excretion products on US were found in the literature. Further studies should investigate additional sample preparation methods, including conjugate cleavage.[26] Ketamine and sildenafil metabolites were only detected in the US samples. Ketamine might also originate from other sources than human consumption, such as excretion of veterinarian medicine. However, ketamine is mainly abused by people and used only for veterinarian purposes of large animals. Frequencies of detected illicit drugs are summarized in Table 2. This high prevalence of COC and MDMA was also seen in PU samples collected from the center of London by Archer *et al.*[10] However, no NPS could be detected in our study. This might be explained most likely by absence of consumption or levels below limit of detection. In the future, more sensitive and targeted screening strategies may overcome such limitations in part.[27] Based on the findings from the screening, MDMA, COC, and MDEA were chosen as substances to test stability of main urinary excretion products in wastewater.

Analytical method for wastewater incubations

As expected, based on previous studies,[28] HILIC separation of the compounds of interest was sufficient and robust. HILIC was chosen as it should be suitable for polar analytes, such as metabolites, and should increase separation power compared to reverse phase columns for these types of compounds due to the different solid phases. For the current method, performance was optimized for method application to stability studies with high concentrations and simple sample preparation. The range for MDMA and BE were designed higher than other analytes (Table 3), as the PU and US screening showed that these two compounds were present in high concentrations in the PU and US samples. A reconstructed ion chromatogram of quantifier transitions of QC_{med} for MDMA and BE and QC_{high} for the remaining analytes is presented in Figure 4. The peak of HMMA-S was tailing but precision and accuracy were within ± 7 % and 95–107%, respectively. Selectivity, linearity, LLOQ, precision, accuracy, and carry-over were assessed for the method validation. All tested parameters were within the ranges defined by the EMA. A summary of the validation data can be found in Table 3.

The LLOQ of the compounds ranged from 2.7 to 20.6 ng/mL and the upper limit of quantification from 340.2 to 2574.6 ng/mL with a minimum of six calibration points. All calibration curves had a squared correlation coefficient higher than 0.99 using 1/x weighing. MDMA-d_5 was used as internal standard for HMMA and HMMA-S.

The back-calculated concentration of all calibration points were within ± 15 % and ± 20 % for the LLOQ.

Precision and accuracy were tested inter- and intra-day using QC_{med}. Additionally, two QC levels were used in the analytical runs as autosampler stability was not assessed. Accuracy ranged from 93 to 106 % and precision (as CV at confidence level 0.05) from 1% to 10%.

Matrix effects were not investigated as all incubations, QC_{high}, and QC_{low} were made from the same batch of wastewater. Recovery was not tested as it is no longer recommended in the latest EMA guidelines. Sample preparation consisted of dilution and all QCs were within limits for accuracy and precision.[22] Neither investigated excretion products nor interfering compounds could be detected in any blank samples.

Stability of urinary metabolites in wastewater

The validated method was used to investigate the stability of the excretion products from the PU and US samples spiked in wastewater. Incubations were designed to give a large initial variance in absolute concentration of COC and MDMA excretion products, based

Table 3. Method validation data: Linear range in ng/mL, number of calibration points for the calibration curve ($R^2 > 0.99$ for all calibration curves), accuracy in %, precision in confidence value at $\alpha = 0.05$, inter-day at 1 concentration level, intra-day at 3 concentration levels.

