Drug Testing and Analysis

WILEY

RESEARCH ARTICLE OPEN ACCESS

Studies on the Stability and Microbial Biotransformation of Five Deschloroketamine Derivatives as Prerequisite for Wastewater-Based Epidemiology Screening

Fabian Frankenfeld | Lea Wagmann | Markus R. Meyer

Department of Experimental and Clinical Toxicology and Pharmacology, Institute of Experimental and Clinical Pharmacology and Toxicology, Center for Molecular Signaling (PZMS), Saarland University, Homburg, Germany

Correspondence: Markus R. Meyer (m.r.meyer@mx.uni-saarland.de)

Received: 25 October 2024 | **Revised:** 25 November 2024 | **Accepted:** 25 November 2024

Keywords: deschloroketamines | LC-HRMS/MS | microbial biotransformation | new psychoactive substances | wastewater

ABSTRACT

Wastewater (WW)-based epidemiology (WBE) is a powerful tool for screening and surveillance of drugs (of abuse) or new psychoactive substances (NPSs) in larger population. Since the drug market changes frequently, it is crucial for WBE to define screening and surveillance biomarkers considering drug metabolism and (microbial) stability. The aims of the presented work were first to identify metabolites, potentially serving as a WBE biomarker of five deschloroketamine derivatives (DCKDs) in rat feces samples after oral administration in addition to already known urinary metabolites, and second to elucidate the microbial biotransformation and WW stability of five DCKDs and their metabolites detected in urine and feces. Microbial biotransformation and stability of DCKD and their metabolites in WW were assessed by incubating parent compounds at 0.1 mg/L or rat urine or rat feces samples in freshly collected, untreated, influent WW over a period of 24 h. All samples were analyzed using liquid chromatography–high-resolution tandem mass spectrometry. All parent compounds, seven Phase I, and one Phase II metabolite were detected in rat feces samples. After WW incubations, all tested DCKD and their metabolites were still detectable at least in trace amounts, but particularly, peak areas of the Phase II *N*- and *O*-glucuronides showed a markable decrease. This is in line with previous findings where Phase II conjugates were identified to be unstable in WW and thus not recommended as a WW biomarker. Hence, incubations demonstrated that the five DCKD and most of their metabolites were sufficiently stable in WW influent and can thus be used as analytical targets in the context of WBE.

1 | Introduction

Wastewater (WW)-based epidemiology (WBE) is used for population surveillance concerning their consumption of drugs, drugs of abuse, and new psychoactive substance (NPS) [1]. WBE provides the means to capture spatial, short- and long-term trends in a population without sampling individual patients [2, 3]. Furthermore, there are no limitations concerning underreporting or personal bias from participants as already observed for (online) surveys on drug use, which are a cheap and easy alternative to WBE [4, 5]. To date, a multitude of studies have been published in which WBE has been used to monitor drug consumption, e.g., the annual European-wide investigation by the sewage analysis CORe group-Europe (SCORE), which was first published in 2011 [6]. However, there are challenges in the development of bioanalytical methods used for such studies, including finding suitable biomarkers for monitoring drugs (of abuse) or NPS. Such biomarkers should fulfill various requirements. First, they should be specific for human consumption and allow to distinguish between actual consumption and disposal into WW [1, 5]. Second, they should not adsorb to the sewer line or filter materials used for sample (pre-) treatment.

Third, biomarkers should be stable in WW (in-sewer stability), which includes hydrolytic stability and stability against biotransformation via microorganisms [5, 7]. Stability data of NPS and their biomarkers in WW are still scarce and need to be assessed by incubating them and their metabolites in WW or activated sludge [8–11].

Ketamine-derived NPSs are emerging on the drug market, with deschloro-*N*-ethyl-ketamine (2-Oxo-PCE) first reported in France in 2016 and deschloroketamine (2-Oxo-PCMe) first reported in the United Kingdom in 2015 [12, 13]. 2-Oxo-PCMe and 2-Oxo-PCE were already identified in human biosamples [14–17]. Though their metabolism and the metabolism of further deschloroketamine derivatives (DCKDs) were partially described in literature, no data concerning their excretion via feces in addition to urine after intake or their stability in WW are available [15, 18, 19]. Hence, this study aimed to investigate the excretion of the five DCKDs—deschloro-*N*-cyclopropyl-ketamine (2-Oxo-PCcP), 2-Oxo-PCE, deschloro-*N*-isopropyl-ketamine (2-Oxo-PCiP), 2-Oxo-PCMe, and deschloro-*N*-propyl-ketamine (2-Oxo-PCPr)—and their metabolites in rat feces samples after oral administration. The microbial biotransformation and WW stability of these DCKDs should be assessed by incubating parent solutions and DCKD metabolites in rat urine and feces samples, obtained after oral administration, using influent WW. Furthermore, suitable biomarkers for future WBE studies should be proposed.

2 | Materials and Methods

2.1 | Reagents and Materials

2-Oxo-PCcP hydrochloride, 2-Oxo-PCE hydrochloride, 2-Oxo-PCiP hydrochloride, 2-Oxo-PCMe hydrochloride, 2-Oxo-PCPr, rat urines, and feces were available from a previous study [15]. Acetonitrile (ACN), ammonium formate, hydrochloric acid (HCl), isopropanol, methanol (MeOH), sodium azide (NaN_3), and all other chemicals were obtained from VWR International GmbH (Darmstadt, Germany). Trimipramine-d3 and ketamine were obtained from LGC Standards GmbH (Wesel, Germany). Water was purified with a Millipore (Merck, Darmstadt, Germany) filtration unit, which purifies water to a resistance of 18.2 Ω × cm. Isolute HCX solid-phase extraction (SPE) cartridges (130 mg, 3 mL) were obtained from Biotage (Uppsala, Sweden). Fresh, untreated, influent WW was obtained from the local WW treatment plant on days without prior rainfalls and directly used for incubations.

