Possibilities and limitations of the sewage-based epidemiology approach in the context of clinical toxicology

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VORWORT

Die nachfolgende Arbeit entstand unter der Anleitung von Herrn Univ.-Prof. Dr. rer. nat. Markus R. Meyer in der Abteilung für Experimentelle und Klinische Toxikologie der Fachrichtung 2.4. Experimentelle und Klinische Pharmakologie und Toxikologie der Universität des Saarlandes in Homburg in der Zeit von August 2020 bis Dezember 2024.

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“It is a profound and necessary truth that the deep things in science are not found because they are useful; they are found because it was possible to find them.”

J. Robert Oppenheimer

ZUSAMMENFASSUNG

Diese Dissertation präsentiert eine umfassende Untersuchung zur Entwicklung, Validierung und Anwendung analytischer Methoden im Kontext der abwasserbasierten (AW) Epidemiologie (ABE). Durch die Entwicklung und Validierung analytischer Methoden für die ABE, sowie die Aufklärung des Metabolismus von fünf neuen psychoaktiven Substanzen (NPS) und die anschließende Untersuchung ihrer Stabilität im AW, verfolgte diese Arbeit das Ziel, die Möglichkeiten und Limitationen von ABE im Kontext von klinischer Toxikologie (KT) zu untersuchen.

Dazu wurden zwei sich ergänzende analytische Methoden für die qualitative und quantitative Analyse von vier Missbrauchsdrogen, einem kognitiven Stimulanz und drei Metaboliten als Biomarker im AW entwickelt und validiert. Weiterhin wurden 24-Stunden AW-Sammelproben und AW-Stichproben analysiert, sowie die erhaltenen Daten anschließend mit einer vorherigen Studie in dieser geographischen Region, sowie Daten aus einer europaweiten Studie verglichen. Des Weiteren wurde der *in vivo* Metabolismus von fünf Deschloroketamin-Derivaten sowie deren Stabilität im AW untersucht. Die daraus erhaltenen Daten sollten genutzt werden, um potenzielle Biomarker der NPS im AW zu etablieren. Die Grenzen der ABE im Kontext KT zeigten sich dadurch, dass die erhobenen Daten zwar auf einer Populationsebene nutzbar sind, allerdings nicht auf den einzelnen Patienten übertragen werden können. Dennoch erwies sich die ABE als nützliches, ergänzendes Werkzeug für klinische Toxikologen.

SUMMARY

This thesis presents a comprehensive investigation into development, validation, and application of analytical methods used for wastewater (WW)-based epidemiology (WBE). By developing and validating analytical methods for WBE, elucidating the metabolism of five new psychoactive substances and investigating their stability in WW, this thesis aimed to elucidate the possibilities and limitations of WBE in the context of clinical toxicology.

For this purpose, two complementary analytical methods for the qualitative and quantitative screening of four drugs of abuse, one cognitive enhancer, and three of their metabolites as biomarkers in WW samples were developed and validated. Furthermore, 24 h composite WW samples and WW gab samples were analyzed, and data then compared to a prior study in the same geographical area as well as to a Europe-wide study. Additionally, the *in vivo* metabolic fate of five deschloroketamine derivatives was elucidated and their microbial biotransformation and WW stability assessed with the aim of finding suitable biomarkers for further WBE studies. Limitations of WBE when used in the context of clinical toxicology were shown, as obtained data is limited to a population size level and cannot be easily transferred to a single patient. Nevertheless, WBE proved to be a useful complementary tool for clinicians.

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1. GENERAL PART

1.1. WASTEWATER-BASED EPIDEMIOLOGY

Wastewater (WW)-based epidemiology (WBE) is an effective tool for the surveillance of populations concerning their (illicit) drug consumption, or the collection of health data (e.g., diseases or diet) in such populations [1, 2]. WBE can be employed as a sole method for surveillance or additionally e.g., in combination with traditional (online) surveys as they are less expensive, easy to perform, and do not require analytical method development and validation. However, surveys are more susceptible to bias as WBE [2]. Data obtained via WBE-based studies may be used to recognize short-, long-term, and spatial trends in (illicit) drug use [3]. Another benefit of WBE is that there is no need for (invasive) sampling of individuals, as samples are collected from WW treatment plants (WWTP) or directly from the sewer system. However, the development of analytical methods in the context of WBE can be challenging. Methods should be able to distinguish between the use of a compound and its disposal via the sewage system. Thus, detection and quantification of metabolites is of importance as well [2]. To be used as a biomarker in WBE, metabolites must fulfil several criteria. Biomarkers should originate from human metabolism, be excreted in consistent amounts, to be present in WW in concentrations adequate for detection. Additionally, they need to be stable in WW, in particular against hydrolysis or microbial biotransformation. Furthermore, biomarkers should show only little or no affinity to particles in WW, the sewage-line itself, or filters used during sample (pre-) treatment [4].

Several studies utilizing WBE have already been published, consisting of the following steps. First, either influent or effluent WW is sampled; influent WW describes WW taken before it reaches a WWTP, effluent WW is WW after the treatment in WWTP. Sampling may be performed by using composite samples, e.g. collected over the course of 24 h, either sampled constantly or in specified time intervals. Sampling over several days or weeks should be considered to gather representative results and to detect fluctuations in the monitored consumption over the week or at weekends [5]. Moreover, grab sampling may be performed as an alternative approach e.g., by

sampling directly from the sewer system [6]. After sample collection, samples may then be stabilized via acidification and/or storage at low temperatures [7]. For sample preparation, solid-phase extraction (SPE) was commonly performed in literature to concentrate samples [3, 5, 7-9]. Furthermore, samples can be filtered as a pretreatment measure to remove particles from WW before the SPE procedure, using e.g., differently coated syringe filters [3, 7]. For analysis liquid chromatography (LC), high performance LC (HPLC) or ultra performance LC (UPLC) coupled with tandem mass spectrometry (MS/MS) or high-resolution MS/MS (HRMS/MS) were described in literature [3, 9, 10]. LC based separation approaches were reported using either reversed-phase (RP) or hydrophilic interaction liquid chromatography (HILIC) coupled to MS/MS or HR/MSMS, thus providing selectivity and sensitivity [3, 11-14]. Methods using gas chromatography-mass spectrometry (GC-MS) were also published [15]. However, GC-MS-based methods are best suited for volatile and non-polar analytes [12].

1.2. DRUGS OF ABUSE, NEW PSYCHOACTIVE SUBSTANCES, AND ABUSED DRUGS

1.2.1. DRUGS OF ABUSE

Compounds with psychoactive properties are consumed by humans for millennia with their use extending from religious, or recreational to even medicinal use. Over time those drugs were used for recreational purposes by large parts of the population with substances ranging from caffeine, or alcohol to cocaine (COC) [16]. After isolation of compounds from plants e.g., morphine, further compounds were obtained by partial synthesis (heroin) or total synthesis e.g., amphetamine (AMPH). Laws to regulate their use were enacted thereafter, e.g., the German Opium Law in 1920, or later the German narcotics law (Betäubungsmittelgesetz) [17-19]. In this day and age, according to the European Drug Agency, approximately 83 million Europeans between the ages of 15 and 64 reported use of such, now illicit, drugs in their life in 2022 [20]. The number of drug users in Europe remained high in the following years [21, 22]. Globally, this trend is also reflected by an estimated 292 million drug users in 2022 by the United Nation Office on Drugs and Crime [23]. Among the most used drugs of abuse (DOA) are

AMPH, cannabis, COC, 3,4-methylendioxymethamphetamine (MDMA), heroin, and other opioids [22]. Analyzing their consumption via WW was first proposed in 2001 by Daughton [24]. Since then, numerous studies have been performed with the aim of estimating DOA consumption via the WBE approach [3, 8, 10, 25]. DOA consumption is estimated by calculation of population normalized mass loads (mg/day/1000 inhabitants), enabling comparison between different sampling days and locations [26]. Furthermore, since WW can be interpreted as pooled urine from all individuals in the sampled WW catchment, metabolism of parent compounds and the following excretion process should be considered for the estimation of substance loads in WW [27, 28]. As already mentioned, metabolites are also crucial for distinguishing between substance consumption and disposal via the sewage system. Moreover, accounting for human metabolism may enable more accurate calculations of substance loads in WW. Thus, Zuccato et al. proposed correction factors for amphetamines, benzoylecgonine (BZE), COC, and heroin considering their excretion in humans, which were then also considered by authors in further studies [3, 27]. Since the first publication of WBE data on DOA use, large scale international studies elucidating DOA consumption were performed. One of these studies is annually performed by the Sewage analysis CORe group-Europe (SCORE), sampling WW in 128 European cities, including multiple German cities [29].

1.2.2. NEW PSYCHOACTIVE SUBSTANCES

New psychoactive substances (NPS) are a class of illicit drugs especially designed to circumvent existing national and international drug legislation and therefore also known as “legal highs” [30, 31]. NPS encompass a wide range of different chemical structures, including synthetic cannabinoids, cathinones, benzodiazepines, opioids, aminoindanes, or arylcylohexamines. New representatives of this drug class are emerging on the drug market frequently and pose a significant challenge for analytical toxicologists. Implementation of NPS into screening methods or substance libraries may be demanding for researchers, due to a lack of reference substances or reference spectra [22, 32]. Data on their metabolic fate is crucial for the detection in human biosamples, underlining the importance of metabolism studies [33]. Although

metabolites may be identified in human biosamples, controlled human studies on the metabolism of NPS are not possible due to ethical concerns [34]. Hence, *in vivo* or *in vitro* models are used to elucidate NPS metabolism [33]. As mentioned above, knowledge on the metabolism of (illicit) drugs is also required to find suitable biomarkers in WW. Since NPS are consumed infrequently and their concentration in WW is generally low, further underlining the need for sensitive and selective analytical methods for their comprehensive assessment in WW samples [35].

1.2.3. ABUSED DRUGS

In addition to DOA and NPS, licit (prescription) drugs from various drug classes, e.g., cognitive enhancers, opioids, or further anesthetics, are abused due to their psychoactive properties [36]. The European drug report lists, amongst others, ketamine as an “increasingly available” substance, which “may be causing harm” [22]. Ketamine is a noncompetitive antagonist targeting the *N*-methyl-D-aspartate receptor, used as an anesthetic in human and veterinary medicine and is also listed on the World Health Organization´s list of essential medicines. However, due to its psychoactive and dissociative properties it has also become a popular recreational drug [37, 38]. As ketamine use is increasing among young people, reflected by the European Web Survey on Drugs (showing that 13 % of the surveyed reported ketamine use), ketamine-derived NPS may gain relevance in the coming years. Additional to survey data, WBE results have shown increased ketamine loads in 12 European cities [22]. The annual amount of ketamine obtained in seizures is also reported to be high with a peak of over 1.7 tons in 2018 [39]. NPS derived from ketamine were already reported on the drug marked in the past, with reports of deschloroketamine (2-oxo-PCMe) in the UK in 2015 and deschloro-*N*-ethyl-ketamine (2-oxo-PCE) in France in 2016 [40, 41].

Cognitive enhancers or nootropic drugs are a class of structurally diverse compounds, including methylphenidate (MPH), modafinil, or piracetam, which are abused to improve a user’s cognitive function and are therefore also known as “smart drugs” [42, 43]. As an example, MPH is indicated for the treatment of attention deficit hyperactivity disorder (ADHD), and its mechanism of action is based on increased concentrations of noradrenaline and dopamine in the central nervous system [42, 43]. In Germany,

MPH is the most frequently prescribed medication for ADHD [44]. Abuse of those drugs for performance enhancement was already reported in literature e.g., for shift workers, military personnel, or students [45]. Cognitive enhancers have already been detected and quantified in WW samples in numerous studies. Furthermore, a review by Wilms et al. concluded that their insufficient removal from WW may have environmental implications [13, 45-47] .

1.3. ROLE OF WASTEWATER-BASED EPIDEMIOLOGY IN CLINICAL TOXICOLOGY

Epidemiological data obtained through WBE approaches are applied in various scientific fields e.g., to monitor the spread of infectious diseases such as COVID-19, measles, or influenza [48]. Additionally, the community exposure to toxic metals via water sources has gained attention [49]. WBE studies were further employed to monitor different chemicals such as per- and polyfluoroalkylated substances which are of great concern due to their possible harmful effects on human health and wildlife [50]. Moreover, (prescription) drugs and personal care products have also been monitored using WBE-based methods [46]. This included studies on (inadequate) removal of drugs and their metabolites from WW, exemplified for MPH by Letzel et al. [51]. Jobling et al. showed the impact of pharmaceuticals on wildlife by investigating feminization of fish due to hormones used as contraceptives [52]. Data obtained utilizing WBE may further be used in the context of clinical toxicology, as monitoring consumptive patterns of DOA and NPS gained increasing interest and is therefore also relevant for e.g., public health or law enforcement [3]. In the past, WBE data on opioids have not only reflected their high consumption rates in the United States, but authors have also concluded that WBE results may be used to forecast related overdoses and fatalities based on measured concentrations [53]. In a pilot study, WBE data on opioid exposure in a community was mapped to identify substance-specific patterns within that community. The resulting maps were used to inform pharmacy-centered public health actions, including the identification of mismatches between prescription volumes and substance concentrations in WW [54]. Additionally, a study by Rice et al. combined WBE with prescription data. Authors were able to explain misleading trends, observed with WBE data with prescription rates of drugs and vice versa. Furthermore, data was

used to identify sources of morphine and then estimate heroin consumption in the monitored community [55].

1.4. ANALYTICAL METHODS IN CONTEXT OF WASTEWATER-BASED EPIDEMIOLOGY

When developing analytical methods for WBE several obstacles need to be overcome. As aforementioned, one of those obstacles is the selection of suitable biomarkers for target compounds in WW. Here, the challenge is not only to test if a metabolite fulfills the in section 1.1. mentioned requirements for biomarkers, but first to obtain reference standards of such compounds before tests may be conducted. This includes not only parent compounds, e.g., NPS, but also their metabolites [35]. Once all target compounds to be included in an analytical method are defined, the selection of an appropriate chromatographic column is a crucial step in method development. Analytical approaches utilizing LC allow for the separation of compounds with a wide range of polarities, especially due to the possibility of changing the analytical setup between RP and HILIC [12]. Although RP-based methods are widely used in bioanalysis, separation of (highly) polar compounds, such as metabolites, is often not sufficient. In contrast to RP columns, HILIC columns provide the means to separate those highly polar compounds [56-58]. Hence, as shown in a review by Senta et al., LC-based analytical methods are the most used in the field of environmental contaminants. Since GC-based separation is predominantly used for non-polar, volatile compounds, inclusion of metabolites in GC-based methods for WBE would require derivatization prior to analysis [59]. MS is widely applied in the field of WBE, with methods published utilizing low-resolution and high-resolution MS (HRMS). For low-resolution MS, ion trap- or triple quadrupole MS (QqQ)-based methods were described in literature. Due to its sensitivity, robustness, and selectivity, QqQ MS was the most reported technique for the quantification in WW samples [6, 60]. Ion trap-based methods were less common due to limitations concerning their robustness compared to QqQ. Concerning HRMS, Orbitrap and time of flight (TOF) analyzers are the most used systems [61]. Both, Orbitrap and TOF, show high mass accuracies (Orbitrap < 5 ppm; TOF < 2 ppm) and are therefore widely used for the screening of (illicit) drugs in WW [61-63]. However, HRMS is limited concerning quantitative approaches due to a

narrower dynamic range compared to QqQ [61]. Additional to the selection of an adequate analytical system, a surrogate matrix for the development and validation process is required, as (influent) WW contains the compounds of interest per se.

2. AIMS AND SCOPES

This thesis aimed to develop and validate suitable analytical procedures in the context of WBE. Furthermore, the metabolism of NPS should be investigated with the purpose of finding suitable screening targets for analysis in the context of clinical toxicology and WBE.

The following steps should be conducted:

- Development and validation of an SPE-based analytical method for the identification and quantification of DOA, cognitive enhancers and their biomarkers in WW
- Evaluation of the performance of two analytical LC columns
- Monitoring of concentrations and mass loads of previously validated analytes in WW samples obtained as grab and 24 h composite samples
- Comparison of obtained loads to previously published data
- Investigation of the *in vivo* metabolic fate of five deschloroketamine derivatives (DCKD)
- Studies on the microbial biotransformation and WW stability of five DCKD and their metabolites

3. METHODS

3.1. ANALYSIS OF DRUGS OF ABUSE AND COGNITIVE ENHANCERS IN WASTEWATER SAMPLES [64, 65]

3.1.1. SAMPLE PREPARATION

Sample preparation was modified based on a previously published procedure [3]. Briefly, 10 mL of WW were fortified with internal standard (IS) mix and then filtered through Phenex-PTFE syringe filters to remove particles. SPE was performed using Isolute HCX cartridges. Priming was performed using 1 mL of methanol (MeOH) and 1 mL of purified water. Then, 10 mL of filtered WW samples, containing IS mix and subsequently washed with purified water, 0.1 M hydrochloric acid, and MeOH (1 mL each). Elution of the target compounds was performed using 1.25 mL of a mixture of MeOH and ammonia. Eluates were partitioned into two aliquots of equal volume and then evaporated to dryness under a gentle stream of nitrogen at 40 °C. Residues were reconstituted using either a mixture of acetonitrile (ACN) and formic acid (HILIC samples) or a mixture of water and formic acid RP C_{18} samples. Final concentrations and composition of mixtures, manufacturers of reagents and materials are given in the respective methods section of the first and second publication included in this thesis [64, 65].