	Linear range, ng/mL	Calibration points	Accuracy, %				Precision, %			
			Inter-day	Intra-day			Inter-day	Intra-day		
			QC_{med} n = 6	QC_{low} n = 6	QC_{med} n = 13	QC_{high} n = 6	QC_{med} n = 6	QC_{low} n = 6	QC_{med} n = 13	QC_{high} n = 6
Benzoylecgonine	10.3-2574.6	9	102	94	95	97	1	4	4	3
Cocaethylene	2.7-340.2	8	95	97	96	98	3	2	3	3
Cocaine	5.4-340.2	7	93	100	99	101	2	3	3	3
Ecgonine Methyl Ester	8.2-340.2	6	98	100	100	100	5	9	5	3
HMEA	5.4-340.2	7	103	95	96	96	2	8	4	5
HMMA-S	9.6-340.2	7	95	106	101	107	4	7	4	6
HMMA	2.7-340.2	8	100	94	94	99	3	6	6	5
MDA	8.2-340.2	6	97	99	100	100	2	5	3	2
MDEA	5.4-340.2	7	97	99	97	98	4	5	4	3
MDMA	20.6-2574.6	8	96	99	98	98	3	10	4	10

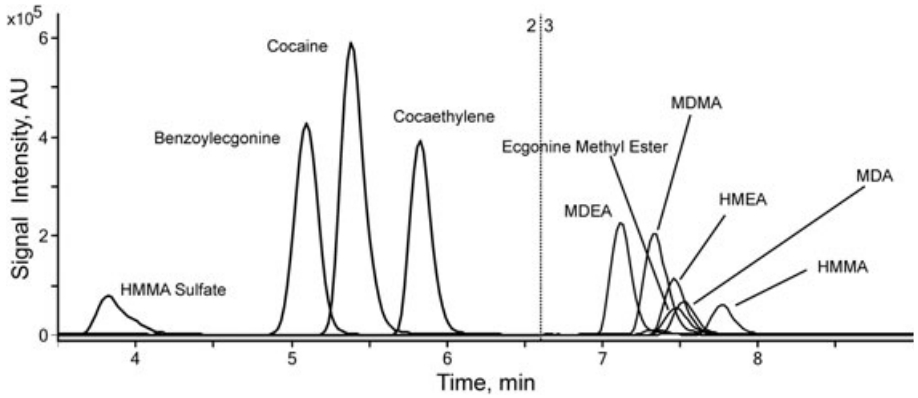

Figure 4. Reconstructed *m/z* chromatograms for the quantifier transitions of BE and MDMA from medium level quality control sample, the rest from high level quality control sample. The line at 6.6 min indicates change in segments.

on concentration estimates from screening of PU and US. Concentrations of MDEA and HMEA were lower than the calibrated range for all incubations.

Sorption of COC, BE, and MDMA to solid particulate matter is less than 5%.[29] Sorption was thus not investigated in the present study, as the more hydrophilic excretion products are expected to adsorb in the same order of magnitude or lower.

A variance analysis of three isolated steps was performed. Biological, sample preparation, and analytical variances from the study are summarized in Table 4. Generally, sample preparation and analytical variance were highest for the two analytes not having deuterated internal standards, i.e., HMMA-S and HMMA.

The confidence values of the stability after 24 h were higher than after 8 h for all investigated compounds. The most pronounced confidence values were from the MDMA metabolites. This variance is likely from the effect of having different absolute and relative levels of excretion products in the incubations.

Stability data after 8 and 24 h are shown in Table 4 and calculated as the percentage remaining relative to the concentration at t0. Variance is given as confidence value at confidence level 0.05, when $n > 3$. Figure 5 shows stability curves over 24 h for the investigated metabolites as average of start concentration in percentage. After 8 h of incubation, COC and EME showed a medium stability, whereas BE and CE had a high stability. After 24 h COC and EME only had one sample in the calibrated range, respectively; BE and CE had a medium stability after 24 h.