2.2 | Pretest Blank Incubations of DCKDs

Blank incubations of parent compounds were performed according to a previously published procedure [11] with minor changes as follows. DCKD parent solutions (0.1 mg/L) were incubated in purified water for 24 h at 22°C. The final volume at the start of each incubation was 4 mL. After 0 and 24 h, an aliquot (2 mL) of each sample was transferred into another reaction tube, and 20 μL each of trimipramine-d3 as the internal standard (IS, 0.1 mg/L) and freshly prepared NaN_3 (0.2%, *w/v*) were added. Samples were then vortexed and centrifuged for 5 min at 18,407 × *g*. Supernatants (100 μL) were transferred into autosampler vials, and 10 μL was injected onto the LC-HRMS/MS system. All concentrations are final concentrations; experiments were performed with 10 replicates per parent compound.

2.3 | WW Incubations of DCKDs

Incubations of parent compounds were performed according to a previously published procedure [11] with minor changes as follows. DCKD parent solutions (0.1 mg/L) were incubated in freshly collected, untreated, influent WW for 24 h at 22°C. The final volume at the start of each incubation was 4 mL. After 0 and 24 h, an aliquot (2 mL) of each sample was transferred into another reaction tube, and 20 μL each of trimipramine-d3, ketamine (0.1 mg/L each, IS), and a freshly prepared NaN_3 solution (0.2%, *w/v*) were added. Samples were then vortexed and centrifuged for 5 min at 18,407 × *g*. Supernatants (100 μL) were transferred into autosampler vials and 10 μL and injected onto the LC-HRMS/MS system. Control incubations were performed by addition of NaN_3 (0.2%, *w/v*) at the start of the incubation, blank incubations were performed using purified water instead of WW. All concentrations are final concentrations, and all experiments were performed in triplicates.

2.4 | WW Incubation of Rat Urines After Administration of DCKDs

Incubations of rat urine samples after administration of DCKD were performed according to previously published procedures [1, 8] with minor changes as follows. Preconcentration of rat urine samples was done by the addition of 1 mL of MeOH to 1 mL of rat urine, vortexing, centrifugation at 18,407 × *g* for 5 min, transfer of supernatants (0.9 mL) into another reaction tube, and evaporation to dryness under a gentle stream of nitrogen at 70°C. Samples were then reconstituted in 50 μL of WW or purified water (blank incubations). Preconcentrated rat urines were incubated in freshly collected, untreated, influent WW for 24 h at 22°C. The final volume at the start of each incubation was 4 mL. After 0 and 24 h, an aliquot (2 mL) of each sample was transferred into another reaction tube, and 20 μL each of trimipramine-d3, ketamine (0.1 mg/L each, IS), and a freshly prepared NaN_3 solution (0.2%, *w/v*) were added. Samples were then vortexed and centrifuged for 5 min at 18,407 × *g*. Supernatants (100 μL) were transferred into autosampler vials, and 10 μL was injected onto the LC-HRMS/MS system. Control incubations were performed by the addition of NaN_3 (0.2%, *w/v*) at the start of the incubation, and blank incubations were performed using purified water instead of WW. All concentrations are final concentrations, and all experiments were performed in triplicates.

2.5 | Feces Extraction for the Detection and Identification of DCKDs and Their Metabolites

Based on a previous work by López-Rabuñal et al. [20], 0.25 g of feces was weighed into reaction tubes. Then, 10 μL of each trimipramine-d3 and ketamine as IS (0.1 mg/L) and 1.5-mL MeOH were added. Samples were homogenized via ultrasonification for 30 min at room temperature and centrifuged for 5 min at

18,407×*g*. The supernatants were evaporated to dryness under a gentle stream of nitrogen at 70°C, and samples were reconstituted using 1 mL of purified water. Samples were then extracted via SPE according to a previously published procedure [21] with minor modifications as follows. Conditioning of HCX cartridges was performed with 1 mL of MeOH and 1 mL of purified water. Then, cartridges were loaded with 1 mL of samples, followed by washing using 1 mL of purified water, 1 mL of HCl (0.1 M), and 2 mL of MeOH. Elution was performed using 1 mL of a MeOH/NH_3 mixture (35%, 98/2, *v/v*). Eluates were evaporated to dryness under a gentle stream of nitrogen at 70°C, and residues were reconstituted in 50 μL of MeOH. A 10 μL aliquot of each sample was injected onto the LC-HRMS/MS system. Rat feces samples could only be extracted once per administered DCKD due to limited amounts of feces available.

2.6 | WW Incubation of Rat Feces After Administration of DCKDs

Incubations of rat feces samples after administration of DCKD were performed according to previously published procedures [1, 8] with minor changes as follows. Preconcentration of rat feces samples was done by the addition of 1.5 mL of MeOH to 0.4 g of rat feces, ultra sonification for 30 min, centrifugation at 18,407×*g* for 5 min, transfer of supernatants into another reaction tube, evaporation to dryness under a gentle stream of nitrogen at 70°C, and reconstitution in 50 μL of WW or purified water (only used for blank incubations). Rat feces extracts were then incubated in freshly collected, untreated, influent WW for 24 h at 22°C. The final volume at the start of each incubation was 4 mL. After 0 and 24 h, an aliquot (2 mL) of each sample was transferred into another reaction tube, and 20 μL each of trimipramine-d3, ketamine (0.1 mg/L each, IS), and a freshly prepared NaN_3 solution (0.2%, *w/v*) were added. Samples were then vortexed and centrifuged for 5 min at 18,407×*g*. Supernatants (100 μL) were transferred into autosampler vials, and 10 μL was injected onto the LC-HRMS/MS system. Control incubations were performed by the addition of NaN_3 (0.2%, *w/v*) at the start of the incubation, and blank incubations were performed using purified water instead of WW. All concentrations are final concentrations; experiments were performed in triplicates.