3.1.2. INSTRUMENT SETTINGS FOR THE ANALYSIS OF WASTEWATER SAMPLES

Sample analysis was performed using a Thermo Fisher Scientific (TF) Dionex UltiMate 3000 consisting of a degasser, a quaternary pump, a DL W2 wash system, and an HCT PAL autosampler. The LC system was coupled to a TF Q Exactive Orbitrap MS, equipped with a heated electrospray ionization II (HESI-II) source. RP separation was performed using a Waters AQUITY UPLC BEH C_{18} column, eluent A consisted of an aqueous ammonium formate solution containing formic acid, eluent B consisted of ammonium formate solution in ACN/MeOH containing formic acid and water. HILIC

separation was performed using a Merck SeQuant ZIC-cHILIC column, eluent C consisted of aqueous ammonium acetate solution, eluent D consisted of ACN containing formic acid. Final compositions of eluents, flow rates, gradients, as well as manufacturers of reagents and materials are given in the methods section of the first and second publication of this thesis [64, 65]. Settings of the HESI-II source and MS conditions are also given in the first and second publication of this thesis [64, 65]. Briefly, analysis via the C_{18} column was performed using only positive ionization mode, MS experiments were performed using parallel reaction monitoring (PRM) with a scheduled inclusion list containing precursor masses of interest and adjusted normalized collision energies. HESI-II source settings after HILIC separation included positive and negative ionization in a single analytical run. MS experiments were also performed in PRM with a scheduled inclusion list.

3.2. STUDIES ON THE METABOLISM AND URINARY DETECTABILITY OF DESCHLOROKETAMINE DERIVATIVES [66]

3.2.1. IN VIVO RAT METABOLISM, URINARY DETECTABILITY AND IN VITRO CONFIRMATION EXPERIMENTS

Six male Wistar rats were used for metabolism experiments in accordance with German laws for animal protection. Studies have been approved by an ethic committee (No 50/2017, Landesamt für Verbraucherschutz, Saarbrücken, Germany). Five DCKD (2-oxo-PCMe, deschloro-*N*-cyclopropyl-ketamine; 2-oxo-PCcP, 2-oxo-PCE, deschloro-*N*-isopropyl-ketamine; 2-oxo-PCiP, and deschloro-*N*-propyl-ketamine; 2-oxo-PCP) were orally administered in an aqueous suspension via gastric intubation in doses of 2 mg/kg body weight (one compound per rat, *n=5*). Rats were housed in metabolism cages and had water *ad libitum* during the collection of urine, which was collected separately from feces. No compound was administered to the sixth rat; its urine was collected as control sample.

To confirm phase I metabolites tentatively identified in rat urine samples, DCKD were incubated in pooled human liver microsomes (pHLM), according to a previous study, with minor modifications [33, 67].

Before LC-HRMS/MS analysis, rat samples were either extracted via urine precipitation (UPP), or SPE. For GC-MS analysis, sample preparation was performed using liquid-liquid extraction after partial urine hydrolysis followed by acetylation (UHyAc).

Experiments on the detectability of DCKD using LC-HRMS/MS or GC-MS were performed in *n=3* respectively, after UPP (LC-HRMS/MS) or UHyAc (GC-MS).

Sample preparation procedures, detectability experiments, and the pHLM incubation protocol are given in detail in the methods section of the third publication of this thesis [66].

3.2.2. INSTRUMENTAL SETTINGS

3.2.2.1. LC-HRMS/MS APPARATUS

Sample analysis was performed using a TF Dionex UltiMate 3000 consisting of a degasser, a quaternary pump, a DL W2 wash system, and an HCT PAL autosampler. The LC system was coupled to a TF Q Exactive Orbitrap MS, equipped with a HESI-II source. Separation of analytes was performed using a TF Accucore Phenyl Hexyl column. Eluent A consisted of an aqueous ammonium formate solution containing formic acid, eluent B consisted of ammonium formate solution in ACN/MeOH, containing formic acid and water. The HESI-II source was operated in positive ionization mode. MS experiments were performed using a full scan mode and data dependent acquisition with an inclusion list containing the masses of expected metabolites. Final compositions of eluents, flow rates, gradients, manufacturers of reagents and materials, final settings of the HESI-II source and MS conditions are given in the methods section of the third publication of this thesis [66].

3.2.2.2. GC-MS APPARATUS

A Hewlett Packard (HP) 5890 II gas chromatograph combined with a HP 5972 MSD mass spectrometer and a HP MS ChemStation were used for GC-MS experiments.

For separation a Macherey Nagel capillary dimethylpolysiloxane column was used. The MS conditions included full scan and electron ionization mode. Detailed settings on the GC-MS apparatus are given in the methods section of the third publication of this thesis [66].

3.3. STUDIES ON THE MICROBIAL BIOTRANSFORMATION AND WASTEWATER STABILITY OF DESCHLOROKETAMINE DERIVATIVES [68]

Biotransformation of DCKD and their metabolites in WW was assessed by incubating the parent compounds at concentrations of 0.1 mg/L or the rat urine or rat feces samples in freshly collected, untreated, influent WW over a period of 24 h at 22 °C. Incubations of rat urines were performed after preconcentrating 1 mL of rat urine via precipitation using MeOH (50:50, v/v), followed by centrifugation, supernatant evaporation to dryness under a stream of nitrogen and reconstitution in WW. Feces samples (0.25 g) were preconcentrated in 1.5 mL MeOH and then homogenized via ultrasonification, followed by centrifugation, supernatant evaporation to dryness under a stream of nitrogen and reconstitution in WW. Incubation was performed in WW for 24 h in amber-colored reaction tubes. After 0 and 24 h, an aliquot of each sample was transferred into another reaction tube, IS and sodium azide (NaN_3) were added. Samples were then vortexed and centrifuged. Supernatants were then transferred into autosampler vials for analysis using LC-HRMS/MS. Control incubations were performed by the addition of NaN_3 at the start of the incubation, blank incubations were performed using purified water instead of WW. Final concentrations and composition of mixtures, manufacturers of reagents and materials are given in the methods section of the fourth publication included in this thesis [68].

Analysis of samples was performed using the same LC-HRMS/MS configuration as in section 3.2.2.1., further settings are listed in the fourth publication of this thesis [68].

4. PUBLICATIONS OF THE RESULTS

The results of this dissertation were published in the following papers:

4.1. Quantitative analysis of drugs of abuse and cognitive enhancers in influent wastewater by means of two chromatographic methods [64]

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Author contribution

F.F.: formal analysis, investigation, data curation, methodology, writing-original draft preparation, writing-review and editing, visualization; C.M.J.: methodology, writing-review and editing; L.W.: conceptualization, writing-review and editing; M.R.M.: conceptualization, resources, writing-review and editing, supervision, project administration.

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RESEARCH ARTICLE

WILEY

Quantitative analysis of drugs of abuse and cognitive enhancers in influent wastewater by means of two chromatographic methods

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Abstract

Sewage-based epidemiology using influent wastewater is used to estimate the consumption trends of (illicit) drugs over a short or long period of time in a subpopulation. The current study aimed to develop two separate methods for the quantitative analysis of selected drugs of abuse (DOA) and cognitive enhancers in influent wastewater using reversed-phase (RP) or hydrophilic interaction liquid chromatography (HILIC) coupled to high-resolution tandem mass spectrometry (LC-HRMS/MS). The performance of RP and HILIC column was evaluated. A simple solid phase extraction was used for sample preparation. Short runtimes of 10 and 15 min on the RP and the HILIC column, respectively, allowed sufficient throughput. A six-point calibration was used for quantification with calibration ranges between 10 and 100 ng/L for all analytes except for benzoylecgonine (BZE, 30–300 ng/L). Method validation was performed according to ICH guideline M10. Analytes such as amphetamine (AMPH), BZE, cocaethylene (CE), cocaine (COC), ethyl sulfate, 4-hydroxy-3-methoxymethamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), methamphetamine, methylphenidate (MPH), and ritalinic acid (RA) were included in method development and validation. Two different column types were necessary for sufficient chromatographic resolution. The analytical setup allowed detection of all other analytes at concentration levels between 1 ng/L for methylphenidate to 10 ng/L for amphetamine. A method for the detection and quantification of DOA, cognitive enhancers, and their biomarkers in wastewater was successfully developed and validated. Moreover, six proof-of-concept samples were analyzed in which AMPH, BZE, COC, MDMA, MPH, and RA were identified and further quantified.

KEYWORDS
cognitive enhancers, drugs of abuse, LC-HRMS/MS, wastewater analysis

1 | INTRODUCTION

Approximately 83 million Europeans aging between 15 and 64 have used illicit drugs in their life. Stimulants such as amphetamines, 3,4-methylenedioxymethamphetamine (MDMA), and cocaine are among the most commonly used drugs of abuse (DOA) in the European union.[1] In addition to DOA, prescription drugs such as methylphenidate, piracetam, and modafinil are abused as cognitive enhancers to improve performances or ease stress.[2]

The extend of current drug abuse and upcoming trends can be monitored by, for example, online surveys, which are easy to perform and cheap. However, one limitation is their highly subjective character, which may lead to under reporting by the participants.[3,4] Hence, complementary and more objective tools, like wastewater-based epidemiology (WBE), are required. This approach allows to monitor drug intake through analyzing compounds of interest after their excretion into wastewater (WW). There is no need of testing individuals,[5] and WBE allows the detection of temporal as well as spatial trends in the abuse of drugs.[6,7]

However, the development of such bioanalytical methods is quite challenging. Hereby, the selection of a suitable biomarker for each drug is crucial. The ideal biomarker is expected to fulfill several criteria. Human specific metabolites should be used to distinguish between human consumption and discarded compounds into WW.[3,5] These metabolites should be excreted in consistent amounts, sufficient for analysis. Furthermore, no sorption to particles, the sewer line itself or filters used for sample (pre-) treatment, should occur. Moreover, biomarkers must be stable under the conditions in WW (in-sewer stability), which includes stability against hydrolysis and stability against biotransformation by microorganisms present in WW.[3,8]

Although reversed-phase (RP) liquid chromatography (LC) is widely used in bioanalysis, separation of highly polar drug metabolites is often not ideal or possible.[9,10] Hydrophilic interaction liquid chromatography (HILIC) as complementary method allows a better separation of polar analytes.[11] The general suitability of HILIC for the separation of hydrophilic DOAs and pharmaceuticals after extracting influent WW using solid phase extraction (SPE) was already demonstrated.[12,13] Particularly very polar pharmaceuticals, which had no retention in classical RP chromatography such as metformin, could be retained using HILIC. Also, the large amount of acetonitrile (ACN) in the mobile phase using HILIC increased the analytical sensitivity.

Aims of this study were first the development of an SPE-based liquid chromatography high-resolution tandem mass spectrometry (LC-HRMS/MS) procedure for identification and quantification of selected DOA, cognitive enhancers, and metabolites in WW. Furthermore, the performance of two analytical LC columns, one RP column and one HILIC column for chromatographic separation of analytes, should be evaluated. Finally, the method should be applied to six proof-of-concept WW grab samples collected over a period of 2 years.

2 | MATERIALS AND METHODS

2.1 | Reagents and materials

Amphetamine (AMPH) sulfate, AMPH-d_5, benzoylecgonine (BZE), BZE-d_3, cocaethylene (CE), CE-d_8, cocaine (COC) hydrochloride (HCl), COC-d_3, ethyl sulfate (EtS) sodium salt, EtS-d_5, 5-hydroxy-indolacetic acid (5-HIAA), 5-HIAA-d_5, 4-hydroxy-3-methoxymethamphetamine (HMMA), MDMA HCl, MDMA-d_5, methamphetamine (METH) HCl, METH-d_5, methylphenidate (MPH) HCl, MPH-d_9, modafinil, modafinilic acid, piracetam, ritalinic acid (RA), and RA-d_{10} were obtained from LGC (Wesel, Germany). All other chemicals were purchased from VWR (Darmstadt, Germany). Water was purified with a Millipore (Merck, Darmstadt, Germany) filtration unit, which purifies water to a resistance of 18.2 $\Omega \times$ cm.

Stock solutions of each compound were prepared at concentrations of 100 µg/L in ACN, calculated for the active compound. The internal standard (IS) solution contained 150 µg/L BZE-d_3, 1 mg/L EtS-d_5, and all other isotopically labeled compounds at concentrations of 50 mg/L in ACN. Calibrator (Cal) and quality control (QC) working solutions were separately prepared in ACN, and final concentrations in surface water (rainwater) as surrogate blank matrix are shown in Table S1.

2.2 | Sample preparation

Sample preparation was performed according to a previously published procedure[6] with minor modifications. WW samples (10 mL) were fortified with IS solution (final concentration 50 ng/L, except BZE-d_3 150 ng/L and EtS-d_5 1 µg/L) and then filtered via Phenex-PTFE 25-mm syringe filters 0.2 mm (Phenomenex, Aschaffenburg, Germany) to remove particles. The SPE was performed using Isolute 200 mg/10 mL (3-mL XL) HCX cartridges (Biotage, Uppsala, Sweden). Cartridges were primed with 1-mL methanol (MeOH) and 1-mL purified water. Cartridges were then loaded with 10 mL of WW and subsequently washed with purified water, 0.1 M hydrochloric acid, and MeOH (1 mL each). Targeted compounds were eluted using a mixture of MeOH and NH_3 (35%, 98/2, *v/v*, 1.25 mL). Eluates were then partitioned into two equal aliquots and evaporated to dryness under a gentle stream of nitrogen at 40°C. The residues were reconstituted using a mixture of ACN and formic acid (50 µL, 99/1, *v/v*, HILIC samples) or a mixture of water and formic acid (50 µL, 99/1, *v/v*, RP C_{18} (C_{18}) samples).

2.3 | Instrumental settings

Samples were analyzed using a Thermo Fisher Scientific (TF, Dreieich, Germany) Dionex UltiMate 3000 consisting of a degasser, a quaternary pump, a DL W2 wash system, and an 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). Calibration was done prior to analysis according to the manufacturer's recommendations using external mass calibration. The final conditions of the LC-system

using the C_{18} column were as follows: Waters AQUITY UPLC BEH C_{18} column (100 × 2.1 mm, 1.7 µm; Massachusetts, USA); gradient elution was done with 2 mM ammonium formate solution containing 0.1% (*v*/*v*) formic acid (eluent A) and 2 mM ammonium formate solution in ACN/MeOH (50/50, *v*/*v*) containing 0.1% (*v*/*v*) formic acid and 1% (*v*/*v*) water (eluent B). The flow rate was at 0.500 mL/min with the following gradient settings: 0–1 min 85% A, 1–3 min to 40% A, 3–6 min to 30% A, 6–8 min 1% A, 8–9.2 min hold 1% A, 9.2–9.21 min to 85% A, 9.21–10 min hold 85% A.

The final conditions of the LC-system with the HILIC column were as follows: Merck SeQuant ZIC-HILIC column (150 × 2.1 mm, 3 µm; Merck, Darmstadt, Germany); gradient elution with 200 mM aqueous ammonium acetate solution (eluent C) and ACN containing formic acid 0.1% (*v*/*v*) (eluent D). The flow rate was at 0.500 mL/min with the following gradient settings: 0–1 min hold 1% C, 1–1.8 min to 10% C, 1.8–9 min hold 10% C, 9–9.5 min to 60% C, 9.5–10.5 min hold 60% C, 10.5–10.6 min to 1% C, 10.6–15 min hold 1% C. Chromatography on both columns was performed at 40°C.

The final HESI-II source and MS conditions were as follows: ionization mode: positive and negative; sheath gas, 60 arbitrary units (AU); auxiliary gas flow rate, 10 AU; spray voltage, 4.00 kV; auxiliary gas heater temperature, 320°C, ion capillary temperature, 320°C; and S-lens RF level, 50.0. Mass spectrometry experiments after C_{18} column separation were performed using parallel reaction monitoring (PRM) in positive mode with a scheduled inclusion list containing the precursor masses of interest, adjusted normalized collision energies (NCEs). The settings for PRM experiments were as follows: resolution 17,500; automatic gain control (AGC) target 2e5; maximum injection time (IT) 250 ms; isolation window, 1 *m*/*z*; high-energy collisional dissociation (HCD) with NCE 30, 40 eV. Mass spectrometry experiments after HILIC column separation were performed using PRM in positive and negative mode in a single analytical run. Settings in positive mode were the same as described for the C_{18} column; settings for negative mode were as follows: resolution 17,500; AGC target 2e5; maximum IT 250 ms; isolation window, 1 *m*/*z*; HCD with NCE 10 eV. TF Xcalibur Qual Browser version 4.1.31.9 was used for data handling. Masses of the precursor ions (*m*/*z*) used for the inclusion list, polarity, and adjusted NCE are listed in Table S2.