Previous stability studies found spiked BE to be stable over 24 h, and spiked EME and COC over 8 h.[5,11] Another study found spiked COC to be stable for 24 h.[12] Spiked CE was found to be stable for 24 h,[12] which is in contrast to the findings of the current study. Different experimental conditions of the stability studies might explain such findings. However, overall findings in the present study were in line with previous findings for investigated COC excretion products.[5,11,12]

Spiked MDMA has a high stability in wastewater[5,11,12]; this was also observed in the present study using urine. Urinary MDA has a marked increase in variation and it should be noted MDA was only within the calibrated range in four of the eight incubations. MDA is one of the compounds where the developed method performs best, based on low variances and high precision and accuracy from the method validation. An F-test comparing normalized biological variance ($n = 3$) for MDA after 24 h with sample preparation variance ($n = 5$) shows that the biological and sample preparation variance are unequal ($\alpha = 0.05$, $\delta_{MDA,BioVar\ t24h} \neq \delta_{MDA,SPVar}$, $F = 17.1$, $p = 0.006$). An F-test comparing the normalized variance from the incubations ($n = 4$) for MDA after 24 h to the biological variance ($n = 3$) after 24 h shows the incubation variance to be unequal to the biological variance ($\alpha = 0.05$, $\delta_{MDA,IncVar,\ 24h} \neq \delta_{MDA,BioVar\ t24h}$,

Table 4. Incubation data: Incubation range in ng/mL. Stability in average of n samples and at the given time point expressed in a percentage of the original concentration remaining, confidence interval at $\alpha = 0.05$. Variance data as standard deviation on normalized data ($\mu = 1$). Biological Variance from IncA, Sample preparation variance and analytical variance from IncC for MDMA excretion products, and IncG for COC excretion products. NA = not available

	Incubation Range		Stability						Variance					
			8 h			24 h			Biological variance				Sample prep variance	Analytical variance
	Min	Max	Average, %	CV	n	Average, %	CV	n	Variance	3 h (n = 3)	12 h (n = 3)	24 h (n = 3)	(n = 5)	(n = 5)
HMMA-S	<LLOQ	177.8	99	14	6	84	17	6	0.259	0.070	0.029	0.029	0.100	0.084
BE	10.2	730.7	88	5	8	78	8	8	0.142	NA	NA	0.014	0.066	0.020
COC	<LLOQ	27.5	74	NA	2	19	NA	1	NA	NA	NA	NA	0.026	0.029
CE	<LLOQ	57.0	88	3	4	56	12	4	0.083	NA	NA	NA	0.048	0.076
EME	<LLOQ	18.7	67	12	3	46	NA	1	NA	NA	NA	NA	0.022	0.024
HMMA	<LLOQ	102.3	102	17	7	107	27	7	0.339	0.027	0.025	0.123	0.061	0.091
MDA	<LLOQ	35.2	85	10	4	103	46	4	0.454	0.084	0.035	0.062	0.007	0.032
MDMA	<LLOQ	433.5	96	2	7	85	4	7	0.075	0.067	0.024	0.029	0.027	0.012