2.7 | Instrumental Settings

Based on a previous study [15], a Thermo Fisher Scientific (TF, Dreieich, Germany) Dionex UltiMate 3000 consisting of a degasser, a quaternary pump, a DL W2 wash system, and a HCT PAL autosampler (CTC Analytics AG, Zwinger, Switzerland). The system was coupled to a TF Q Exactive orbitrap mass spectrometer, equipped with a heated electrospray ionization II source (HESI-II). According to the manufacturer's recommendations, calibration was performed prior to analysis using external mass calibration. The conditions of the LC system were as follows: TF Accucore Phenyl Hexyl column (100×2.1 mm ID, 2.6 μm particle size) and gradient elution eluents A and B. The flow rate was set as follows: 0–11.5 min at 0.500 mL/min, 11.5–13.5 min at 0.800 mL/min with the following gradient settings: 0–1.0 min hold 1% B, 1–10 min to 99% B, 10–11.5 min hold 99% B, and 11.5–13.5 min hold 1% B. The HESI-II source conditions were as follows: ionization mode: positive; heater temperature, 320°C; ion transfer capillary temperature, 320°C, ionization mode, positive; sheath gas, 60 arbitrary units (AU); auxiliary gas, 10 AU; spray voltage, 4.00 kV; and S lens RF level, 60.0. Mass spectrometry experiments for identification of metabolites in rat feces were performed using full scan mode and data-dependent acquisition (DDA) with an inclusion list containing the masses of the expected metabolites. For full data scan, the settings were as follows: resolution, 35,000 at *m/z* 200; automatic gain control (AGC) target, 1e6; maximum injection time (IT), 120 ms; and scan ranges: *m/z* 130–530. The settings for the DDA with the respective inclusion lists were as follows: resolution, 17,000 at *m/z* 200; AGC target, 2e5; maximum IT, 250 ms; loop count, 5; isolation window, 1.0 *m/z*; stepped normalized collision energy, 17.5%, 35%, 52.5%; and pick others, enabled. The HESI-II source settings and mass spectrometry experiments for the microbial stability experiments were the same as for the identification of metabolites in feces. A separate inclusion list containing masses of parent compounds and metabolites previously identified in urine [15] or feces was used. ChemSketch 2018 2.1 (ACD/Labs, Toronto, Canada) was used to draw the structures of the hypothetical metabolites and for the calculations of the exact masses. Xcalibur Qual Browser version 4.1.31.9 (TF, Dreieich, Germany) was used for data handling. Two-sided *t*-tests (TOST) were performed using MS Excel version 2408 (Microsoft, Redmond, USA).

3 | Results and Discussion

3.1 | Pretest Blank Incubations for the Stability Assessment of DCKDs

To test for the number of replicates required for WW incubations of DCKD and whether normalization of the obtained peak areas to an IS might be necessary, a pretest using blank incubations with purified water was performed. Differences in peak areas between t_{0h} and t_{24h}, of DCKD in purified water, and their coefficients of variation (CV) are listed in Table S1. Chemical structures of parent compounds detected in this study are given in Figures S1–S5. Negative values indicate a decrease in peak area; positive values indicate an increase in peak area of the respective compound over 24 h. Values and CVs obtained without normalization to an IS showed changes in peak areas between −4.6% and 3.5%, and CVs below 10%. Normalization of DCKD peak areas to an IS led to values between 20% and 54%, with CVs between 27% and 36%. Furthermore, changes in peak areas of five DCKDs were also calculated for $n = 3$ using three randomly selected sample sets of each DCKD from the initial experiment carried out with $n = 10$. Calculations were performed without and with IS normalization. DCKD peak area changes varied between −1.1% and 7.2%, (CVs were below 10%) without IS normalization, compared to increases in peak areas between 5% and 54% (CVs between 5% and 50%) with IS normalization. High CVs using the IS normalization can be due to high variations in IS areas, e.g., resulting from fluctuations during the LC-HRMS/MS analysis. As CVs of DCKD areas were below 10% for $n = 10$ and $n = 3$ and IS-normalized values showed considerably higher CVs, normalization was not considered as beneficial for the analysis. Thus, all further experiments were performed in triplicates, and calculations were performed

without IS normalization. Nevertheless, IS was added in further experiments to check the performance of the LC-HRMS/MS system. Observed, minor but not significant differences in peak areas of DCKD over 24 h during blank incubations could be explained by fluctuations during the LC-HRMS/MS analysis. Incubations were performed over 24 h since the residence time of WW in sewer systems can vary between different catchments or within the same system [22]. A study by Ort et al. showed residence times between 1 and 12 h for WW collected in different European cities [3]. The 24 h period was then chosen to account for very long WW residence times.

3.2 | WW Stability and Studies on Microbial Biotransformation of DCKDs

Stability of DCKD, given as differences in peak areas between t_{0h} and t_{24h} after WW, control, and blank incubations of parent compound solutions, is given in Figure 1 and is listed in Table S2 including their CVs and results of a TOST comparing WW and control incubations. Chemical structures of parent compounds detected after WW incubations of parent compound solutions are given in Figures S1–S5. Peak area differences were between 0.4% and 6.4% (WW incubations), −3.3% and 1.2% (control incubations), and −2.6% and 8.2% (blank incubations). CVs ranged between 1.7 and 8.8, indicating reproducible results for all investigated compounds. *p* values obtained after a TOST comparing WW and control incubations were above 0.05 for all compounds except 2-Oxo-PCPr. As no significant differences in peak areas between WW and control incubations were observed for 2-Oxo-PCcP, 2-Oxo-PCE, 2-Oxo-PCiP, and 2-Oxo-PCMe and values were comparable with those obtained in the blank incubations, these analytes could be considered as stable under the tested WW conditions. 2-Oxo-PCPr showed a significant difference between WW and control incubations in the TOST. However, peak areas showed only a minor increase and were comparable to those obtained in the blank incubation (Section 3.1). Furthermore, the observed increase in peak areas of 2-Oxo-PCPr might be attributed to variations during the analysis, which only led to minor differences in peak areas between both sample sets (t_{0h} and t_{24h}). Hence, 2-Oxo-PCPr could also be used as a potential screening target in WW. WW incubations were also screened for metabolites or degradation products originating from microbial biotransformation of the DCKD. Such products might include cleavage of Phase II metabolites (leading to the aglycons) or molecules originating from reductive metabolism (i.e., phenols originating from a reduced keto function). No metabolites or degradation products could be identified for any of the five DCKDs indicating their stability. WW used for this study consisted of freshly collected, untreated influent WW grab samples. In general, it should be noted that the composition of WW samples always differs depending on the sewer site [23]. Furthermore, the microbial composition of WW is complex and varies due to location and time [24]. Hence, using WW collected at other locations, i.e., in larger cities, at WW treatment plants located near industry, or in smaller communities could lead to differences in the observed degradation of parent compounds or metabolites.