2.4 | Method validation

The method was validated according to the ICH guideline M10 on bioanalytical method validation and study sample analysis.[14] For identification, MS^2 spectra were compared to a database,[15] and for quantification, peak area ratios of the quantifier ions of analytes and IS in MS^2 (see Table S2) were used.

2.4.1 | Calibration curve and lower limits of quantitation

The calibration curve consisted of six calibration standards (Cal 1–6), prepared by spiking aged surface water, as blank matrix, with different calibrator solutions and the IS mix. QC lower limit of quantification (LLOQ), QC low, QC medium (mid), and QC high were prepared by spiking blank matrix with different QC spike solutions, prepared separately from the calibration standards (final concentrations in are listed in Table S1) and IS. Calibrators and QC samples were extracted as described above (Section 2.2). Each analytical run consisted of Cal 1–6, QC LLOQ, QC low, QC mid, QC high, a blank matrix sample, and a zero sample (blank matrix spiked with IS). Back calculated concentrations of the calibration standards should be within ±15% of the nominal value (±20% for QC LLOQ), and at least 75% of the calibrators must fulfill this criterion. The limit of detection was set to be equal to the LLOQ, and the LLOQ was accepted in case the back calculated concentrations were within ±20%.

2.4.2 | Selectivity and carry-over

Selectivity was tested by extraction (see Section 2.2) of six different sources of surface water (blank matrix), which were analyzed individually on both columns as described in Section 2.3. Acceptance criteria (AC) are met if the analyte response is less than 20% of the LLOQ or 5% of the IS. Carry-over was tested by injecting blank extracts after QC high samples ($n = 3$) on both columns; no carry-over greater than 20% of the LLOQ for the analytes or 5% for the IS should be observed to fulfill AC.

2.4.3 | Dilution integrity

Dilution integrity was determined by spiking blank matrix with analyte concentrations 10 times the QC high and diluting these 1:10 with blank matrix ($n = 5$). Accuracy and precision of all analytes should be within ±15%.

2.4.4 | Matrix factors (MFs), recovery, and studies on co-eluting analytes

MFs and recovery were determined using blank matrix from six different sources. Three sets of samples at QC low and QC high were prepared ($n = 6$): set 1 consisted of pure solutions of analytes and IS, set 2 consisted of blank matrix spiked with analytes and IS after the extraction, and set 3 consisted of blank matrix spiked before the extraction. Pure solutions of analytes and IS were prepared in ACN and formic acid (99/1, *v*/*v*, HILIC samples) or a mixture of water and formic acid (99/1, *v*/*v*, C_{18} samples). Sample preparation of set 2 was performed as described in Section 2.2 without IS fortification prior to SPE. Sample preparation of set 3 was performed as described in Section 2.2.

For each analyte and IS, the MF was calculated, according to the guideline of the European Medicines Agency,[16] using the ratio of set 1 and set 2, and the IS-normalized MF of each analyte was calculated by dividing the analytes MF by the MF of the corresponding IS. The IS-normalized MF of HMMA was calculated by using MDMA-d_5 as IS, because no isotopically labeled analog of HMMA was available to be included in this study.

Additionally, the recovery (RE) according to Matuszewski et al.[17] was tested. RE was calculated via the peak area ratio of set 3 to set

2 for each analyte and IS. Since for every analyte, except HMMA, a corresponding isotopically labeled analog was available, the IS-normalized RE was calculated additionally, as described for the IS-normalized MF, CVs should not exceed 15%.

Ion suppression or enhancement was tested according to Remane et al. at concentrations of 10 µg/L for all co-eluting analytes and IS,[18] CVs for all co-eluting analytes and IS should be within 15%.

2.4.5 | Stability

Stability of the stock solution was tested over 6 weeks; samples were stored at −22°C, and aliquots were analyzed once a week ($n = 3$). As the extracts for both columns were prepared using the same stock solution, stability was only determined using the HILIC column. Furthermore, stability in the autosampler was tested for processed QC low and QC high samples ($n = 3$, 24 h at 10°C), as well as freeze thaw stability (3 cycles, 24 h per cycle at −22°C) and long-term stability over 106 days at −22°C for unprocessed samples. Concentrations should be within ±15% of nominal values for both QC levels when analyzed directly after the sample preparation and after the applied storage conditions via a freshly prepared calibration. Short-term stability and benchtop stability were not applicable for this study as the samples were directly frozen after sampling and hence not tested.

2.4.6 | Accuracy and precision

Accuracy and precision were evaluated for QC LLOQ, QC low, QC mid, and QC high samples. Within-run accuracy and precision experiments consisted of five sets of QC samples, measured within a single analytical run. Between-run accuracy and precision were determined with three different runs on two different days. QC concentrations were back calculated via calibration curves, and AC for accuracy experiments are met if mean concentrations are within ±15% of the nominal values (±20% for QC LLOQ). For precision experiments, the coefficient of variation (CV) should be within ±15% for all QC samples (±20% for QC LLOQ) to meet the AC.

2.5 | WW sample collection, treatment, and application of the method

Proof of concept WW grab samples ($n = 6$ in total) were obtained between June 2021 and March 2023, at two different sampling points. Samples were acidified with acetic acid (0.1% *v*/*v*) and stored at −22°C until the final sample preparation.

3 | RESULTS AND DISCUSSION

3.1 | Samples preparation

As described in Section 2.2, sample preparation was performed according to Meyer et al. with minor modifications.[6] We were able to use the same sample preparation (the same extract) prior to analysis by C18 and HILIC. This resulted in lower costs and higher sample throughput in contrast to individual sample preparation prior to C18 and HILIC. Best peak shapes were obtained on the C_{18} column if the analytes were reconstituted in a mixture of water and formic acid (50 µL, 99/1, *v*/*v*), and on the HILIC column with a reconstitution mixture containing ACN and formic acid (50 µL, 99/1, *v*/*v*). Therefore, two different extracts were necessary for the analysis using two different columns (see Figures 1 and 2). A volume of 50-mL reconstitution solvent was used to increase the concentration factor up to 100. The extraction and/or separation of 5-HIAA, modafinil, modafinilic acid, and piracetam was not reproducible, and these analytes were thus not included in the method validation.

3.2 | Method validation

Aged rainwater was used as blank matrix although it does not necessarily reflect the complexity of influent WW. However, influent WW cannot be used as blank matrix for validation as it contains the analytes of interest per se. Thus, a surrogate matrix must be used, and aged rainwater seems to be one reasonable alternative although there are still some limitations.

3.2.1 | Selectivity and carry-over

Analysis of six blank samples showed no interfering signals in PRM runs with either the C_{18} or HILIC column. Furthermore, on both columns, no carry-over of analytes in blank runs after the QC high ($n = 3$) could be observed. Additionally, blank samples should be injected between study samples and after Cal 6, as potential carry-over was only tested up to concentrations of QC high.

3.2.2 | Dilution integrity

The selection of the concentration range was based on previously studies and expected concentrations. Higher concentrations during validation might have increased the risk of carry-over and were thus avoided. Instead, the dilution integrity was tested to also allow the reliable quantification of higher concentrations after dilution. All analytes met the AC for the 1:10 dilution after analysis on both columns except for HMMA and EtS (Table S3). Relative mean concentration and CV of HMMA were >15% (55%, CV 22%), and for EtS, the IS could not be detected.

3.2.3 | MFs, recovery, and studies on co-eluting analytes

IS-normalized MF, RE, and IS-normalized RE are listed in Tables 1 and 2. After C_{18} separation, CVs obtained for IS-normalized MF of QC low

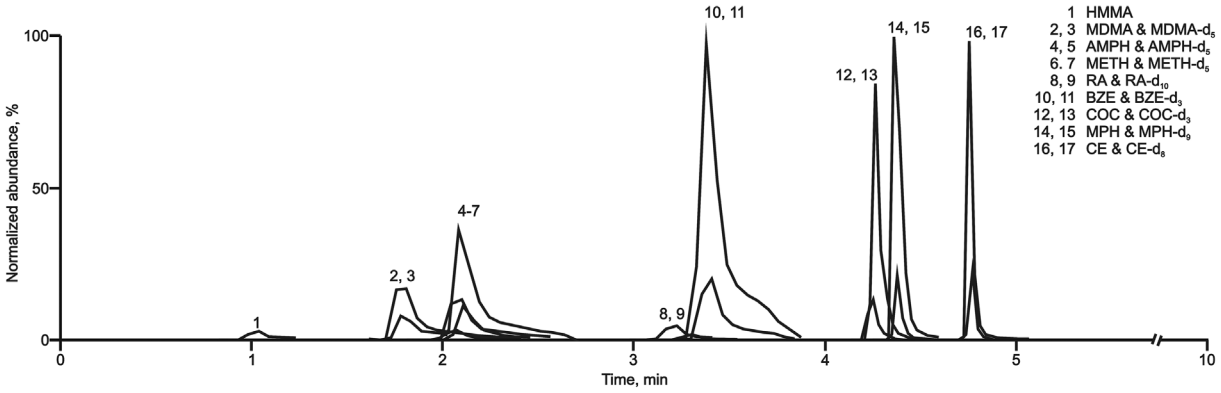

FIGURE 1 Extracted quantifier ion chromatogram of an extracted lower limit of quantification (LLOQ) sample after separation using the C_{18} column; AMPH, amphetamine; BZE, benzoylecgonine; CE, cocaethylene; COC, cocaine; HMMA, 4-hydroxy-3-methoxymethamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; MPH, methylphenidate; RA, ritalinic acid.

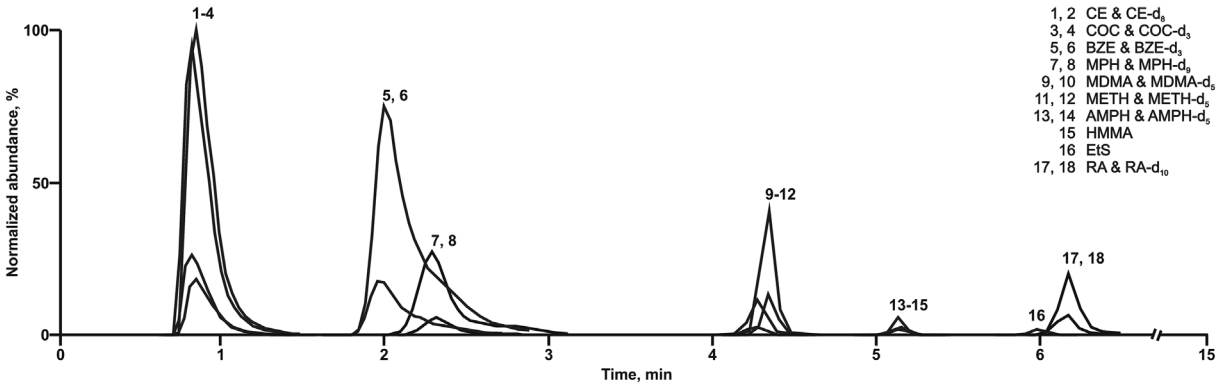

FIGURE 2 Extracted quantifier ion chromatogram of an extracted lower limit of quantification (LLOQ) sample after separation using the hydrophilic interaction liquid chromatography column; AMPH, amphetamine; BZE, benzoylecgonine; CE, cocaethylene; COC, cocaine; EtS, ethyl sulfate; HMMA, 4-hydroxy-3-methoxymethamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; MPH, methylphenidate; RA, ritalinic acid.

TABLE 1 Internal standard (IS)-normalized matrix factor (MF), recovery (RE), IS-normalized RE, and their coefficients of variation (CV) calculated for quality control (QC) low and QC high ($n = 6$), analyzed on the C_{18} column.

	QC low			QC high		
Analyte	IS-normalized MF, % (CV, %)	RE, % (CV, %)	IS-normalized RE, % (CV, %)	IS-normalized MF, % (CV, %)	RE, % (CV, %)	IS-normalized RE, % (CV, %)
AMPH	94 (5)	73 (33)	112 (8)	101 (4)	80 (9)	100 (8)
BZE	101 (3)	48 (30)	97 (13)	98 (3)	54 (16)	105 (7)
CE	102 (8)	15 (26)	148 (7)	105 (10)	14 (28)	87 (13)
COC	100 (8)	19 (31)	103 (13)	98 (7)	26 (30)	108 (9)
EtS	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
HMMA	29 (14)	105 (51)	203 (13)	28 (13)	135 (11)	369 (25)
MDMA	99 (2)	55 (40)	111 (9)	96 (12)	63 (16)	111 (6)
METH	125 (10)	57 (35)	148 (27)	102 (13)	61 (16)	169 (23)
MPH	100 (3)	43 (40)	105 (13)	93 (11)	46 (18)	104 (7)
RA	100 (2)	69 (20)	119 (11)	96 (5)	62 (15)	105 (6)

Abbreviations: AMPH, amphetamine; BZE, benzoylecgonine; CE, cocaethylene; COC, cocaine; EtS, ethyl sulfate; HMMA, 4-hydroxy-3-methoxymethamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; MPH, methylphenidate; n. a., not available; QC: quality control; RA, ritalinic acid.

TABLE 2 Internal standard (IS)-normalized matrix factor (MF), recovery (RE), IS-normalized RE, and their coefficients of variation (CV) calculated for quality control (QC) low and QC high ($n = 6$), analyzed on the hydrophilic interaction liquid chromatography column.

	QC low			QC high		
Analyte	IS-normalized MF, % (CV, %)	RE, % (CV, %)	IS-normalized RE, % (CV, %)	IS-normalized MF, % (CV, %)	RE, % (CV, %)	IS-normalized RE, % (CV, %)
AMPH	102 (11)	147 (75)	107 (14)	99 (3)	393 (48)	99 (2)
BZE	121 (25)	75 (44)	109 (46)	100 (2)	148 (46)	107 (4)
CE	94 (12)	98 (24)	121 (10)	100(1)	139 (32)	107 (5)
COC	97 (8)	99 (25)	122 (15)	100 (1)	143 (34)	107 (4)
EtS	81 (45)	92 (217)	IS n.d.	100 (4)	1 (18)	IS n.d.
HMMA	15 (61)	196 (94)	150 (103)	134 (10)	415 (48)	434 (48)
MDMA	100 (5)	108 (110)	110 (9)	101 (5)	123 (40)	112 (4)
METH	102 (6)	185 (122)	110 (14)	103 (6)	116 (40)	110 (7)
MPH	96 (6)	104 (41)	116 (9)	99 (2)	146 (34)	106 (5)
RA	97 (2)	930 (208)	109 (10)	97 (2)	129 (16)	107 (5)

Abbreviations: AMPH, amphetamine; BZE, benzoylecgonine; CE, cocaethylene; COC, cocaine; EtS, ethyl sulfate; HMMA, 4-hydroxy-3-methoxymethamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; MPH, methylphenidate; QC, quality control; RA, ritalinic acid.

and QC high were below 15%, except for HMMA (see Table 1). After HILIC separation, HMMA, BZE, and EtS did not meet the AC given by the ICH.[14]

CVs of the IS-normalized RE were within 15% for most analytes. METH (C_{18} column), BZE, EtS (HILIC column), and HMMA (both columns) did not meet the AC. In comparison, CVs of RE calculated according to Matuszewski et al. were above 15% for all analytes, which could be due to variations in the extractions or fluctuations during the measurements. Because there was a corresponding IS for each analyte except for HMMA, it was expected that the IS-normalized RE would show lower CVs than the RE. Differences in meeting the AC for RE between both columns might be explained with varying column performances during method validation. Furthermore, it could be influenced by the different reconstitution solvents used for both columns.

Ion suppression or enhancement was tested according to Remane et al.[18]; CVs for all co-eluting analytes and IS were below 15% (Table S4). The co-eluting analytes can be identified in Figures 1 and 2.

3.2.4 | Stability

Analysis of stability samples was performed after HILIC separation. Stability of the stock solution was given over a 6-week interval at −22°C. Values of the stability in the autosampler are listed in Table S5. All analytes (except EtS and HMMA) passed the criteria for the stability in the autosampler. Relative mean concentrations and CVs of the freeze and thaw stability are listed in Table S6. HMMA, METH, and EtS did not meet the AC set by the ICH; METH only fulfilled the ICH criteria for the first two freeze and thaw cycles and failed the third cycle in QC low samples.[14] Values for the long-term stability are given in Table S7. HMMA did not meet the AC set by the ICH guidelines.[14] Furthermore, MDMA failed the requirements for long-term stability as well as EtS and HMMA.