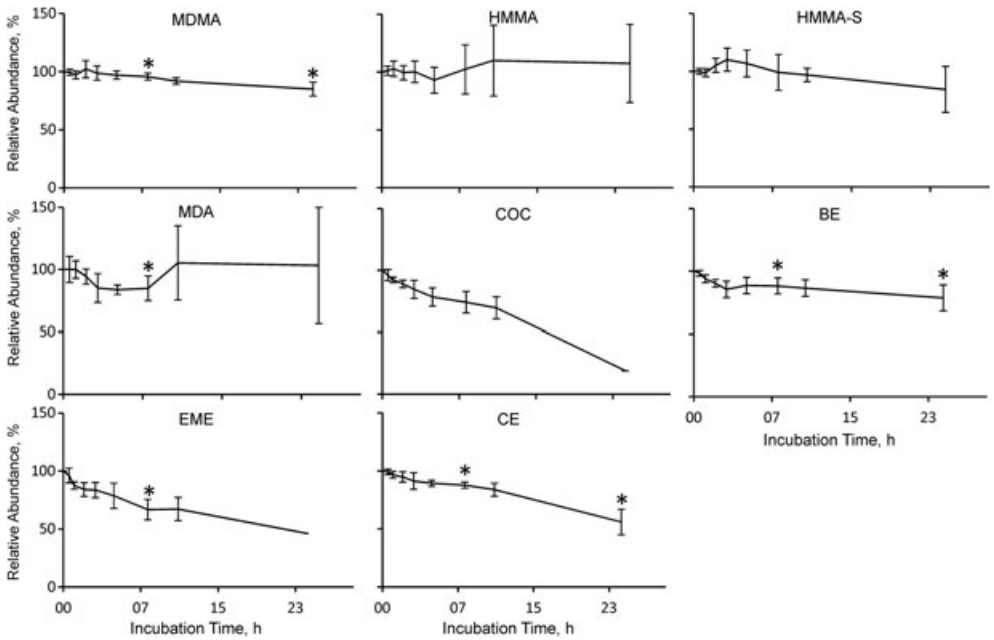

Figure 5. Stability curves showing normalized averages in a percentage of the original concentration remaining, against incubation time in h. Error bars show confidence value (when $n > 3$, $\alpha = 0.05$). Stars indicate confidence intervals below 100%, thus a significant decrease.

$F = 168.9$, $p = 0.006$). The increased variation could be caused by different biotransformation pathways, such as *N*-demethylation of MDMA and *O*-demethylation to 3,4-dihydroxyamphetamine, as it was observed for MDPV.[4] Previous studies have found spiked MDA to be stable in wastewater.[12] Further studies should be encouraged for MDA to allow a better understanding of what happens with urinary MDA in wastewater, preferably using urine from MDA-only users.

Investigated MDMA excretion products have been shown to be stable for at least three freeze-thaw cycles in urine as well for six months at -20 °C.[30] However, conjugate cleaving during pre-concentration might have occurred in the present study. The average concentration of HMMA in the samples after 24 h was 107 ± 27% ($\alpha = 0.05$, $n = 7$). The HMMA increase could be explained by cleaving of the corresponding phase II metabolites or by biotransformation of MDMA and 3,4-dihydroxymethamphetamine into HMMA. The increase is however not significant due to the large variation. The pathway *O*-demethylenylation with subsequent *O*-methylation could also be a pathway relevant for MDMA, as it shares the methylenedioxy moiety with MDPV.[4]

HMMA-S showed a high stability and cleaving of the sulfate (and possibly glucuronide) would be of importance to use HMMA as a wastewater target for assessing consumption of MDMA. Stability of HMMA-S corresponds to findings by Gomes *et al.,* who found conjugated estrogen sulfates to have a high stability for 8 h of incubation in wastewater.[14] In contrast, estrogen glucuronides incubated in wastewater were completely deconjugated after 8 h of incubation in wastewater at 17 °C; furthermore, the opioid metabolite, morphine-3-glucuronide, has been shown to be unstable in wastewater after 24 h.[12,13] HMMA conjugate cleavage might be based on acidic hydrolysis, as previous studies have shown urinary HMMA concentrations to increase more than 30-fold after acidic hydrolysis of 1 mL urine at 160 °C for 20 min.[31] As wastewater analysis requires volumes in the range 50–100 mL per sample, acidic hydrolysis under elevated temperatures should be worth investigating in the future.

Conclusions

Screening of pooled urine and urinated soil samples showed COC and MDMA to be the main consumed illicit drugs in the contributing festival population. A HILIC-MS/MS method was developed and validated to assess the stability of COC, MDMA, and MDEA excretion products. Assessing the stability of targets for illicit drug consumption with urinary excretion products should give a better understanding on how transformations in the sewer system affects concentrations of investigated targets. For the first time, HMMA and HMMA-S were both shown to be stable in wastewater after 8 and 24 h of incubation. HMMA should thus be a suitable biomarker to monitor MDMA consumption in WBE studies. Further studies should investigate conjugate cleavage of HMMA-S and glucuronide and the detectability of HMMA in real wastewater samples.

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