3.3 | WW Stability of DCKD Metabolites Obtained From Rat Urine

Stability of DCKD and their metabolites, given as differences in peak areas between t_{0h} and t_{24h} after WW, control, and blank incubations of rat urines after oral administration of DCKD listed in Table S3 including their CVs and results of a TOST. Figure 2 shows results exemplified for 2-Oxo-PCE. Experiments were performed with rat urine samples after oral administration of DCKD, as no reference compounds of the previously identified DCKD metabolites were available. IDs of DCKD metabolites used in the following and Supporting Information S1 are the same as described in a previous publication; their chemical structures are given in their respective metabolic pathways (Figures S1–S5) [15]. DCKD metabolites already described in the previous publication but not listed in this study were not detected after WW, control, or blank incubations, which was most likely due to the dilution of these already in rat urine low–abundant metabolites. Those metabolites are also not included in Figures S1–S5.

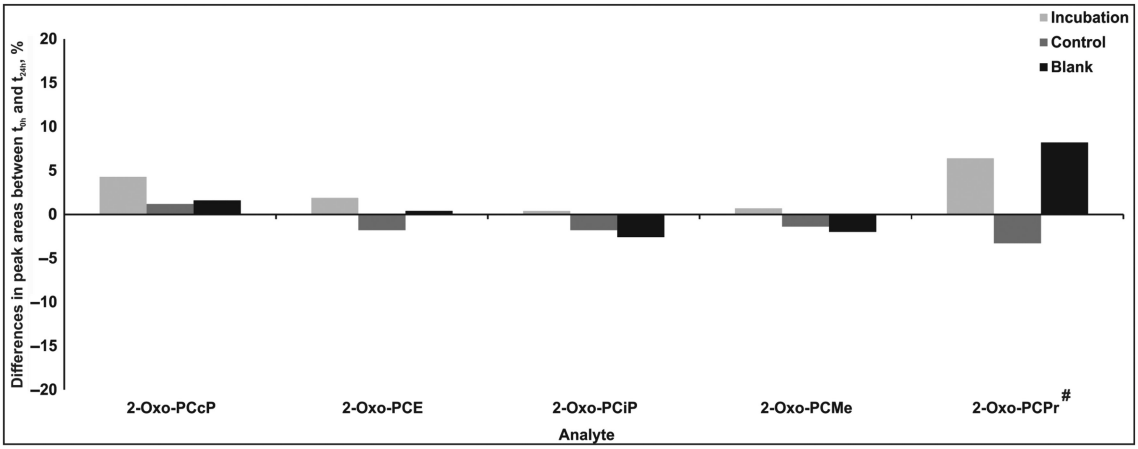

FIGURE 1 | Stability of deschloroketamine derivatives, as pure substances, in wastewater, control, and blank incubations over 24 h. Stability values are given as differences in peak areas between t_{0h} and t_{24h}, negative values indicating a decrease in peak area, positive values indicating an increase in peak area. 2-Oxo-PCcP, deschloro-*N*-cyclopropyl-ketamine; 2-Oxo-PCE, deschloro-*N*-ethyl-ketamine; 2-Oxo-PCiP, deschloro-*N*-isopropyl-ketamine; 2-Oxo-PCMe, deschloroketamine; 2-Oxo-PCPr, deschloro-*N*-propyl-ketamine; #, $p < 0.05$ in the two-sided *t*-test comparing incubation and control.

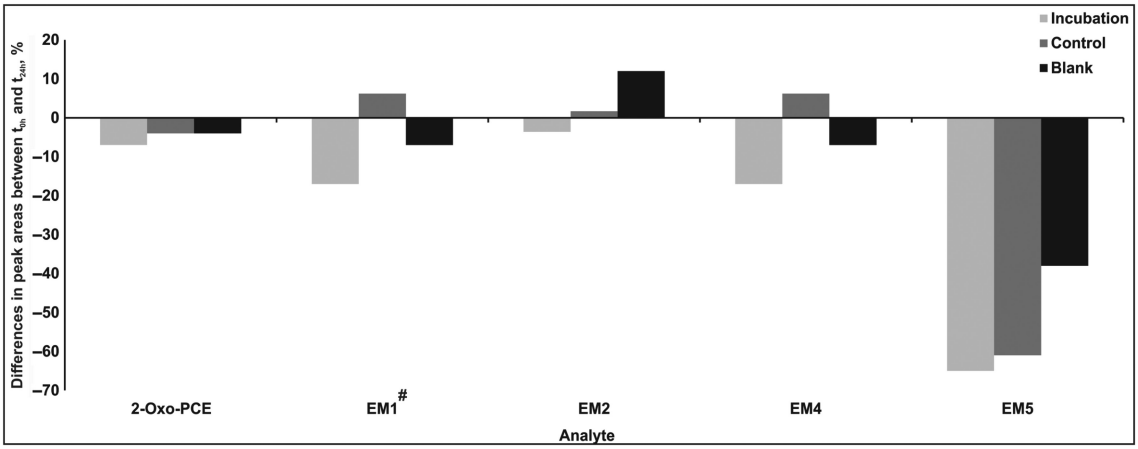

FIGURE 2 | Stability of deschloroketamine derivatives, and their metabolites obtained from rat urine, after wastewater, control, and blank incubations over 24 h, exemplified for 2-Oxo-PCE. Stability values are given as differences in peak areas between t_{0h} and t_{24h}, negative values indicating a decrease in peak area, positive values indicating an increase in peak area. 2-Oxo-PCE, deschloro-*N*-ethyl-ketamine; EM, 2-Oxo-PCE metabolite; EM1, *N*-dealkylation; EM2, hydroxylation + oxidation to a ketone; EM4, hydroxylation isomer 2; EM5, *N*-dealkylation + glucuronidation; #, $p < 0.05$ in the two-sided *t*-test comparing incubation and control.