3.2.5 | Accuracy and precision

A linear calibration model was used for all analytes. Within- and between-run accuracy and precision results are shown in Tables 3 and 4. All analytes, except HMMA, showed relative mean concentrations within ±15% of the nominal concentration for QC low, QC mid, QC high, and ±20% for QC LLOQ, as well as CVs < 15% after C_{18} separation. AMPH, EtS, and HMMA did not meet the AC after HILIC separation most likely due to poor peak shape at low concentrations and lack of a labeled standard of HMMA. The poor peak shape can lead to issues with reproducible determination of peak areas. The remaining analytes fulfilled the AC set by the ICH.[14]

HMMA did not pass any of the validation experiments, with either column. A possible explanation might be that no corresponding isotope labeled IS was available for this analyte. Although MDMA-d_5 is the deuterated analog of HMMAs parent compound, it could not compensate for variations during extractions or analysis. Furthermore, EtS-d_5 could not be detected in any of the validation experiments, even though it could be detected in the spike solution used for the whole validation process. This was likely due to a poor extraction performance, as EtS-d_5 could be detected in stock stability samples.

TABLE 3 Within- and between-day accuracy (A) and precision (P), of quality control (QC) lower limit of quantification (LLOQ), QC low, QC mid, and QC high ($n = 5$), analyzed via the C_{18} column. EtS was not included in the C_{18} method.

	Relative mean concentration (A), % (CV (P), %							
	Within-run				Between-run			
Analyte	QC LLOQ	QC low	QC mid	QC high	QC LLOQ	QC low	QC mid	QC high
AMPH	112 (9)	100 (11)	101 (9)	106 (7)	102 (12)	100 (4)	101 (3)	108 (4)
BZE	111 (2)	109 (2)	103 (3)	109 (2)	104 (1)	102 (4)	99 (5)	109 (1)
CE	91 (6)	104 (6)	95 (9)	108 (8)	95 (9)	99 (6)	96 (1)	108 (2)
COC	93 (10)	101 (3)	101 (5)	107 (2)	92 (9)	100 (3)	100 (3)	107 (6)
EtS	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
HMMA	51 (4)	73 (11)	83 (18)	156 (22)	75 (31)	105 (13)	123 (15)	152 (4)
MDMA	93 (2)	103 (3)	108 (4)	111 (2)	92 (9)	98 (10)	102 (10)	112 (6)
METH	88 (3)	96 (7)	111 (4)	97 (5)	96 (9)	97 (11)	103 (8)	102 (3)
MPH	118 (3)	100 (7)	107 (4)	112 (2)	97 (5)	97 (2)	102 (5)	109 (7)
RA	105 (3)	105 (6)	93 (10)	108 (2)	101 (8)	95 (13)	95 (4)	110 (5)

Abbreviations: AMPH, amphetamine; BZE, benzoylecgonine; CE, cocaethylene; COC, cocaine; CV, coefficient of variation; EtS, ethyl sulfate; HMMA, 4-hydroxy-3-methoxymethamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; mid, medium; MPH, methylphenidate; n. a., not available; QC, quality control; RA, ritalinic acid.

TABLE 4 Within- and between-day accuracy (A) and precision (P), of quality control (QC) lower limit of quantification (LLOQ), QC low, QC mid, and QC high ($n = 5$), analyzed via the HILIC column.

	Relative mean concentration (A), % (CV (P), %							
	Within-run				Between-run			
Analyte	QC LLOQ	QC low	QC mid	QC high	QC LLOQ	QC low	QC mid	QC high
AMPH	n.d.	90 (4)	100 (8)	106 (10)	n.d.	96 (36)	100 (19)	106 (12)
BZE	108 (2)	107 (2)	102 (5)	107 (5)	106 (2)	101 (5)	100 (5)	111 (4)
CE	98 (1)	106 (2)	99 (6)	110 (1)	95 (3)	100 (4)	98 (5)	112 (2)
COC	102 (9)	106 (2)	100 (6)	110 (3)	97 (4)	100 (5)	98 (5)	111 (2)
EtS	IS n. d.	IS n. d.	IS n. d.	IS n. d.	IS n. d.	IS n. d.	IS n. d.	IS n. d.
HMMA	84 (10)	93 (10)	112 (26)	100 (11)	166 (38)	116 (133)	148 (129)	137 (133)
MDMA	83 (2)	102 (3)	109 (3)	109 (3)	90 (14)	96 (9)	102 (10)	111 (5)
METH	99 (5)	101 (7)	103 (7)	111 (1)	105 (3)	96 (4)	102 (6)	112 (6)
MPH	94 (1)	106 (2)	99 (8)	109 (1)	93 (7)	100 (4)	99 (6)	108 (6)
RA	104 (4)	103 (3)	96 (10)	111 (3)	102 (5)	94 (9)	96 (4)	113 (4)

Abbreviations: AMPH, amphetamine; BZE: benzoylecgonine; CE, cocaethylene; COC, cocaine; CV, coefficient of variation; EtS, ethyl sulfate; HMMA, 4-hydroxy-3-methoxymethamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; mid, medium; MPH, methylphenidate; n.d., not detected; RA, ritalinic acid; QC, quality control.

3.3 | Application of the method to analyze WW samples for proof-of-concept

Tables 5 and 6 show the results of the analysis of six WW grab samples obtained from two different sites (one and two). Analysis of the samples allowed the identification and quantification of the DOA and the cognitive enhancers included in the presented method. AMPH, METH, MDMA, COC, and its metabolite BZE were mainly detected in the samples originating from site two. Detecting only little concentrations of METH compared with AMPH is in accordance with previously published data by Ort et al.[7] describing the differences in AMPH and METH distribution in Europe. In samples from both sites, MPH and its metabolite RA could be identified, which could originate from patients with ADHD, as MPH is the most commonly prescribed medication for ADHD in Germany.[19] However, MPH also is misused as a cognitive enhancer.[20]

TABLE 5 Concentrations of drugs of abuse and cognitive enhancers in wastewater grab samples, analyzed using the C_{18} column.

		Analyte concentration, ng/L									
Sampling month	Sampling site	AMPH	BZE	CE	COC	EtS	HMMA	MDMA	METH	MPH	RA
JUN 21	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
	two	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	13	18
DEC 21	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	23
	two	88	<30	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	14
APR 22	one	n. d.	<30	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	<10	14
	two	>100	<30	n. d.	22	n. d.	n. d.	>100	19	<10	>100
AUG 22	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	<10	n. d.	n. d.	n. d.
	two	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
NOV 22	one	n. d.	120	n. d.	17	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
	two	>100	56	n. d.	<10	n. d.	n. d.	n. d.	n. d.	n. d.	>100
MAR 23	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	44	n. d.	15	>100
	two	56	n. d.	n. d.	14	n. d.	n. d.	<10	n. d.	n. d.	n. d.

Abbreviations: AMPH, amphetamine; BZE, benzoylecgonine; CE, cocaethylene; COC, cocaine; EtS, ethyl sulfate; HMMA, 4-hydroxy-3-methoxymethamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; MPH, methylphenidate; n. d., not detected; one, sampling site one; RA, ritalinic acid; two, sampling site two.

TABLE 6 Concentrations of DOA and cognitive enhancers in wastewater grab samples, analyzed using the hydrophilic interaction liquid chromatography column.

		Analyte concentration, ng/L									
Sampling month	Sampling site	AMPH	BZE	CE	COC	EtS	HMMA	MDMA	METH	MPH	RA
JUN 21	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
	two	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	18
DEC 21	one	n. d.	<30	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	<10	24
	two	>100	40	n. d.	17	n. d.	n. d.	n. d.	n. d.	n. d.	17
APR 22	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	<10	14
	two	>100	<30	n. d.	22	n. d.	n. d.	>100	n. d.	n. d.	>100
AUG 22	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	<10	n. d.	n. d.	23
	two	27	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	11
NOV 22	one	n. d.	121	n. d.	17	n. d.	n. d.	n. d.	n. d.	n. d.	36
	two	n. d.	57	n. d.	<10	n. d.	n. d.	n. d.	n. d.	n. d.	>100
MAR 23	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	44	n. d.	15	>100
	two	56	n. d.	n. d.	12	n. d.	n. d.	<10	n. d.	n. d.	28

Abbreviations: AMPH, amphetamine; AW, sampling site one; BZE, benzoylecgonine; CE, cocaethylene; COC, cocaine; DOA, drugs of abuse; EtS, ethyl sulfate; HKW, sampling site two; HMMA, 4-hydroxy-3-methoxymethamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; MPH, methylphenidate; n. d., not detected; one, sampling site one; RA, ritalinic acid; two, sampling site two.

3.4 | LC-HRMS/MS analysis and impact of the different LC columns

For both columns, sufficient chromatographic separation should be achieved within short runtimes. Using the C_{18} column, an analytical run was performed within 10 min, including a washout phase. Furthermore, only three pairs of coeluting analytes were present for the C_{18} compared with the HILIC. Separation of analytes was possible within the first 5 min of a run. For the HILIC column, separation of analytes was performed within 15 min, also including a washout phase. However, baseline separation of analytes could not be achieved as there were five clusters of coeluting analytes. Figures 1 and 2 show the chromatographic separation of all analytes and IS included in the method validation at the LLOQ.

Different MS settings were tested during method development, whereas the PRM mode with adjusted collision energies and time windows was able to provide highest sensitivity with the lowest LOIs (see Table S2).

Although most analytes fulfilled all validation criteria across both columns, differences were observed. In terms of analysis time, the C_{18}

column allowed shorter runtimes compared with the HILIC. Furthermore, three analytes did not fulfill all validation criteria using the C_{18} column (METH, MDMA, and HMMA), whereas five analytes did not fulfill at least one criterium after HILIC separation (AMPH, BZE, HMMA, MDMA, and EtS). Regarding the peak shape, the HILIC column showed better peak shapes compared with the C_{18} column. Because HILIC columns are better suited for analysis of polar compounds, it might be expected that the performance separation of metabolites would be better compared with the C_{18} column. When considering the accuracy and precision results, relative mean concentrations after analysis across both columns showed no major differences. Only for AMPH, the results of the QC LLOQ after HILIC separation deviated from the C_{18} column. Comparing the concentrations determined in the proof-of concept samples (see Tables 5 and 6), differences between the values obtained with both columns for the same samples were observed. Concentrations for AMPH and RA were in general higher when quantified after HILIC separation compared with C_{18} separation. MPH and COC concentrations were slightly higher when analyzed after C_{18} separation.

4 | CONCLUSIONS

Two methods for qualitative and quantitative analysis of four DOA, one cognitive enhancer, and three of their biomarkers in WW was successfully developed and validated using RP-LC and HILIC. One common simple sample preparation via SPE was used, and analysis via LC-HRMS/MS was possible within short run times of 10 (C_{18} column) or 15 min (HILIC column). Validation results showed advantages of the C_{18} over the HILIC column, as only METH and HMMA did not fulfill all validation parameters via the C_{18}, whereas AMPH, BZE, EtS, and HMMA did not fulfill the criteria set by the ICH using the HILIC. Thus, only HMMA could not by reliably quantified. The used SPE did not allow the extraction of EtS, but its analysis should be possible by dilute and shoot as shown by Mastroianni et al.[21] or Rodríguez-Álvarez et al.[22] A two-column setup is necessary for the analysis of all analytes included in this method, because on neither column, all analytes met the criteria set by the ICH (see Table S8). Evaluation of proof-of-concept samples allowed the quantification of several analytes in the WW grab samples. The presented method is thus ready to be used for monitoring trends in the consumption of DOA and cognitive enhancer in WW samples.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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REFERENCES

1. EMCDDA. *European Drug Report 2022*. Publications of of the European Union; 2022.
2. Wilms W, Wozniak-Karczewska M, Corvini PF, Chrzanowski L. Nootropic drugs: methylphenidate, modafinil and piracetam—population use trends, occurrence in the environment, ecotoxicity and removal methods—a review. *Chemosphere*. 2019;233:771-785. doi:10.1016/j.chemosphere.2019.06.016
3. Gracia-Lor E, Castiglioni S, Bade R, et al. Measuring biomarkers in wastewater as a new source of epidemiological information: current state and future perspectives. *Environ Int*. 2017;99:131-150. doi:10.1016/j.envint.2016.12.016
4. Castiglioni S, Zuccato E, Chiabrando C, Fanelli R, Bagnati R. Mass spectrometric analysis of illicit drugs in wastewater and surface water. *Mass Spectrom Rev*. 2008;27(4):378-394. doi:10.1002/mas.20168
5. Mardal M, Kinyua J, Ramin P, 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 Test Anal*. 2017;9(1):106-114. doi:10.1002/dta.1957
6. Meyer MR, Vollerthun T, Hasselbach R. Prevalence and distribution patterns of amphetamine and methamphetamine consumption in a federal state in southwestern Germany using wastewater analysis. *Drug Alcohol Depend*. 2015;156:311-314. doi:10.1016/j.drugalcdep.2015.09.006
7. Ort C, van Nuijs AL, Berset JD, et al. Spatial differences and temporal changes in illicit drug use in Europe quantified by wastewater analysis. *Addiction*. 2014;109(8):1338-1352. doi:10.1111/add.12570
8. Chen C, Kostakis C, Gerber JP, Tscharke BJ, Irvine RJ, White JM. Towards finding a population biomarker for wastewater epidemiology studies. *Sci Total Environ*. 2014;487:621-628. doi:10.1016/j.scitotenv.2013.11.075
9. Nguyen HP, Schug KA. The advantages of ESI-MS detection in conjunction with HILIC mode separations: fundamentals and applications. *J Sep Sci*. 2008;31(9):1465-1480. doi:10.1002/jssc.200700630
10. Gray N, Heaton J, Musenga A, Cowan DA, Plumb RS, Smith NW. Comparison of reversed-phase and hydrophilic interaction liquid chromatography for the quantification of ephedrines using medium-resolution accurate mass spectrometry. *J Chromatogr a*. 2013;1289:37-46. doi:10.1016/j.chroma.2013.03.021
11. Subirats X, Abraham MH, Roses M. Characterization of hydrophilic interaction liquid chromatography retention by a linear free energy relationship. Comparison to reversed- and normal-phase retentions. *Anal Chim Acta*. 2019;1092:132-143. doi:10.1016/j.aca.2019.09.010
12. van Nuijs AL, Tarcomnicu I, Simons W, et al. Optimization and validation of a hydrophilic interaction liquid chromatography-tandem mass spectrometry method for the determination of 13 top-prescribed pharmaceuticals in influent wastewater. *Anal Bioanal Chem*. 2010;398(5):2211-2222. doi:10.1007/s00216-010-4101-1
13. van Nuijs AL, Tarcomnicu I, Bervoets L, et al. Analysis of drugs of abuse in wastewater by hydrophilic interaction liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem*. 2009;395(3):819-828. doi:10.1007/s00216-009-3017-0
14. EMA. *ICH Guideline M10 on Bioanalytical Method Validation and Study Sample Analysis*. In Vol EMA/CHMP/ICH/172948/2019. 2022. European Medicines Agency; 2022.
15. Maurer HH, Meyer MR, Helfer AG, Weber AA. *Maurer/Meyer/Helfer/-Weber MMHW LC-HR-MS/MS library of drugs, poisons, and their metabolites*. Wiley-VCH; 2018.

16. EMA. *Guideline on Bioanalytical Method Validation*. In. Vol EMEA/CHMP/EWP/192217/2009 Rev.1 Corr.2. 2015. European Medicines Agency; 2011.
17. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal Chem*. 2003;75(13):3019-3030. doi:10.1021/ac020361s
18. Remane D, Meyer MR, Wissenbach DK, Maurer HH. Ion suppression and enhancement effects of co-eluting analytes in multi-analyte approaches: systematic investigation using ultra-high-performance liquid chromatography/mass spectrometry with atmospheric-pressure chemical ionization or electrospray ionization. *Rapid Commun Mass Spectrom*. 2010;24(21):3103-3108. doi:10.1002/rcm.4736
19. Schwabe U, Ludwig WD. *Arzneiverordnungs-Report 2020*. Springer; 2020. 10.1007/978-3-662-62168-4
20. Finger G, Silva ER, Falavigna A. Use of methylphenidate among medical students: a systematic review. *Rev Assoc Med Bras (1992)*. 2013;59(3):285-289. doi:10.1016/j.ramb.2012.10.007
21. Mastroianni N, Lopez de Alda M, Barcelo D. Analysis of ethyl sulfate in raw wastewater for estimation of alcohol consumption and its correlation with drugs of abuse in the city of Barcelona. *J Chromatogr a*. 2014;1360:93-99. doi:10.1016/j.chroma.2014.07.051
22. Rodriguez-Alvarez T, Racamonde I, Gonzalez-Marino I, et al. Alcohol and cocaine co-consumption in two European cities assessed by wastewater analysis. *Sci Total Environ*. 2015;536:91-98. doi:10.1016/j.scitotenv.2015.07.016

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Quantitative Analysis of Drugs of Abuse and Cognitive Enhancers in Influent Wastewater by Means of Two Chromatographic Methods.

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Highlights:

- Quantification of drugs of abuse and cognitive enhancers in wastewater
- Method development using two different analytical columns
- Comparison of analytical columns concerning their analytical power

Keywords: Wastewater analysis, drugs of abuse, cognitive enhancers, LC-HRMS/MS

Table S1

Final concentrations of the calibrators (Cal) and quality control (QC) samples, lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) in surface water, concentrations are given in ng/L (except for Ethyl sulfate (EtS), µg/L); mid: medium; AMPH: amphetamine; BZE: benzoylecgonine; CE: cocaethylene; COC: cocaine; EtS: ethyl sulfate; HMMA: 4-hydroxy-3-methoxymethamphetamine; MDMA: 3,4-methylenedioxymethamphetamine; METH: methamphetamine; MPH: methylphenidate; RA: ritalinic acid

Analyte	Cal 1 (LLOQ)	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6 (ULOQ)	QC LLOQ	QC low	QC mid	QC high
AMPH	10	20	40	60	80	100	10	30	50	90
BZE	30	60	120	180	240	300	30	90	150	270
CE	10	20	40	60	80	100	10	30	50	90
COC	10	20	40	60	80	100	10	30	50	90
EtS	200	400	800	1200	1600	2000	200	600	1000	1800
HMMA	30	60	120	180	240	300	30	90	150	270
MDMA	10	20	40	60	80	100	10	30	50	90
METH	10	20	40	60	80	100	10	30	50	90
MPH	10	20	40	60	80	100	10	30	50	90
RA	10	20	40	60	80	100	10	30	50	90

Table S2

Analytes and internal standards included in the method validation, the *m/z* of their precursor and quantifier ions, HILIC: hydrophilic interaction liquid chromatography; limit of identification (LOI) and retention times (RT) on both columns; AMPH: amphetamine; BZE: benzoylecgonine; CE: cocaethylene; COC: cocaine; EtS: ethyl sulfate; HMMA: 4-hydroxy-3-methoxymethamphetamine; MDMA: 3,4-methylenedioxymethamphetamine; METH: methamphetamine; MPH: methylphenidate; n. a.: not available; n. t.: not tested; RA: ritalinic acid

Analyte	Precursor ion, *m/z*	Quantifier ion, *m/z*	LOI, [ng/L]	RT (HILIC, min)	RT (C_{18}, min)	NCE [eV]	Ionization
AMPH	136.1121	91.0542	10	5.36	1.75	40	positive
AMPH-d_5	141.1435	93.0672	n. t.	5.36	1.75	40	positive
BZE	290.1387	168.1019	1	2.26	3.25	30	positive
BZE-d_3	293.1575	171.1205	n. t.	2.26	3.25	30	positive
CE	318.1700	196.1332	1	0.92	4.72	30	positive
CE-d_8	326.2202	204.1834	n. t.	0.92	4.72	30	positive
COC	304.1543	182.1175	1	0.95	4.33	30	positive
COC -d_3	307.1732	185.1363	n. t.	0.95	4.33	30	positive
EtS	124.9914	96.9590	5	5.87	n. a.	10	negative
EtS-d_5	130.0228	97.9650	n. t.	5.87	n. a.	10	negative
HMMA	196.1332	165.0910	1	5.44	1.02	40	positive
MDMA	194.1176	163.0753	1	4.64	2.04	40	positive
MDMA-d_5	199.1489	165.0879	n. t.	4.64	2.04	40	positive
METH	150.1277	91.0542	1	4.64	2.04	40	positive
METH-d_5	155.1591	92.0608	n. t.	4.64	2.04	40	positive
MPH	234.1489	84.0814	1	1.95	4.20	30	positive
MPH-d_9	243.2053	93.1377	n. t.	1.95	4.20	30	positive
RA	220.1332	84.0814	1	6.32	3.17	30	positive
RA-d_{10}	230.1960	93.1377	n. t.	6.32	3.17	30	positive

Table S3

Dilution integrity after dilution with surface water 1:10 (n=5); AMPH: amphetamine; BZE: benzoylecgonine; CE: cocaethylene; COC: cocaine; CV: coefficient of variation; EtS: ethyl sulfate; HILIC: hydrophilic interaction liquid chromatography; HMMA: 4-hydroxy-3-methoxymethamphetamine; MDMA: 3,4-methylenedioxymethamphetamine; METH: methamphetamine; MPH: methylphenidate; n. a.: not applicable; n.d.: not detected; RA: ritalinic acid

Analyte	Relative mean concentration, % (CV, %)	
	C_{18}	HILIC
AMPH	104 (7)	107 (3)
BZE	101 (4)	100 (2)
CE	103 (5)	100 (3)
COC	105 (2)	101 (3)
EtS	n. a.	IS n.d.
HMMA	114 (3)	55 (22)
MDMA	108 (4)	113 (1)
METH	102 (4)	105 (5)
MPH	104 (5)	101 (2)
RA	107 (9)	96 (8)

Table S4

Ion enhancement or suppression of all coeluting analytes and internal standards, analyzed for both columns; AMPH: amphetamine; BZE: benzoylecgonine; CE: cocaethylene; CV: coefficient of variation; COC: cocaine; EtS: ethyl sulfate; HILIC: hydrophilic interaction liquid chromatography; HMMA: 4-hydroxy-3-methoxymethamphetamine; MDMA: 3,4-methylenedioxymethamphetamine; METH: methamphetamine; MPH: methylphenidate; n. a.: not available; n. c.: no coeluting observed for this column; RA: ritalinic acid

Analyte	Ion enhancement / suppression, % (CV, %)	
	C_{18}	HILIC
AMPH	13 (7)	3 (5)
AMPH-d_5	3 (2)	-12 (3)
BZE	1 (15)	-4 (9)
BZE-d_3	1 (5)	-15 (5)
CE	55 (5)	2 (8)
CE-d_8	2 (5)	-9 (7)
COC	5 (13)	-10 (7)
COC -d_3	-2 (3)	-6 (4)
EtS	n. a.	-25 (8)
EtS-d_5	n. a.	-38 (8)
HMMA	n. c.	6 (6)
MDMA	14 (4)	-4 (5)
MDMA-d_5	-10 (13)	-3 (6)
METH	4 (14)	2 (9)
METH-d_5	-9 (5)	9 (1)
MPH	30 (6)	7 (4)
MPH-d_9	3 (6)	-7 (5)
RA	7 (2)	3 (14)
RA-d_{10}	-6 (3)	16 (14)

Table S5

Stability in the autosampler over 24h at 10°C, on both columns; AMPH: amphetamine; BZE: benzoylecgonine; CE: cocaethylene; COC: cocaine; CV: coefficient of variation; EtS: ethyl sulfate; HILIC: hydrophilic interaction liquid chromatography; HMMA: 4-hydroxy-3-methoxymethamphetamine; IS: internal standard; MDMA: 3,4-methylenedioxymethamphetamine; METH: methamphetamine; MPH: methylphenidate; n. a.: not available; n.d.: not detected; RA: ritalinic acid; QC: quality control

	Relative mean concentration, % (CV, %)							
Analyte	C_{18} QC low		C_{18} QC high		HILIC QC low		HILIC QC high	
	0h	24h 10°C	0h	24h 10°C	0h	24h 10°C	0h	24h 10°C
AMPH	93 (4)	101 (4)	110 (2)	107 (3)	103 (7)	98 (7)	104 (4)	100 (6)
BZE	111 (1)	107 (2)	110 (2)	113 (1)	111 (2)	113 (0)	113 (1)	110 (3)
CE	109 (3)	111 (1)	114 (1)	114 (1)	109 (5)	107 (1)	114 (0)	108 (2)
COC	87 (2)	102 (4)	110 (3)	108 (5)	109 (2)	104 (1)	114 (1)	109 (2)
EtS	n. a.	n. a.	n. a.	n. a.	IS n. d.	IS n. d.	IS n. d.	IS n. d.
HMMA	112 (19)	97 (4)	159 (6)	81 (10)	109 (92)	67 (54)	126 (13)	234 (107)
MDMA	110 (2)	107 (4)	113 (2)	111 (2)	105 (5)	89 (3)	111 (2)	95 (14)
METH	145 (13)	99 (5)	n. d.	n. d.	114 (0)	110 (5)	110 (2)	104 (13)
MPH	109 (3)	111 (1)	103 (5)	115 (2)	107 (2)	105 (6)	111 (2)	108 (4)
RA	107 (2)	93 (8)	111 (2)	100 (7)	111 (2)	114 (1)	110 (2)	111 (2)

Table S6

Freeze thaw stability over three 24h cycles at -22°C (n=3), analyzed using the hydrophilic interaction liquid chromatography (HILIC) column; AMPH: amphetamine; BZE: benzoylecgonine; CE: cocaethylene; COC: cocaine; CV: coefficient of variation; EtS: ethyl sulfate; HMMA: 4-hydroxy-3-methoxymethamphetamine; MDMA: 3,4-methylenedioxymethamphetamine; METH: methamphetamine; MPH: methylphenidate; n.d.: not detected; RA: ritalinic acid; QC: quality control

	Relative mean concentration, % (CV, %)							
Analyte	QC low				QC high			
	0 h	24 h -24 °C	48 h -24 °C	72 h -24 °C	0 h	24 h -24 °C	48 h -24 °C	72 h -24 °C
AMPH	99 (7)	97 (3)	108 (4)	100 (2)	104 (4)	109 (3)	113 (1)	98 (10)
BZE	112 (2)	110 (1)	100 (9)	106 (4)	113 (1)	103 (14)	109 (7)	99 (11)
CE	110 (5)	100 (2)	98 (9)	104 (3)	114 (1)	113 (1)	110 (4)	115 (2)
COC	110 (2)	98 (4)	99 (7)	103 (3)	114 (1)	113 (1)	105 (10)	95 (12)
EtS	IS n.d.	IS n.d.	IS n.d.	IS n.d.	IS n.d.	IS n.d.	IS n.d.	IS n.d.
HMMA	181 (56)	73 (21)	75 (2)	138 (52)	126 (13)	28 (66)	142 (57)	64 (21)
MDMA	107 (5)	93 (5)	105 (8)	95 (11)	111 (2)	86 (1)	107 (3)	98 (9)
METH	114 (1)	110 (15)	93 (4)	229 (73)	110 (2)	111 (1)	105 (13)	126 (7)
MPH	109 (2)	103 (5)	96 (4)	104 (5)	111 (2)	104 (10)	113 (1)	97 (12)
RA	112 (2)	113 (1)	99 (11)	111 (2)	110 (2)	113 (1)	111 (4)	98 (10)

Table S7

Long term stability, samples were stored 106 days (d) at -22°C and analyzed on hydrophilic interaction liquid chromatography (HILIC) column; AMPH: amphetamine; BZE: benzoylecgonine; CE: cocaethylene; COC: cocaine; CV: coefficient of variation; EtS: ethyl sulfate; HMMA: 4-hydroxy-3-methoxymethamphetamine; MDMA: 3,4-methylenedioxymethamphetamine; METH: methamphetamine; MPH: methylphenidate; n.d.: not detected; RA: ritalinic acid; QC: quality control

	Relative mean concentration, % (CV, %)			
Analyte	QC low		QC high	
	0 d	106 d	0 d	106 d
AMPH	99 (7)	107 (2)	99 (7)	107 (2)
BZE	112 (2)	102 (4)	112 (2)	102 (4)
CE	110 (5)	100 (4)	110 (5)	100 (4)
COC	110 (2)	99 (3)	110 (2)	99 (3)
EtS	IS n. d.	IS n. d.	IS n. d.	IS n. d.
HMMA	147 (36)	88 (20)	147 (36)	88 (20)
MDMA	107 (5)	19 (70)	107 (5)	19 (70)
METH	114 (10)	92 (11)	114 (10)	92 (11)
MPH	109 (2)	100 (5)	109 (2)	100 (5)
RA	112 (2)	95 (5)	112 (2)	95 (5)

Table S8

Fulfillment of all validation parameters on both columns; AMPH: amphetamine; BZE: benzoylecgonine; CE: cocaethylene; COC: cocaine; EtS: ethyl sulfate; HILIC: hydrophilic interaction liquid chromatography; HMMA: 4-hydroxy-3-methoxymethamphetamine; MDMA: 3,4-methylenedioxymethamphetamine; METH: methamphetamine; MPH: methylphenidate; n. a.: not applicable; RA: ritalinic acid

Analyte	All validation parameters fulfilled	
	C_{18}	HILIC
AMPH	yes	no
BZE	yes	no
CE	yes	yes
COC	yes	yes
EtS	n. a.	no
HMMA	no	no
MDMA	yes	yes
METH	no	yes
MPH	yes	yes
RA	yes	yes

4.2. Prevalence of Drugs of Abuse and Cognitive Enhancer Consumption Monitored in Grab Samples and Composite Wastewater via Orbitrap Mass Spectrometry Analysis [65]

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Author contribution

F.F.: formal analysis, investigation, data curation, methodology, writing-original draft preparation, writing-review and editing, visualization; C.M.J.: methodology, writing-review and editing; L.W.: conceptualization, writing-review and editing; M.R.M.: conceptualization, resources, writing-review and editing, supervision, project administration.

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MDPI

Brief Report

Prevalence of Drugs of Abuse and Cognitive Enhancer Consumption Monitored in Grab Samples and Composite Wastewater via Orbitrap Mass Spectrometry Analysis

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Abstract: Wastewater (WW)-based epidemiology is an approach for the objective surveillance of the consumption of (illicit) drugs in populations. The aims of this study were to monitor drugs of abuse, cognitive enhancers, and their metabolites as biomarkers in influent WW. Data obtained from different sampling points and mean daily loads were compared with previously published data. The prevalence of analytes was monitored in WW grab samples collected monthly over 22 months at two sampling points and 24 h composite WW samples collected over 2 weeks at a WW treatment plant in the same city. Quantification was performed using a previously validated and published method based on solid-phase extraction followed by liquid chromatography coupled with high-resolution tandem mass spectrometry. Grab samples allowed for frequent detection of ritalinic acid and sporadic detection of drugs of abuse. The daily mean loads calculated for 24 h WW composite samples were in accordance with data published in an international study. Furthermore, loads of amphetamine and methamphetamine increased compared with those observed in a previously published study from 2014. This study showed frequent quantification of ritalinic acid in the grab samples, while drugs of abuse were commonly quantified in the composite WW samples. Daily mean loads were in accordance with trends reported for Germany.

Keywords: wastewater-based epidemiology; wastewater surveillance; drugs of abuse; cognitive enhancers; LC-HRMS/MS

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1. Introduction

The availability and prevalence of illicit drugs remains high. The drug-use report of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported that approximately 83 million Europeans aged between 15 and 64 have used illicit drugs in their life [1,2]. Amongst the most used drugs of abuse (DOA) in the European union are stimulants such as amphetamine (AMPH), 3,4-methylendioxymethamphetamine (MDMA), and cocaine (COC). In addition to DOA, prescription drugs such as methylphenidate (MPH) are abused as cognitive enhancers for stress release or performance enhancement [3]. Serious health risks are associated with acute and chronic abuse [4]. Hence, surveillance of consumption patterns and trends is of importance. Wastewater (WW)-based epidemiology is an approach to monitoring drug consumption in a population, allowing monitoring of spatial, short-, and long-term trends [5,6]. Through this approach, intake of (illicit) drugs can be monitored after their excretion into WW by detecting parent compounds and/or metabolites as biomarkers. Different sampling techniques were described in the literature; they included composite sampling, which are typically average samples collected over 24 h, or grab samples only showing the load at one single time point. Additionally, sampling over consecutive days is advised to obtain representative results [7]. In 2011, the first Europe-wide WW investigation was conducted by the sewage analysis CORe group—Europe (SCORE), providing data about illicit drug consumption in 19 European cities. Since then,

annual reports have been published, including data from several German cities [8]. The aim of this study was the local monitoring of concentrations and loads of amphetamine, benzoylecgonine (BZE), cocaethylene (CE), cocaine, MDMA, methamphetamine (METH), methylphenidate, and ritalinic acid (RA) in influent WW collected as grab or 24 h composite samples. Data obtained at the different sampling points and mean daily loads calculated for 24 h composite samples should be compared with previously published data to identify drug consumption trends.

2. Results and Discussion

Concentrations of AMPH, BZE, and MDMA were quantified using a C_{18} column and CE, COC, METH, MPH, and RA using a HILIC column. The development, validation, and results, based on which the concentration values of the monitored compounds were determined, have already been published [9]. The current method was operated at 5 ppm mass tolerance in positive ESI mode only, as all analytes could be detected sufficiently. It should be noted that some compounds such as ethyl sulfate can only be detected sufficiently by using negative ESI. Furthermore, negative ESI is often less prone to matrix effects than positive ESI [10]. The in-sewer (WW "surviving" time) stability of all analytes was already tested and found to be sufficient [11–13]. For preparation of the blank samples, purified water and surface water were used and they were found to be free of analytes.