2-Oxo-PCcP showed an increased peak area after WW incubations (55%) of rat urines with a high CV (23%), compared to a modest 7% increase (CV 4.2%) in the control and a minor −0.8% decrease (CV 20%) in the blank incubations. Such an increase of the parent compound might be explained by unspecific reductive microbial biotransformation of several hydroxy metabolites [25]. Metabolites CM1 (*N*-dealkylation), CM2 (*N*-dealkylation + acetylation), and CM5 (*N*-dealkylation + glucuronidation) showed reduced peak areas after 24-h WW incubations. Peak areas of metabolite CM4 (hydroxylation isomer 2) were increased by 600% in WW incubations (CV 14%), compared to a high increase of 520% in control incubations (CV 13%) and a slight −14% decrease in blank incubations (CV 2%). A decrease in peak areas of glucuronides was expected since glucuronidated compounds showed to be instable in WW [26, 27]. Hence, an increase in peak areas of the corresponding Phase I metabolite (CM1) might also be expected. As both compounds showed decreased peak areas after WW incubations, a degradation of CM1 was assumed. The observed decrease of CM5 in control incubations might be explained by residual activity of microorganisms in control incubations. This was also described in the literature where NaN_3 was used to deactivate microorganisms in WW during stability studies on several drugs of abuse [28]. A TOST comparison of WW incubations and control incubations revealed that *p* values obtained for CM1, CM4, and CM5 were below 0.05, indicating a significant difference between both sets. Considering the observed peak area changes after 24 h, CM1 and CM5 were assumed to be instable in WW. As no major decreases in WW were observed for the parent compound, CM2 and CM4 could be assumed as stable in the tested conditions.

2-Oxo-PCE showed minor decreased peak areas after WW incubations of rat urine (−7%, CV 2.5%, see Figure 2). The obtained values were only slightly below those obtained after control (−4%, CV 6%) and blank incubations (−4%, CV 4%). For Phase I metabolites, EM1 (*N*-dealkylation, −17%), EM2 (hydroxylation + oxidation to a ketone, −3.6%), and EM4 (hydroxylation isomer 2, 3%) also only slight changes in peak areas were observed. The peak area of Phase II metabolite EM5 (*N*-dealkylation + glucuronidation) showed a major decrease of −65%, which was in line with the results obtained for CM5 as already discussed above. Furthermore, peak areas in control and blank incubations showed lower peak areas after 24 h, which could indicate a general instability of this analyte. CVs of all incubations were below 15%, except the control incubation of EM5 (32%). According to a TOST, the only significant difference between WW and control incubations was observed for EM1. As already mentioned before, slight changes in peak areas could be attributed to variations during the measurement, as minor changes in peak areas were also observed for parent compounds during blank incubations. Considering the obtained results, only EM5 was assumed to be instable in WW. However, it should be considered that *N*-dealkylation metabolites are not substance-specific for one of the studied DCKD and therefore only provide a general indication towards DCKD consumption. Thus, substance-specific metabolites should be used as biomarkers to allow for precise identification of the respective parent compound. This not only enables differentiation between the DCKD described in this study, but also potential further representatives of this class of NPS that might enter the ever-changing NPS market in the future.

2-Oxo-PCiP showed only minor changes (−3.6%, CV 10%) after WW incubations. Metabolites IM1 (*N*-dealkylation) and IM8 (*N*-dealkylation + glucuronidation) showed high decreases in peak areas of −35% (IM1, CV 19%) and −71% (IM8, CV 10%). This is in accordance with the results obtained for *N*-dealkylation and *N*-dealkylation + glucuronidation metabolites of 2-Oxo-PCcP and 2-Oxo-PCE. Peak areas of IM6 (hydroxylation isomer 3) were strongly increased (320%, CV 7.7%), compared to major decrease in peak areas of the corresponding Phase II glucuronide IM9 (−100%, CV 7.8%). The decrease of peak areas of glucuronides was already observed for glucuronides of 2-Oxo-PCcP and 2-Oxo-PCE and is in line with the literature on WW stability of glucuronides [26, 27]. Increased peak areas of IM6 might be due to the cleavage of its corresponding Phase II metabolite IM9. As

IM1, IM8, and IM9 showed a high degradation of peak areas after WW incubations, these metabolites were assumed unstable in WW. The TOST performed for all analytes showed no significant changes for IM8, which was due to also reduced peak areas in control incubations (−71%). Although the TOST performed for 2-Oxo-PCiP and IM6 indicated significant differences between WW and control incubations, the parent compound was assumed to be stable in WW as results of rat urine WW incubations were comparable to the prior experiments (Sections 3.1 and 3.2). IM6 also could be considered stable, since peak areas increased, possibly due to cleavage of IM9.

2-Oxo-PCMe showed increased peak areas after WW incubations of rat urines (21%, CV 3%). Metabolites MM1 (*N*-dealkylation), MM2 (*N*-dealkylation + hydroxylation), MM6 (hydroxylation isomer 2), and MM7 (*N*-dealkylation + acetylation) showed only slight differences in peak areas (between −7.5% and 20%) after 24-h WW incubations. Higher decreases in peak areas were observed for MM4 (hydroxylation isomer 1 + oxidation to a ketone, −28%) and MM8 (*N*-dealkylation + glucuronidation, −80%). The observed degradation of the *N*-glucuronide was in line with literature on glucuronides in WW [26, 27]. All CVs except for MM1 control incubation (17%) and MM7 WW incubation (22%) were below 15%. Results of a TOST showed significant differences between WW and control experiments for 2-Oxo-PCMe, MM4, MM6, and MM8. As MM4 and MM8 were the only analytes with highly reduced peak areas, those were assumed to be instable in WW; other metabolites and the parent compound were assumed to be stable and could be used as potential WW biomarkers. As already discussed above, slight changes in peak areas could be due to fluctuations during the LC-HRMS/MS analysis and should not indicate any instabilities. Furthermore, when considering a metabolite as a biomarker, only substance-specific metabolites should be used.

2-Oxo-PCPr and metabolites PM2 (hydroxylation isomer 1 + oxidation to a ketone), PM6 (dihydroxylation + monooxidation to a ketone), and PM7 (hydroxylation isomer 2 + oxidation to carboxylic acid) showed only minor decreases in peak areas (between −15% and −9.3%) with CVs below 15%. PM4 (hydroxylation isomer 2) showed increased peak areas of 1200% (CV 8.3%), which might be a result of the cleavage of PM11 (hydroxylation isomer 2 + glucuronidation, −100%). CVs of WW and control incubations of PM11 were high (73% and 91%), due to poor signal intensities. PM1 (*N*-dealkylation, −27%) and PM10 (*N*-dealkylation + glucuronidation, −61%) also showed a major decrease in peak areas. PM9 (dihydroxylation isomer 2, 170%) showed a high increase in its peak area, but since peak areas in its control incubations were also increased by 210%, results should not be considered for the stability evaluation. As already discussed, instabilities of glucuronides were in accordance with literature data [26, 27]. TOST showed no significant differences between WW and control incubations of PM2, PM6, and PM7 ($p > 0.05$). Hence, these metabolites were assumed to be stable in WW. The TOST performed for 2-Oxo-PCPr showed a *p* value below 0.05. Nevertheless, 2-Oxo-PCPr was considered stable under WW conditions, as a decrease of −9.3% was considered as only a minor change in peak area and the observed change in peak area could be due to variations during the analysis. Furthermore, MM1 and MM2 are not substance-specific; hence, their use as biomarkers is limited.

3.4 | Identification DCKDs and Their Metabolites in Rat Feces After Oral Administration of DCKDs

As WW does not only contains compounds and their metabolites excreted via urine but also those excreted via feces, the excretion pattern of the five DCKDs in rat feces was investigated. Parent compounds and metabolites detected in rat feces samples obtained after oral administration of five DCKDs are listed in Table S4. Chemical structures of parent compounds and metabolites detected in rat feces are given in their respective metabolic pathways (Figures S1–S5) [15]. Identification was performed as described in a previous publication [15]. Phase I metabolites included *N*-dealkylation (CM1, EM1, IM1, MM1, and PM1), hydroxylation (PM4), and hydroxylation with further oxidation to a carboxylic acid (PM7). The only Phase II metabolite was identified as *N*-dealkylation of 2-Oxo-PCMe with further glucuronidation (MM8). No additional metabolites to those already identified in rat urine were found.

3.5 | WW Stability of DCKD Metabolites Obtained From Rat Feces

Stability of the five DCKDs and their metabolites after WW feces incubation is given as differences in peak areas between t_{0h} and t_{24h} after WW, control, and blank incubations of DCKD and is listed in Table S4 including their CVs and results of a TOST. Chemical structures of parent compounds and metabolites detected after WW incubations of rat feces are given in their respective metabolic pathways (Figures S1–S5) [15]. Figure 3 shows differences in peak areas exemplified for 2-Oxo-PCPr and its metabolites. Experiments were performed with rat feces samples after oral administration of DCKD, as no reference compounds of the previously identified DCKD metabolites were available.

2-Oxo-PCcP, 2-Oxo-PCE, and 2-Oxo-PCMe showed increased peak areas ranging from 5.3% to 20%, with CVs below 15% for all except 2-Oxo-PCE (27%). Peak areas of 2-Oxo-PCiP (−7.8, CV 21%) and 2-Oxo-PCPr (−14%, CV 17%) were only slightly decreased. Differences in peak areas of *N*-dealkylation metabolites varied between different feces samples; CM1 and EM1 showed increased peak areas after WW incubations with 140% (CM1) and 130% (EM1); IM1 (−3.0%), MM1 (3.1%), and PM1 (37%) showed considerably smaller differences in peak areas. Furthermore, peak areas of PM4 (hydroxylation isomer 2, −2.2%) and PM7 (hydroxylation isomer 2 + oxidation to carboxylic acid, −17%) were also only slightly decreased. MM8 (*N*-dealkylation + glucuronidation), the only Phase II metabolite detected in this experiment, showed less degradation (−17%) compared to rat urine experiments (Section 3.3). Considering the differences in peak areas obtained for rat feces incubations, all DCKDs and most metabolites were assumed to be stable in WW. As already discussed above, slight differences in peak areas might be due to fluctuations during the analysis and do not necessarily reflect instabilities. High increases in peak areas of CM1 and EM1 could indicate that the respective Phase II metabolites were present at t_{0h}, but not detectable in WW, and then cleaved during the incubation. However, results from rat urines showed that degradation of *N*-dealkylation + glucuronidation metabolites did not result in higher peak areas of the corresponding Phase I metabolites. According to the TOST performed,

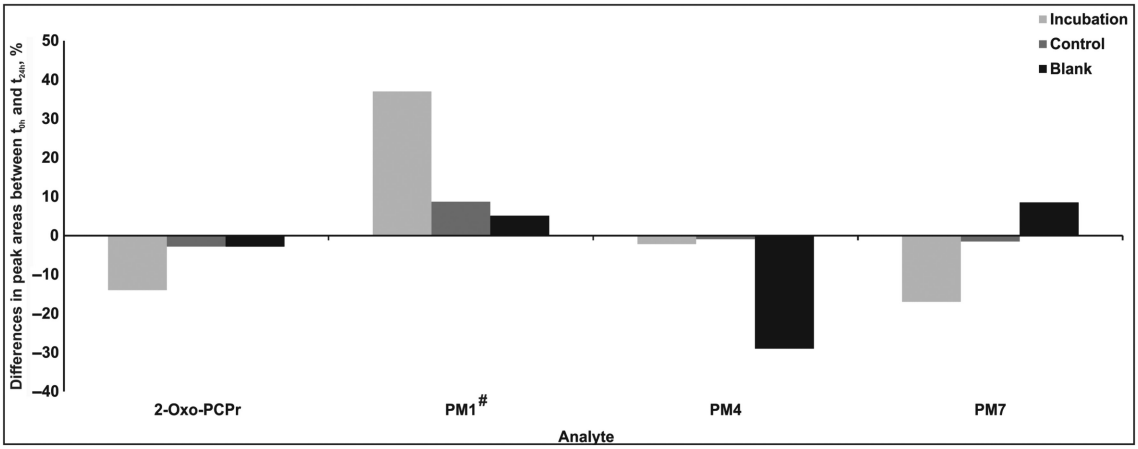

FIGURE 3 | Stability of deschloroketamine derivatives, and their metabolites obtained from rat feces, after wastewater, control, and blank incubations over 24 h, exemplified for 2-Oxo-PCPr. Stability values are given as differences in peak areas between t_{0h} and t_{24h}, negative values indicating a decrease in peak area, positive values indicating an increase in peak area. 2-Oxo-PCPr, deschloro-*N*-propyl-ketamine; PM, 2-Oxo-PCPr metabolite; PM1, *N*-dealkylation; PM4, hydroxylation isomer 2; PM7, hydroxylation isomer 2 + oxidation to a carboxylic acid; #, $p < 0.05$ in the two-sided *t*-test comparing incubation and control.

the only significant differences between WW and control incubations were observed for PM1. Considering the results obtained for glucuronides in Section 3.3. MM8 should not be proposed as a potential screening target. Furthermore, considering results obtained after WW incubation of feces samples, parent compounds, PM4, and PM7 are assumed to be stable in WW and could be used as potential biomarkers of DCKD in WW.