2.1. Wastewater Grab Samples

Quantification results of all (n = 44) grab samples are summarized in Table S1 in the Electronic Supplementary Material (ESM). Figure 1 compares the 2022 results obtained at both sites. Methylphenidate (as MPH or RA) was the most frequently detected compound (39/44 samples). MPH concentrations ranged between 11 and 34 ng/L, while its metabolite RA ranged from 10 to 3869 ng/L. Cocaine (as COC or BZE) was the second most frequently detected in a total of 23/44 samples. COC ranged between 12 and 729 ng/L and BZE between 56 and 246 ng/L. AMPH appeared in 9/44 samples with concentrations between 56 and 1814 ng/L. MDMA was detected in 8/44 samples and quantified between 15 and 1155 ng/L. CE and METH could not be detected. Quantified values below the respective lower limits of quantification (LLOQ, 30 ng/L for BZE and 10 ng/L for MDMA) were specified as < "LLOQ" in Table S1. Calculated values below the LLOQ were not considered in Figure 1 and the comparison of the sampling sites. The frequent detection of RA may be due to the predominant prescription of MPH for ADHD patients in Germany [14]. However, misuse of MPH as a cognitive enhancer was also reported [15]. Moreover, Letzel et al. reported low removal rates of RA during sewage treatment, leading to RA concentrations in the ng/L range in effluent WW [11]. Effluent WW is released into rivers and other bodies; it can also be used to treat drinking water and then discharged into the sewage system, which ultimately might influence quantification results [16]. The absence of METH in the samples is in accordance with previously published data, showing only low METH concentrations in the western part of Germany [5,6]. As grab samples do not allow for normalization of concentrations and are only representative for the time of sampling, comparison with data from other studies is not possible. Furthermore, according to Ort et al., the absence of analytes in grab samples does not prove their total absence at this sampling site, since only the time point of sampling is represented [7].

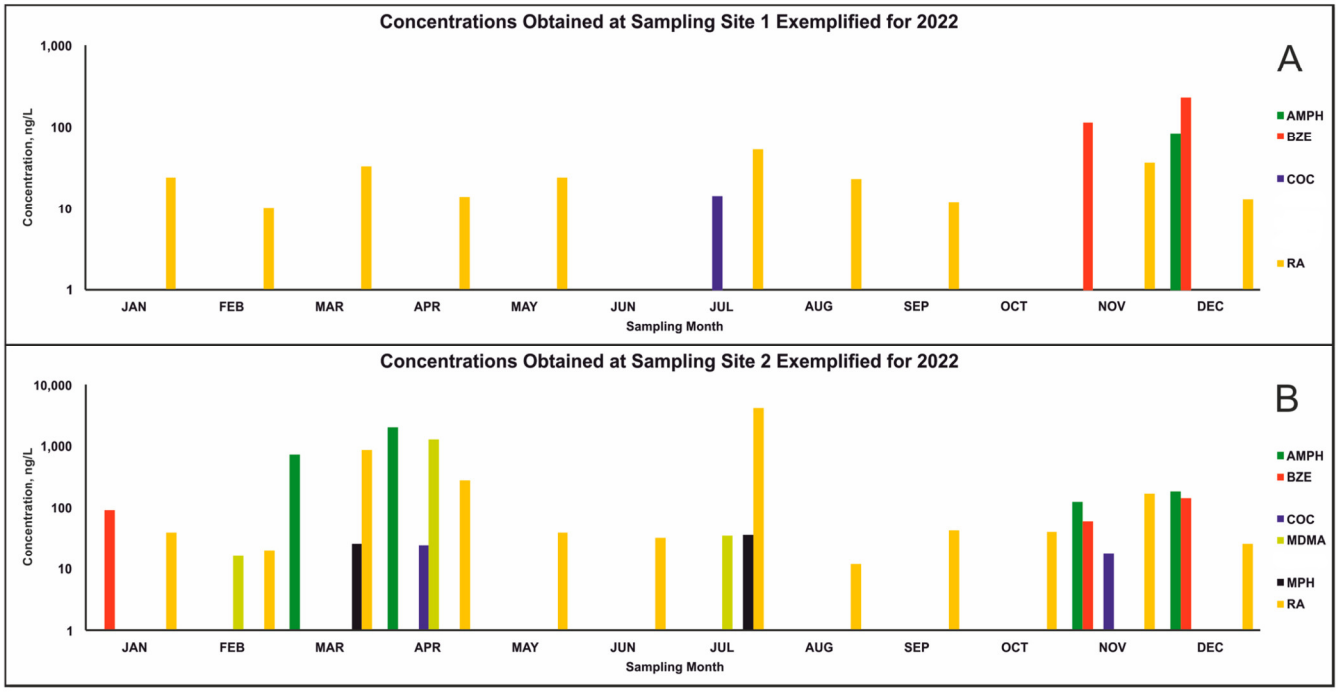

Figure 1. Concentrations of drugs of abuse, one cognitive enhancer, and their metabolites as biomarkers in wastewater grab samples exemplified for 2022, in logarithmic scale. (**A**) Results of sampling site one; (**B**), results of sampling site two. AMPH—amphetamine; BZE—benzoylecgonine; CE—cocaethylene; COC—cocaine; MDMA—3,4-methylenedioxymethamphetamine; METH—methamphetamine; MPH—methylphenidate; RA—ritalinic acid.

2.2. Twenty-Four Hour Composite Wastewater Samples

Daily loads (mg/day/1000 inhabitants) of all analytes, as well as daily, weekday, and weekend mean values are provided in Table S2 (ESM) and compared in Figure 2. AMPH, BZE, COC, and RA were found in all samples, while MDMA and METH were found in 5/14 and 11/14 samples, respectively. CE and MPH could not be detected. Comparison with a study by Meyer et al. showed an increase in the mean daily load of AMPH to 149 mg/day/1000 inhabitants compared with the median daily load of 46 mg/day/1000 inhabitants found in 2014 [6]. This observation is consistent with data provided by the EMCDDA and SCORE network [8]. Daily mean loads of AMPH in Saarbrucken (SB), which is also located in the federal state of Saarland, showed an even higher daily mean value of 346 mg/day/1000 inhabitants. Data from 2017 onwards showed increasing daily mean loads for AMPH in SB up to 2023, and highest AMPH loads in Europe were, amongst others, detected in German cities [8]. The daily mean load of METH was 5 mg/day/1000 inhabitants in this study, also showing an increase compared with the median published by Meyer et al. with 1.5 mg/day/1000 inhabitants [6]. These values are comparable to those obtained for SB (1.6 mg/day/1000 inhabitants), but below loads in the eastern part of Germany, e.g., Chemnitz, with 346 mg/day/1000 inhabitants [8]. MDMA (4 mg/day/1000 inhabitants) was also below the load obtained in SB (26 mg/day/1000 inhabitants) and below the lowest German daily mean (Munich, 7 mg/day/1000 inhabitants). Zuccato et al. proposed correction factors for AMPH and related substances and for BZE/COC, considering their excretion in humans [17]. Using these factors, the corrected daily mean load of AMPH would be 492 mg/day/1000 inhabitants, METH at 11.5 mg/day/1000 inhabitants, and MDMA at 6 mg/day/1000 inhabitants. Adjustment of METH and MDMA loads does not lead to a relevant change in comparison with loads provided by the EMCDDA, but the adjusted load of AMPH is higher compared with SB in the EMCDDA study [8]. Furthermore, a potential overestimation of AMPH due to METH metabolism, resulting in up to 7% AMPH, was discussed by van Nuijs et al. [18]. Due

to low METH loads and the mentioned distribution of AMPH and METH in Germany, an overestimation should not be of relevance. The daily mean load of COC was below the values provided for SB in the EMCDDA report, with 46 mg/day/1000 inhabitants in Homburg compared with 245 mg/day/1000 inhabitants in SB and below the lowest daily mean in Germany (Chemnitz, 57 mg/day/1000 inhabitants) [8]. Considering the daily mean load of BZE (86 mg/day/1000 inhabitants), the ratio of BZE to COC was 0.53. A study by Castiglioni et al. showed similar ratios of both analytes in WW samples in Italy and the USA [19]. Using the correction factor for BZE to estimate COC loads described by Zuccato et al., the mean daily load of COC would be at 200 mg/day/1000 inhabitants [17]. This corrected value is higher compared with the aforementioned load of COC using the quantified COC concentrations and closer to the load described for SB. Comparison of RA daily means (29 mg/day/1000 inhabitants) with a study by Letzel et al. showed comparable results for influent WW obtained at a WWTP in Southern Bavaria serving 26,000 inhabitants (23.7 mg/day/1000 inhabitants) [11]. During the reported sampling period, weekday means of AMPH, BZE, and COC were slightly higher compared with weekend means. This might be explained by the fact that there were no festivals on either weekend or that the only holiday was on a weekday (Thursday, 05/18/23).

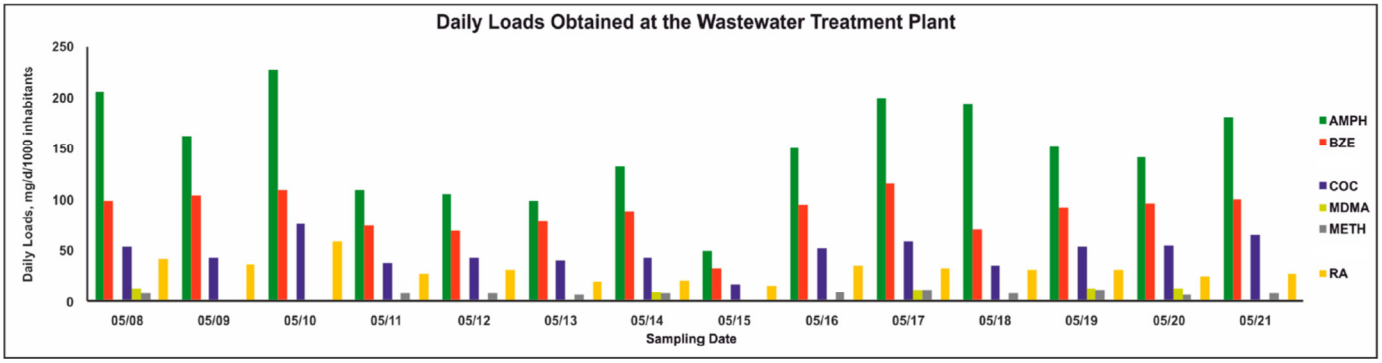

Figure 2. Daily loads of drugs of abuse, one cognitive enhancer, and their metabolites as biomarkers in 24 h composite wastewater samples throughout weeks 19 and 20 in May 2023. AMPH—amphetamine; BZE—benzoylecgonine; CE—cocaethylene; COC—cocaine; MDMA—3,4-methylenedioxymethamphetamine; METH—methamphetamine; MPH—methylphenidate; RA—ritalinic acid.

2.3. Comparison of Grab Samples and 24 h Composite Samples

Comparison of qualitative results obtained in both grab and 24 h composite samples revealed that AMPH, BZE, COC, and RA were continuously detectable in all 24 h composite samples. Grab samples allowed only sparse detection of most analytes, except RA, which could be detected in almost all samples (39/44). As already discussed in Section 2.1, this might be due to low removal rates [11]. METH was not detected using grab samples but was quantified in 24 h composite samples. This could be explained by only seldom and occasional consumption of METH within the monitored community. This would mean that METH was not detected in grab samples at this sampling time but may have been detected at another point in time. As CE was neither detected in grab nor 24 h composite samples, it is possible that no formation of CE took place during the monitored sampling time.

3. Materials and Methods

3.1. Reagents and Materials

Analytes and their deuterated analogues, used as internal standards (IS), were obtained from LGC (Wesel, Germany). All other chemicals were purchased from VWR (Darmstadt, Germany). Water was purified with a Millipore (Merck, Darmstadt, Germany) filtration unit, which purifies water to a resistance of 18.2 Ω × cm. Stock solutions, calibrator, IS mix, and quality control working solutions were prepared as described elsewhere [9].

3.2. Sample Preparation

Sample preparation via solid-phase extraction was performed according to a previously published procedure [9]: 10 mL WW samples were fortified with IS mix solution (final concentration 50 ng/L, except BZE-d_3 150 ng/L). Particles were removed using Phenex-PTFE 25-mm syringe filters 0.2 mm (Phenomenex, Aschaffenburg, Germany) and SPE was performed using Isolute 200 mg/10 mL (3 mL XL) HCX cartridges (Biotage, Uppsala, Sweden). The cartridges were primed with 1 mL methanol (MeOH) and 1 mL purified water, then cartridges were loaded with 10 mL of WW followed by a washing step using purified water, 0.1 M hydrochloric acid, and MeOH (1 mL each). Compounds were eluted using 1.25 mL of a MeOH/NH_3 mixture (35%, 98/2, v/v). Eluates were partitioned into two equal aliquots and evaporated to dryness under a gentle stream of nitrogen at 40 °C. Residues were reconstituted using a mixture of water and formic acid (50 µL, 99/1, v/v, RP C_{18} samples) or a mixture of acetonitrile and formic acid (50 µL, 99/1, v/v, HILIC samples). Calibration and quality control samples were prepared accordingly; final concentrations of calibrators and quality controls are given in Table S3.

3.3. Instrumental Settings

Samples were analyzed according to a previously published procedure with minor changes [9], using a Thermo Fisher Scientific (TF, Dreieich, Germany) Dionex UltiMate 3000 consisting of a degasser, a quaternary pump, a DL W2 wash system, and an HCT PAL autosampler (CTC Analytics AG, Zwinger, Switzerland). The system was coupled with a TF Q Exactive orbitrap mass spectrometer equipped with a heated electrospray ionization II source (HESI-II). Calibration was performed prior to analysis according to the manufacturer's recommendations using external mass calibration. The final conditions of the LC-system using the C_{18} column were as follows: Waters AQUITY UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 µm; Milford, MA, USA); gradient elution was performed with 2 mM ammonium formate solution containing 0.1% (v/v) formic acid (eluent A) and 2 mM ammonium formate solution in ACN/MeOH (50/50, v/v) containing 0.1% (v/v) formic acid and 1% (v/v) water (eluent B). The flow rate was 0.500 mL/min with the following gradient settings: 0–1 min 85% A, 1–3 min to 40% A, 3–6 min to 30% A, 6–8 min 1% A, 8–9.2 min hold 1% A, 9.2–9.21 min to 85% A, 9.21–10 min hold to 85% A.

The final conditions of the LC system with the HILIC column were as follows: Merck SeQuant ZIC-cHILIC column (150 × 2.1 mm, 3 µm; Merck, Darmstadt, Germany); gradient elution with 200 mM aqueous ammonium acetate solution (eluent C) and ACN containing formic acid 0.1% (v/v) (eluent D). The flow rate was at 0.5 mL/min with the following gradient settings: 0–1 min hold to 1% C, 1–1.8 min to 10% C, 1.8–9 min hold to 10% C, 9–9.5 min to 60% C, 9.5–10.5 min hold to 60% C, 10.5–10.6 min to 1% C, and 10.6–15 min hold to 1% C. Chromatography on both columns was performed at 40 °C.

The final HESI-II source and MS conditions were as follows: ionization mode, positive; sheath gas, 60 arbitrary units (AU); auxiliary gas flow rate, 10 AU; spray voltage, 4.00 kV; auxiliary gas heater temperature, 320 °C; ion capillary temperature, 320 °C; and S-lens RF level, 60.0. Mass spectrometry experiments after C_{18} column separation were performed using parallel reaction monitoring (PRM) in positive mode with a scheduled inclusion list containing the precursor masses of interest, adjusted normalized collision energies (NCE). The settings for PRM experiments were as follows: resolution, 17,500; automatic gain control target, 2×10^5; maximum injection time, 250 ms; isolation window, 1 m/z; high-energy collisional dissociation with NCE 30, 40 eV. Mass spectrometry experiments after HILIC column separation were performed using PRM in positive mode as described for the C_{18} column. TF Xcalibur Qual Browser version 4.1.31.9 was used for data handling. Masses of the precursor ions (m/z) used for the inclusion list, polarity, and adjusted NCE were previously described [9] and are also given in Table S4. The MS2 spectra of all analytes are shown in Figure S1.

3.4. Sampling

3.4.1. Wastewater Grab Samples

WW grab samples (n = 44 in total) were obtained between June 2021 and March 2023 at two different sewers (sampling sites one and two) in the city of Homburg, Germany. Samples (80 mL total sample volume each) were acidified with acetic acid (0.1%, v/v) and stored at −22 °C until sample preparation. If quantification results were above the calibration range, samples were diluted 1:10 with blank surface water and then analyzed according to Sections 3.2 and 3.3.

3.4.2. Twenty-Four Hour Composite Wastewater Samples

In total, fourteen 24 h composite influent WW samples were obtained using an Endres + Hauser stationary water samples type asp-station 2000. The sampling point was located immediately after the grit chamber, and the total sample volume was 600 mL and consisted of twelve 50 mL samples collected every two hours. The sample interval was from 8 am to 8 am, throughout weeks 19 and 20 in May 2023 at the WW treatment plant (WWTP) in Homburg, Germany. Finally, 80 mL of each 24 h composite sample was acidified and stored as described in Section 3.4.1.

4. Conclusions

In the presented study, we analyzed concentrations of DOA, one cognitive enhancer, and their metabolites in influent WW samples by comparing grab samples from two different sampling sites and 24 h composite samples. RA, AMPH, BZE, and COC were the most frequently quantified analytes in grab samples. Compared with a previous study [6], 24 h composite samples revealed increasing loads of AMPH and METH at the same WWTP, which is in line with data published for other cities in Germany [8]. However, METH loads were lower compared with cities in the eastern part of Germany, due to the European AMPH/METH borders location Germany [6]. Furthermore, loads of COC and MDMA were also in accordance with values obtained in German cities [8]. Comparison of grab samples and 24 h composite samples indicated that composite samples provided more consistent detection and quantification results of analytes. These findings show the importance of the sampling strategies used in the context of WW-based epidemiology. The rise of DOA loads observed in Homburg in this study also underlines the importance of continuous WW surveillance to observe trends even in smaller communities.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules29163870/s1, Figure S1: MS2 spectra of all analytes; Table S1: Concentrations of drugs of abuse, one cognitive enhancer, and their metabolites as biomarkers in wastewater grab samples between June 2021 and March 2023.; Table S2: Daily loads of drugs of abuse, one cognitive enhancer, and their metabolites as biomarkers as well as daily, weekday, and weekend mean values in 24 h composite wastewater samples throughout weeks 19 and 20 in May 2023.; Table S3: Final concentrations of the calibrators (Cal) and quality control (QC) samples, lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) in surface water; Table S4: Analytes and internal standards included in the method validation, the m/z of their precursor and quantifier ions.

Author Contributions: Conceptualization, M.R.M. and L.W.; formal analysis, F.F.; investigation, F.F.; methodology, F.F. and C.M.J.; resources, M.R.M.; data curation, F.F.; writing—original draft preparation, F.F.; writing—review and editing, F.F., L.W., C.M.J. and M.R.M.; visualization, F.F.; supervision, M.R.M.; project administration, M.R.M. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

References

1. EMCDDA. *European Drug Report 2022*; Publications of of the European Union: Lisbon, Portugal, 2022.
2. EMCDDA. *European Drug Report 2023*; Publications of of the European Union: Lisbon, Portugal, 2023.
3. Wilms, W.; Woźniak-Karczewska, M.; Corvini, P.F.; Chrzanowski, Ł. Nootropic drugs: Methylphenidate, modafinil and piracetam—Population use trends, occurrence in the environment, ecotoxicity and removal methods—A review. *Chemosphere* **2019**, *233*, 771–785. [CrossRef] [PubMed]
4. Fox, T.P.; Oliver, G.; Ellis, S.M. The destructive capacity of drug abuse: An overview exploring the harmful potential of drug abuse both to the individual and to society. *ISRN Addict.* **2013**, *2013*, 450348. [CrossRef] [PubMed]
5. Ort, C.; van Nuijs, A.L.; Berset, J.D.; Bijlsma, L.; Castiglioni, S.; Covaci, A.; de Voogt, P.; Emke, E.; Fatta-Kassinos, D.; Griffiths, P.; et al. Spatial differences and temporal changes in illicit drug use in Europe quantified by wastewater analysis. *Addiction* **2014**, *109*, 1338–1352. [CrossRef] [PubMed]
6. Meyer, M.R.; Vollerthun, T.; Hasselbach, R. Prevalence and distribution patterns of amphetamine and methamphetamine consumption in a federal state in southwestern Germany using wastewater analysis. *Drug Alcohol Depend.* **2015**, *156*, 311–314. [CrossRef] [PubMed]
7. Ort, C.; Lawrence, M.G.; Rieckermann, J.; Joss, A. Sampling for pharmaceuticals and personal care products (PPCPs) and illicit drugs in wastewater systems: Are your conclusions valid? *A critical review. Environ. Sci. Technol.* **2010**, *44*, 6024–6035. [CrossRef] [PubMed]
8. EMCDDA. *Wastewater Analysis and Drugs—A European Multi-City Study*; Publications of of the European Union: Lisbon, Portugal, 2024.
9. Frankenfeld, F.; Wagmann, L.; Jacobs, C.M.; Meyer, M.R. Quantitative analysis of drugs of abuse and cognitive enhancers in influent wastewater by means of two chromatographic methods. *Drug Test. Anal.* **2023**. [CrossRef] [PubMed]
10. Helfer, A.G.; Meyer, M.R.; Michely, J.A.; Maurer, H.H. Direct analysis of the mushroom poisons alpha- and beta-amanitin in human urine using a novel on-line turbulent flow chromatography mode coupled to liquid chromatography-high resolution-mass spectrometry/mass spectrometry. *J. Chromatogr. A* **2014**, *1325*, 92–98. [CrossRef] [PubMed]
11. Letzel, M.; Weiss, K.; Schüssler, W.; Sengl, M. Occurrence and fate of the human pharmaceutical metabolite ritalinic acid in the aquatic system. *Chemosphere* **2010**, *81*, 1416–1422. [CrossRef] [PubMed]
12. Castiglioni, S.; Bijlsma, L.; Covaci, A.; Emke, E.; Hernández, F.; Reid, M.; Ort, C.; Thomas, K.V.; van Nuijs, A.L.N.; de Voogt, P.; et al. Evaluation of uncertainties associated with the determination of community drug use through the measurement of sewage drug biomarkers. *Environ. Sci. Technol.* **2013**, *47*, 1452–1460. [CrossRef] [PubMed]
13. van Nuijs, A.L.; Abdellati, K.; Bervoets, L.; Blust, R.; Jorens, P.G.; Neels, H.; Covaci, A. The stability of illicit drugs and metabolites in wastewater, an important issue for sewage epidemiology? *J. Hazard. Mater.* **2012**, *239–240*, 19–23. [CrossRef] [PubMed]
14. Ludwig, W.D.; Mühlbauer, B.; Seifert, R. *Arzneiverordnungs-Report 2023*; Springer: Berlin, Germany, 2023.
15. Finger, G.; Silva, E.R.; Falavigna, A. Use of methylphenidate among medical students: A systematic review (1992). *Rev. Assoc. Med. Bras.* **2013**, *59*, 285–289. [CrossRef] [PubMed]
16. DVGW. *Drug Residues in the Water Cycle; an Assessment from the Perspective of Drinking Water Supply*; DVGW information WATER No. 54; Deutscher Verein des Gas- und Wasserfachs: Bonn, Germany, 2015.
17. Zuccato, E.; Chiabrando, C.; Castiglioni, S.; Bagnati, R.; Fanelli, R. Estimating community drug abuse by wastewater analysis. *Environ. Health Perspect.* **2008**, *116*, 1027–1032. [CrossRef] [PubMed]
18. van Nuijs, A.L.; Castiglioni, S.; Tarcomnicu, I.; Postigo, C.; Lopez de Alda, M.; Neels, H.; Zuccato, E.; Barcelo, D.; Covaci, A. Illicit drug consumption estimations derived from wastewater analysis: A critical review. *Sci. Total Environ.* **2011**, *409*, 3564–3577. [CrossRef] [PubMed]
19. Castiglioni, S.; Bagnati, R.; Melis, M.; Panawennage, D.; Chiarelli, P.; Fanelli, R.; Zuccato, E. Identification of cocaine and its metabolites in urban wastewater and comparison with the human excretion profile in urine. *Water Res.* **2011**, *45*, 5141–5150. [CrossRef] [PubMed]

Prevalence of Drugs of Abuse and Cognitive Enhancer Consumption Monitored in Grab and Composite Wastewater by Means of Orbitrap Mass Spectrometry Analysis

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Table S1. Concentrations of drugs of abuse, one cognitive enhancer, and their metabolites as biomarkers in wastewater grab samples between June 2021 and March 2023. AMPH, amphetamine; BZE, benzoylecgonine; CE, cocaethylene; COC, cocaine; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; MPH, methylphenidate; n. d., not detected; RA, ritalinic acid; #, back calculated concentration obtained after 1:10 dilution of samples; *, concentration remaining above the calibration range after 1:10 dilution; concentrations below the respective lower limit of quantification (LLOQ) are given as "< LLOQ"

Sampling month	Sampling site	Analyte concentration, ng/L							
		AMPH	BZE	CE	COC	MDMA	METH	MPH	RA
06/21	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
	two	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	18
07/21	one	n. d.	< 30	n. d.	n. d.	n. d.	n. d.	n. d.	85
	two	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
08/21	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	16
	two	n. d.	< 30	n. d.	n. d.	n. d.	n. d.	n. d.	17
09/21	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	11	586#
	two	720#	246	n. d.	729#	n. d.	n. d.	n. d.	55
10/21	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	36
	two	1,385#*	n. d.	n. d.	22	n. d.	n. d.	n. d.	366#
11/21	one	n. d.	94	n. d.	12	16	n. d.	n. d.	23
	two	n. d.	241	n. d.	15	n. d.	n. d.	n. d.	483#
12/21	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	25
	two	88	< 30	n. d.	17	n. d.	n. d.	n. d.	17
01/22	one	n. d.	< 30	n. d.	n. d.	n. d.	n. d.	n. d.	24
	two	n. d.	83	n. d.	n. d.	n. d.	n. d.	n. d.	36
02/22	one	n. d.	< 30	n. d.	n. d.	n. d.	n. d.	n. d.	10
	two	n. d.	< 30	n. d.	n. d.	15	n. d.	n. d.	19
03/22	one	n. d.	< 30	n. d.	n. d.	n. d.	n. d.	n. d.	33
	two	665#	< 30	n. d.	n. d.	n. d.	n. d.	24	783#
04/22	one	n. d.	< 30	n. d.	n. d.	n. d.	n. d.	n. d.	14
	two	1,814#*	< 30	n. d.	23	1155#*	n. d.	n. d.	261#

Table S1. continued

Sampling month	Sampling site	Analyte concentration, ng/L							
		AMPH	BZE	CE	COC	MDMA	METH	MPH	RA
05/22	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	24
	two	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	36
06/22	one	n. d.	n. d.	n. d.	n. d.	< 10	n. d.	n. d.	n. d.
	two	n. d.	< 30	n. d.	n. d.	n. d.	n. d.	n. d.	30
07/22	one	n. d.	n. d.	n. d.	15	n. d.	n. d.	n. d.	53
	two	n. d.	n. d.	n. d.	n. d.	33	n. d.	34	3869[#]*
08/22	one	n. d.	n. d.	n. d.	n. d.	< 10	n. d.	n. d.	23
	two	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	11
09/22	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	12
	two	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	40
10/22	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
	two	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	38
11/22	one	n. d.	120	n. d.	n. d.	n. d.	n. d.	n. d.	36
	two	113[#]	56	n. d.	17	n. d.	n. d.	n. d.	155[#]
12/22	one	87	239	n. d.	n. d.	n. d.	n. d.	n. d.	13
	two	168[#]	131	n. d.	n. d.	n. d.	n. d.	n. d.	24
01/23	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
	two	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	31
02/23	one	n. d.	128	n. d.	n. d.	n. d.	n. d.	n. d.	18
	two	n. d.	n. d.	n. d.	n. d.	44	n. d.	n. d.	21
03/23	one	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	769[#]
	two	56	n. d.	n. d.	12	< 10	n. d.	n. d.	28

Table S2. Daily loads of drugs of abuse, one cognitive enhancer, and their metabolites as biomarkers as well as daily, weekday and weekend mean values in 24-h composite wastewater samples throughout weeks 19 and 20 in May 2023. AMPH, amphetamine; BZE, benzoylecgonine; CE, cocaethylene; COC, cocaine; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; MPH, methylphenidate; n. d., not detected; RA, ritalinic acid, -; not applicable.

Sampling date	Day	Daily loads, mg/day/1000 inhabitants							
		AMPH	BZE	CE	COC	MDMA	METH	MPH	RA
05/08/23	Monday	203	98	n. d.	51	12	6	n. d.	40
05/09/23	Tuesday	161	102	n. d.	42	n. d.	n. d.	n. d.	35
05/10/23	Wednesday	226	108	n. d.	73	n. d.	n. d.	n. d.	57
05/11/23	Thursday	107	72	n. d.	36	n. d.	6	n. d.	25
05/12/23	Friday	103	67	n. d.	41	n. d.	6	n. d.	30
05/13/23	Saturday	97	76	n. d.	39	n. d.	5	n. d.	18
05/14/23	Sunday	131	87	n. d.	41	8	6	n. d.	19
05/15/23	Monday	47	31	n. d.	15	n. d.	n. d.	n. d.	14
05/16/23	Tuesday	149	93	n. d.	51	n. d.	8	n. d.	34
05/17/23	Wednesday	196	114	n. d.	56	11	10	n. d.	31
05/18/23	Thursday	191	69	n. d.	33	n. d.	6	n. d.	30
05/19/23	Friday	150	91	n. d.	51	12	10	n. d.	30
05/20/23	Saturday	140	95	n. d.	53	11	5	n. d.	24
05/21/23	Sunday	179	99	n. d.	63	n. d.	7	n. d.	25
Daily mean	-	149	86	-	46	4	5	-	29
Weekday mean	-	153	84	-	45	3	5	-	33
Weekend mean	-	137	89	-	49	5	6	-	21

Table S3

Final concentrations of the calibrators (Cal) and quality control (QC) samples, lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) in surface water, concentrations are given in ng/L; mid: medium; AMPH: amphetamine; BZE: benzoylecgonine; CE: cocaethylene; COC: cocaine; MDMA: 3,4-methylenedioxymethamphetamine; METH: methamphetamine; MPH: methylphenidate; RA: ritalinic acid

Analyte	Cal 1 (LLOQ)	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6 (ULOQ)	QC LLOQ	QC low	QC mid	QC high
AMPH	10	20	40	60	80	100	10	30	50	90
BZE	30	60	120	180	240	300	30	90	150	270
CE	10	20	40	60	80	100	10	30	50	90
COC	10	20	40	60	80	100	10	30	50	90
MDMA	10	20	40	60	80	100	10	30	50	90
METH	10	20	40	60	80	100	10	30	50	90
MPH	10	20	40	60	80	100	10	30	50	90
RA	10	20	40	60	80	100	10	30	50	90

Table S4

Analytes and internal standards included in the method validation, the *m/z* of their precursor and quantifier ions, HILIC: hydrophilic interaction liquid chromatography; limit of identification (LOI) and retention times (RT) on both columns; AMPH: amphetamine; BZE: benzoylecgonine; CE: cocaethylene; COC: cocaine; MDMA: 3,4-methylenedioxymethamphetamine; METH: methamphetamine; MPH: methylphenidate; n. t.: not tested; RA: ritalinic acid

Analyte	Precursor ion, *m*	Quantifier ion, *m/*	LOI, [ng/L]	RT (HILIC, min	RT (C_{18}, min	NCE [eV
AMPH	136.1121	91.0542	10	5.36	1.75	40
AMPH-d_5	141.1435	93.0672	n. t.	5.36	1.75	40
BZE	290.1387	168.1019	1	2.26	3.25	30
BZE-d_3	293.1575	171.1205	n. t.	2.26	3.25	30
CE	318.1700	196.1332	1	0.92	4.72	30
CE-d_8	326.2202	204.1834	n. t.	0.92	4.72	30
COC	304.1543	182.1175	1	0.95	4.33	30
COC -d_3	307.1732	185.1363	n. t.	0.95	4.33	30
MDMA	194.1176	163.0753	1	4.64	2.04	40
MDMA-d_5	199.1489	165.0879	n. t.	4.64	2.04	40
METH	150.1277	91.0542	1	4.64	2.04	40
METH-d_5	155.1591	92.0608	n. t.	4.64	2.04	40
MPH	234.1489	84.0814	1	1.95	4.20	30
MPH-d_9	243.2053	93.1377	n. t.	1.95	4.20	30
RA	220.1332	84.0814	1	6.32	3.17	30
RA-d_{10}	230.1960	93.1377	n. t.	6.32	3.17	30