4 | Conclusions

Following WW incubations of DCKD and/or their metabolites as a parent compound solution, in rat urines and in rat feces, the DCKD parent compounds showed no analytically relevant decrease in peak areas, except for 2-Oxo-PCcP. Hence, all other DCKD could be suggested as potential screening targets in WW. However, only screening for parent compounds does not allow to discriminate between actual intake of the compounds and disposal. Thus, compound specific metabolites should be added to the screening procedure. Among them, most Phase I metabolites showed only slight differences in peak areas indicating their stability as screening targets as well. Notable exceptions here were observed for *N*-dealkylation metabolites CM1, IM1, and PM1 with higher decreases in peak areas. The use of *N*-dealkylation metabolites as screening targets is nevertheless limited due to their lack of specificity for a particular DCKD. Hydroxylation or hydroxylation + oxidation metabolites could be used as substance-specific targets in WW, mostly showing small decreases or increases in peak areas. Cleavage of their corresponding Phase II metabolites could lead to a (high) increase in peak areas, which must be considered when metabolites are used for quantification but do not negatively affect qualitative screenings. Phase II glucuronides were instable in WW, with the only exception being MM8 after rat feces incubations. Hence, glucuronides are not recommended as screening targets for DCKD in WW samples. Future studies should focus on the detectability of DCKD and its metabolites in real WW samples. It should also be considered as a limitation of this study that experiments performed only give indications concerning the stability of tested DCKD and metabolites. To further sharpen biomarker recommendation for DCKD, experiments are needed to assess whether, e.g., adhesion to sewer lines or filter materials during sample preparation can be observed.

Acknowledgments

The authors would like to thank the people at the WWTP, Anush Abelian, Jason Wallach, Adeboye Adejare, Simon D. Brand, Aline C. Vollmer, K. Simon Wellenberg, Juel Maalouli Schaar, Sascha K. Manier, Tanja M. Gampfer, Selina Hemmer, Matthias D. Kroesen, Tilman F. Arnst, Philip Schippers, Anna-Lena Gehl, Claudia Fecher-Trost, Gabriele Ulrich, Armin A. Weber, Carsten Schröder, and Selina Wolter for their support and/or helpful discussion. The graphical abstract was created with BioRender.com. Open Access funding enabled and organized by Projekt DEAL.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data may be made available upon reasonable request from the authors.

References

1. M. Mardal, J. Kinyua, P. Ramin, et al., "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," *Drug Testing and Analysis* 9, no. 1 (2017): 106–114, https://doi.org/10.1002/dta.1957.

2. M. R. Meyer, T. Vollerthun, and R. Hasselbach, "Prevalence and Distribution Patterns of Amphetamine and Methamphetamine Consumption in a Federal State in Southwestern Germany Using Wastewater Analysis," *Drug and Alcohol Dependence* 156 (2015): 311–314, https://doi.org/10.1016/j.drugalcdep.2015.09.006.

3. C. Ort, A. L. van Nuijs, J. D. Berset, et al., "Spatial Differences and Temporal Changes in Illicit Drug Use in Europe Quantified by Wastewater Analysis," *Addiction* 109, no. 8 (2014): 1338–1352, https://doi.org/10.1111/add.12570.

4. S. Castiglioni, E. Zuccato, C. Chiabrando, R. Fanelli, and R. Bagnati, "Mass Spectrometric Analysis of Illicit Drugs in Wastewater and Surface Water," *Mass Spectrometry Reviews* 27, no. 4 (2008): 378–394, https://doi.org/10.1002/mas.20168.

5. E. Gracia-Lor, S. Castiglioni, R. Bade, et al., "Measuring Biomarkers in Wastewater as a New Source of Epidemiological Information: Current State and Future Perspectives," *Environment International* 99 (2017): 131–150, https://doi.org/10.1016/j.envint.2016.12.016.

6. EUDA, "Wastewater Analysis and Drugs—A European Multi-City Study," 2024, https://www.emcdda.europa.eu/publications/html/pods/waste-water-analysis_en.

7. C. Chen, C. Kostakis, J. P. Gerber, B. J. Tscharke, R. J. Irvine, and J. M. White, "Towards Finding a Population Biomarker for Wastewater Epidemiology Studies," *Science of the Total Environment* 487 (2014): 621–628, https://doi.org/10.1016/j.scitotenv.2013.11.075.

8. M. Mardal and M. R. Meyer, "Studies on the Microbial Biotransformation of the Novel Psychoactive Substance Methylenedioxypyrovalerone (MDPV) in Wastewater by Means of Liquid Chromatography-High Resolution Mass Spectrometry/Mass Spectrometry," *Science of the Total Environment* 493 (2014): 588–595, https://doi.org/10.1016/j.scitotenv.2014.06.016.

9. A. Wick, M. Wagner, and T. A. Ternes, "Elucidation of the Transformation Pathway of the Opium Alkaloid Codeine in Biological Wastewater Treatment," *Environmental Science & Technology* 45, no. 8 (2011): 3374–3385, https://doi.org/10.1021/es103489x.

10. M. Mardal, M. Bischoff, M. Ibanez, U. Ruffing, F. Hernandez, and M. R. Meyer, "Microbial Biotransformation of Five Pyrrolidinophenone-Type Psychoactive Substances in Wastewater and a Wastewater Isolated Pseudomonas Putida Strain," *Drug Testing and Analysis* 9, no. 10 (2017): 1522–1536, https://doi.org/10.1002/dta.2165.

11. M. Mardal, B. Miserez, R. Bade, et al., "3-Fluorophenmetrazine, a Fluorinated Analogue of Phenmetrazine: Studies on In Vivo Metabolism in Rat and Human, In Vitro Metabolism in Human CYP Isoenzymes and Microbial Biotransformation in *Pseudomonas Putida* and Wastewater Using GC and LC Coupled to (HR)-MS Techniques," *Journal of Pharmaceutical and Biomedical Analysis* 128 (2016): 485–495, https://doi.org/10.1016/j.jpba.2016.06.011.

12. EMCDDA, "Europol 2016 Annual Report on the implementation of Council Decision 2005/387/JHA," accessed September 27, 2024, https://www.emcdda.europa.eu/system/files/publications/4724/TDAN17001ENN_PDFWEB.pdf2016.

13. EMCDDA, "Europol 2015 Annual Report on the implementation of Council Decision 2005/387/JHA," accessed September 27, 2024, https://www.emcdda.europa.eu/publications/implementation-reports/2015_en2015.

14. S. Riess, M. Cheze, A. Muckensturm, N. Klinger, O. Roussel, and V. Cirimele, "2-Fluorodeschloroketamine Consumption: About Two Deaths and a Case of Self-Mutilation," *Journal of Analytical Toxicology* 48, no. 5 (2024): 398–404, https://doi.org/10.1093/jat/bkae021.

15. F. Frankenfeld, L. Wagmann, A. Abelian, et al., "In Vivo and In Vitro Metabolic Fate and Urinary Detectability of Five Deschloroketamine Derivatives Studied by Means of Hyphenated Mass Spectrometry," *Metabolites* 14, no. 5 (2024): 270, https://doi.org/10.3390/metabo14050270.

16. N. Theofel, P. Moller, E. Vejmelka, et al., "A Fatal Case Involving *N*-Ethyldeschloroketamine (2-Oxo-PCE) and Venlafaxine," *Journal of Analytical Toxicology* 43, no. 2 (2019): e2–e6, https://doi.org/10.1093/jat/bky063.

17. M. H. Y. Tang, Y. K. Chong, C. Y. Chan, et al., "Cluster of Acute Poisonings Associated With an Emerging Ketamine Analogue, 2-Oxo-PCE," *Forensic Science International* 290 (2018): 238–243, https://doi.org/10.1016/j.forsciint.2018.07.014.

18. K. Hajkova, B. Jurasek, J. Cejka, et al., "Synthesis and Identification of Deschloroketamine Metabolites in Rats' Urine and a Quantification Method for Deschloroketamine and Metabolites in Rats' Serum and Brain Tissue Using Liquid Chromatography Tandem Mass Spectrometry," *Drug Testing and Analysis* 12, no. 3 (2020): 343–360, https://doi.org/10.1002/dta.2726.

19. I. A. Larabi, F. Zerizer, A. Ameline, et al., "Metabolic Profiling of Deschloro-*N*-Ethyl-Ketamine and Identification of New Target Metabolites in Urine and Hair Using Human Liver Microsomes and High-Resolution Accurate Mass Spectrometry," *Drug Testing and Analysis* 13, no. 6 (2021): 1108–1117, https://doi.org/10.1002/dta.3007.

20. A. López-Rabuñal, D. Di Corcia, E. Amante, et al., "Simultaneous Determination of 137 Drugs of Abuse, New Psychoactive Substances, and Novel Synthetic Opioids in Meconium by UHPLC-QTOF," *Analytical and Bioanalytical Chemistry* 413, no. 21 (2021): 5493–5507, https://doi.org/10.1007/s00216-021-03533-y.

21. J. Welter, M. R. Meyer, E. U. Wolf, W. Weinmann, P. Kavanagh, and H. H. Maurer, "2-Methiopropamine, a Thiophene Analogue of Methamphetamine: Studies on Its Metabolism and Detectability in the Rat and Human Using GC-MS and LC-(HR)-MS Techniques," *Analytical and Bioanalytical Chemistry* 405, no. 10 (2013): 3125–3135, https://doi.org/10.1007/s00216-013-6741-4.

22. C. Zillien, L. Posthuma, E. Roex, and A. Ragas, "The Role of the Sewer System in Estimating Urban Emissions of Chemicals of Emerging Concern," *Reviews in Environmental Science and Biotechnology* 21, no. 4 (2022): 957–991, https://doi.org/10.1007/s11157-022-09638-9.

23. C. Ort, M. G. Lawrence, J. Rieckermann, and A. Joss, "Sampling for Pharmaceuticals and Personal Care Products (PPCPs) and Illicit Drugs in Wastewater Systems: Are Your Conclusions Valid? A Critical Review," *Environmental Science & Technology* 44, no. 16 (2010): 6024–6035, https://doi.org/10.1021/es100779n.

24. M. Kokko, S. Epple, J. Gescher, and S. Kerzenmacher, "Effects of Wastewater Constituents and Operational Conditions on the Composition and Dynamics of Anodic Microbial Communities in Bioelectrochemical Systems," *Bioresource Technology* 258 (2018): 376–389, https://doi.org/10.1016/j.biortech.2018.01.090.

25. G. Fuchs, "Anaerobic Metabolism of Aromatic Compounds," *Annals of the New York Academy of Sciences* 1125 (2008): 82–99, https://doi.org/10.1196/annals.1419.010.

26. A. P. W. Banks, F. Y. Lai, J. F. Mueller, G. Jiang, S. Carter, and P. K. Thai, "Potential Impact of the Sewer System on the Applicability of Alcohol and Tobacco Biomarkers in Wastewater-Based Epidemiology," *Drug Testing and Analysis* 10 (2018): 530–538, https://doi.org/10.1002/dta.2246.

27. V. Kumar, A. C. Johnson, N. Nakada, N. Yamashita, and H. Tanaka, "De-Conjugation Behavior of Conjugated Estrogens in the Raw Sewage, Activated Sludge and River Water," *Journal of Hazardous Materials* 227-228 (2012): 49–54, https://doi.org/10.1016/j.jhazmat.2012.04.078.

28. I. Gonzalez-Marino, J. B. Quintana, I. Rodriguez, and R. Cela, "Determination of Drugs of Abuse in Water by Solid-Phase Extraction, Derivatisation and Gas Chromatography-Ion Trap-Tandem Mass Spectrometry," *Journal of Chromatography A* 1217, no. 11 (2010): 1748–1760, https://doi.org/10.1016/j.chroma.2010.01.046.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.