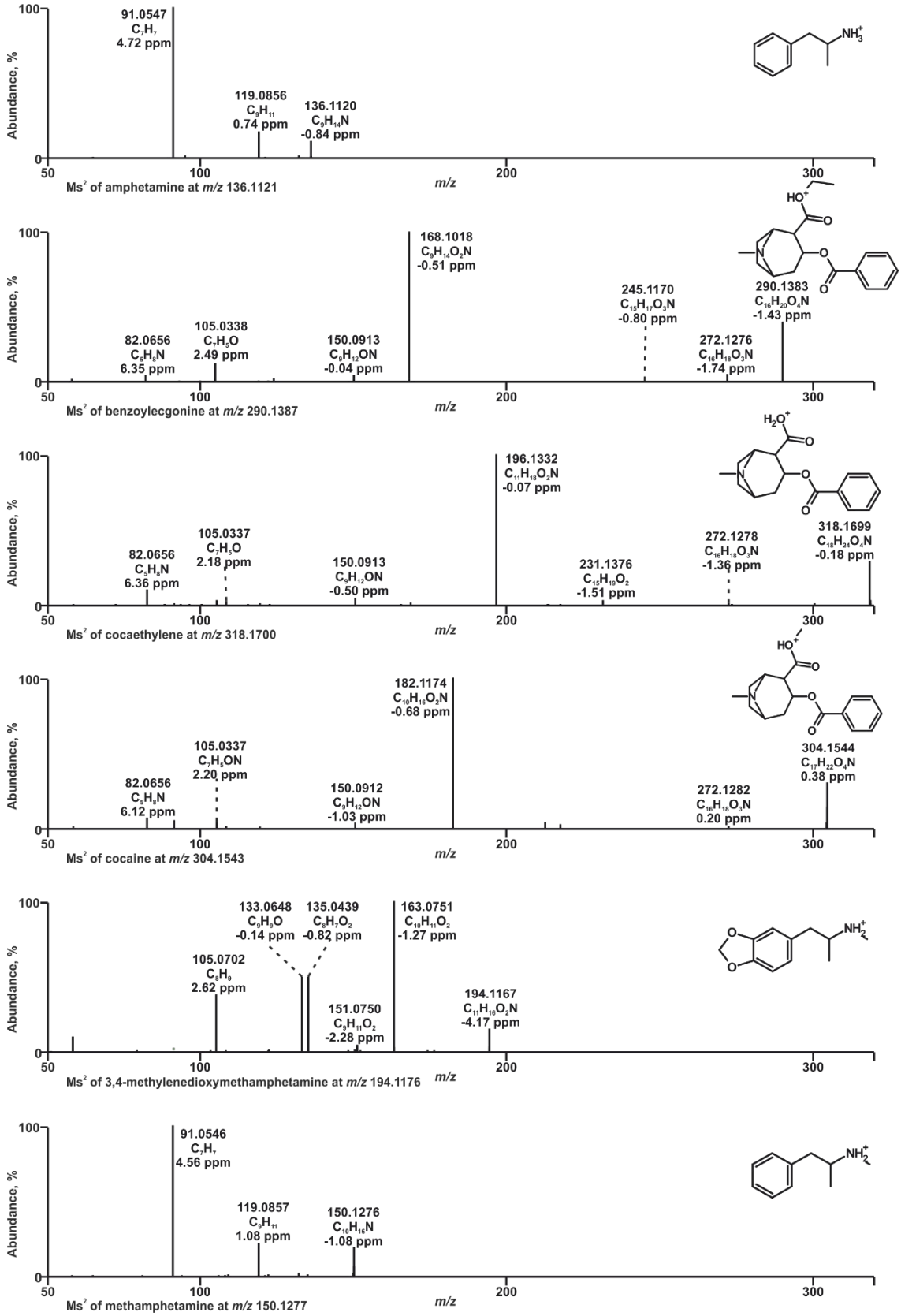

Figure S1. HRMS^2 spectra of the compounds of interest.

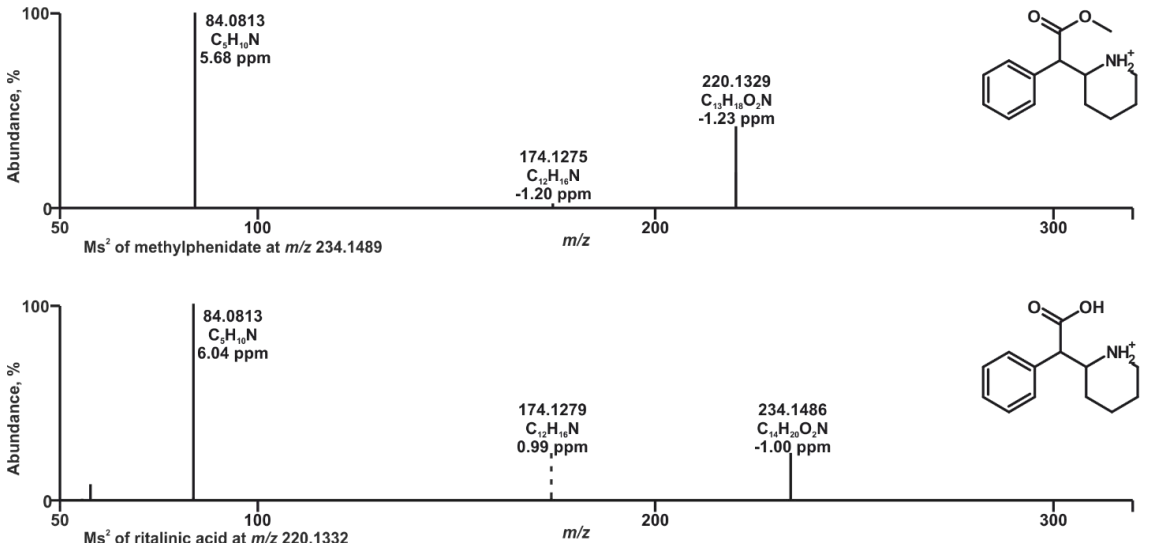

Figure S2. Continued.

4.3. In Vivo and In Vitro Metabolic Fate and Urinary Detectability of Five Deschloroketamine Derivatives Studied by Means of Hyphenated Mass Spectrometry [66]

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Author contribution

F.F.: formal analysis, investigation, data curation, writing-original draft preparation, writing-review and editing, visualization; L.W.: writing-review and editing; A.Ab.: writing-review and editing, design and synthesis of compounds; J.W.: writing-review and editing, design and synthesis of compounds; A.Ad.: writing-review and editing, design and synthesis of compounds; S.D.B.: resources, writing-review and editing; M.R.M.: conceptualization, resources, writing-review and editing, supervision, project administration.

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Article

In Vivo and In Vitro Metabolic Fate and Urinary Detectability of Five Deschloroketamine Derivatives Studied by Means of Hyphenated Mass Spectrometry

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Abstract: Ketamine derivatives such as deschloroketamine and deschloro-*N*-ethyl-ketamine show dissociative and psychoactive properties and their abuse as new psychoactive substances (NPSs) has been reported. Though some information is available on the biotransformation of dissociative NPSs, data on deschloro-*N*-cyclopropyl-ketamine deschloro-*N*-isopropyl-ketamine and deschloro-*N*-propyl-ketamine concerning their biotransformation and, thus, urinary detectability are not available. The aims of the presented work were to study the in vivo phase I and II metabolism; in vitro phase I metabolism, using pooled human liver microsomes (pHLMs); and detectability, within a standard urine screening approach (SUSA), of five deschloroketamine derivatives. Metabolism studies were conducted by collecting urine samples from male Wistar rats over a period of 24 h after their administration at 2 mg/kg body weight. The samples were analyzed using liquid chromatography high-resolution tandem mass spectrometry (LC-HRMS/MS) and gas chromatography–mass spectrometry (GC-MS). The compounds were mainly metabolized by *N*-dealkylation, hydroxylation, multiple oxidations, and combinations of these metabolic reactions, as well as glucuronidation and *N*-acetylation. In total, 29 phase I and 10 phase II metabolites were detected. For the LC-HRMS/MS SUSA, compound-specific metabolites were identified, and suitable screening targets could be recommended and confirmed in pHLMs for all derivatives except for deschloro-*N*-cyclopropyl-ketamine. Using the GC-MS-based SUSA approach, only non-specific acetylated *N*-dealkylation metabolites could be detected.

Keywords: new psychoactive substance; deschloroketamine; deschloro-*N*-ethyl-ketamine; deschloro-*N*-isopropyl-ketamine; deschloro-*N*-cyclopropyl-ketamine; deschloro-*N*-propyl-ketamine; metabolism; in vivo; in vitro; LC-HRMS/MS

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1. Introduction

According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) in Europe, over 83 million adults have used illicit drugs at least once in their life. Data from the European Web Survey on Drugs reported that, in 2021, next to the use of lysergic acid diethylamide and new psychoactive substances (NPSs), 13% of those surveyed consumed ketamine [1]. Furthermore, the annual amounts of seized ketamine have been at relatively high levels since its peak in 2018, with over 1.7 tons seized [2]. Ketamine is known as an anesthetic and analgesic and is listed on the World Health Organization's list of essential medicines [3,4]. Ketamine can also induce psychoactive and dissociative effects caused by the antagonism of *N*-methyl-D-aspartate receptors [5,6]. Structurally

related compounds, such as phencyclidine (also known as PCP or angel dust), show similar effects that have been associated with their recreational use [6]. NPSs derived from ketamine are emerging on the drug market, with deschloroketamine (2-oxo-PCMe) first reported in the UK in 2015 [7] and deschloro-*N*-ethyl-ketamine (2-oxo-PCE) first reported in France in 2016 [8]. Both substances were patented by Stevens in 1961 [9] and are potent dissociative agents with ethanol-like effects in lower doses, with noticeable dissociative effects described for 2-oxo-PCMe and analgesic as well as dissociative effects described for 2-oxo-PCE [4]. Hájková et al. described the metabolism of 2-oxo-PCMe in rats after its subcutaneous administration [10], Larabi et al. studied the metabolism of 2-oxo-PCE in human liver microsomes (HLMs) [11], and Tang et al. reported cases of acute poisonings in humans associated with the intake of 2-oxo-PCE. These authors tentatively identified three metabolites of 2-oxo-PCE. However, detailed knowledge on the metabolism of 2-oxo-PCE has remained limited, supporting the importance of investigating the metabolism of emerging NPSs in the context of forensic and clinical toxicology [12].

Hence, this study aimed to investigate the in vivo metabolic fate of five deschloroketamine derivatives: 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, see Figure 1). Sample preparations were followed by an analysis using liquid chromatography high-resolution tandem mass spectrometry (LC-HRMS/MS) or gas chromatography coupled with mass spectrometry (GC-MS). Furthermore, in vivo phase I metabolites should be confirmed by human in vitro data after incubations using pooled HLMs (pHLMs). The limits of identification (LOI) in human urine samples, as well as standard urine screening approaches (SUSA) by LC-HRMS/MS and GC-MS, were investigated.

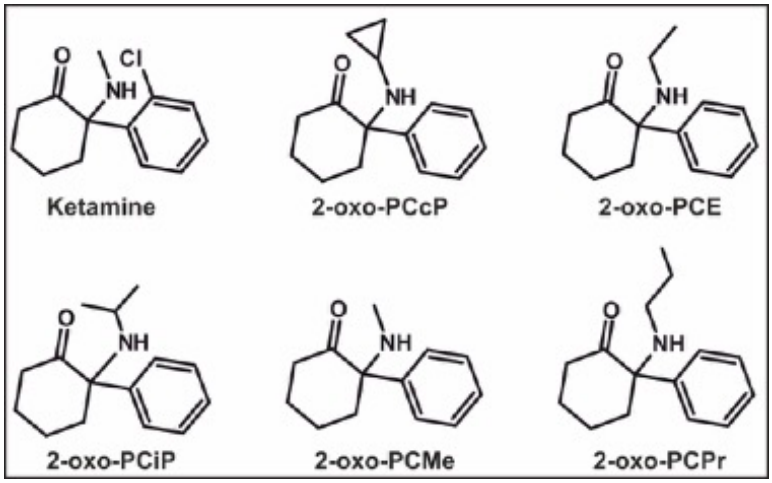

Figure 1. Structures of ketamine and deschloroketamine derivatives 2-oxo-PCcP, 2-oxo-PCE, 2-oxo-PCiP, 2-oxo-PCMe, and 2-oxo-PCPr.

2. Materials and Methods

2-Oxo-PCcP hydrochloride, 2-oxo-PCE hydrochloride, 2-oxo-PCiP hydrochloride, 2-oxo-PCMe hydrochloride, and 2-oxo-PCPr were synthesized by the standard methods and analytically characterized by ^{1}H and ^{13}C nuclear magnetic resonance (NMR), high-performance liquid chromatography (HPLC) (>95% pure on HPLC and NMR), and high-resolution mass spectrometry (HRMS.) Acetonitrile (ACN), ammonium formate, dichloromethane (DCM), ethyl acetate, hydrochloric acid (HCl), isopropanol, methanol (MeOH), sodium hydroxide solution (NaOH), and all other chemicals were obtained from VWR International GmbH (Darmstadt, Germany). Diazepam-d5 was obtained from LGC Standards GmbH (Wesel, Germany). $NADP^{+}$ was obtained from Biomol (Hamburg, Germany), superoxide dismutase, magnesium chloride ($MgCl_2$), isocitrate, and isocitrate dehydrogenase were from Merck (Darmstadt, Germany). pHLMs (20 mg microsomal protein/mL, 330 pmol total CYP/mg protein) were obtained from Corning (Amsterdam,

the Netherlands). All enzyme-containing preparations were thawed at 37 °C after delivery, aliquoted, snap frozen in liquid nitrogen and stored at −80 °C until use. 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).

2.1. Rat Urine Samples

Six male Wistar rats (Charles River, Sulzfeld, Germany) were used for our metabolism studies in accordance with German laws for animal protection. These studies have been approved by an ethics committee (No 50/2017, Landesamt für Verbraucherschutz, Saarbrücken, Germany). The compounds were orally administered (one rat per compound, n = 5) in an aqueous suspension via gastric intubation in doses of 2 mg/kg body weight (BW). After administration, rats were housed in metabolism cages for 24 h. Rats had water ad libitum during the collection of urine, which was collected separately from feces using metabolism cages. No compounds were administered to the sixth rat, and its urine was used as control.

2.2. Sample Preparations

2.2.1. Urine Conjugate Cleavage for the Identification of Phase I Metabolites by LC-HRMS/MS

According to a previously published procedure, with minor modifications [13], 2 mL of rat urine (adjusted to pH 5.2 with acetic acid, 1 M, approximately 50 µL) was incubated at 56 °C for 3 h with 50 µL of a glucuronidase (EC no. 3.2.1.31, Merck) and arylsulfatase (EC no. 3.1.6.1, Merck) mixture from *Helix pomatia*. This procedure was performed in duplicate per rat (twelve samples in total); one aliquot of cleaved urine samples (UGLUC) was extracted via liquid–liquid extraction (LLE) and the second via solid-phase extraction (SPE).

2.2.2. Urine LLE for the Identification of Phase I Metabolites by LC-HRMS/MS

LLE was performed as described elsewhere, with minor modifications [14]. A volume of 2 mL of UGLUC was acidified using 1 mL of HCl (37%), and then 2 mL of ammonium sulfate solution (30%) and 1 mL of NaOH solution (10 M) were added (final pH 8–9). This mixture was extracted using 5 mL of ethyl acetate/DCM/isopropanol (3:1:1, *v*/*v*/*v*), followed by manually shaking for 20 s and centrifugation for two minutes at 3000× *g*. The organic layer was transferred into a glass flask, evaporated to dryness at 60 °C, and reconstituted with 100 µL of MeOH. A 10 µL aliquot of each sample was injected onto the LC-HRMS/MS system.

2.2.3. Urine SPE for the Identification of Phase I Metabolites by LC-HRMS/MS

As described elsewhere, with minor modifications [13], HCX cartridges were conditioned with 1 mL of MeOH and 1 mL of purified water. The cartridges were loaded with 2 mL of UGLUC and washed with 1 mL of purified water, 1 of mL HCl (0.1 M), and 2 mL of MeOH. Target compounds were eluted using 1 mL of a MeOH/NH_3 mixture (35%, 98/2, *v*/*v*). The eluates were evaporated to dryness at 70 °C under a gentle stream of nitrogen, and residues were dissolved in 50 µL of MeOH. A 10 µL aliquot of each sample was injected onto the LC-HRMS/MS system.

2.2.4. Urine Precipitation for the Identification of Phase II Metabolites by LC-HRMS/MS

Urine samples were prepared as described elsewhere, with minor modifications [15]. A volume of 0.1 mL of native urine was mixed with 0.5 mL of ACN containing 0.1 mg/mL of diazepam-d_5. The mixture was shaken in a rotary shaker for 2 min at 2000 rpm, centrifuged at 18,407× *g*, and then 0.5 mL was transferred into a glass vial and evaporated to dryness at 70 °C under a gentle stream of nitrogen. The residue was dissolved in 50 µL of a mixture of 2 mM ammonium formate solution containing 0.1% (*v*/*v*) formic acid (eluent A) and 2 mM ammonium formate solution in acetonitrile/methanol (50:50, *v*/*v*) containing 0.1%

(*v*/*v*) formic acid and 1% (*v*/*v*) water (eluent B; 1:1, *v*/*v*) and 10 µL was injected onto the LC-HRMS/MS system.

2.2.5. Urine SPE for the Identification of Phase II Metabolites by LC-HRMS/MS

Native rat urine samples were extracted via SPE as described in Section 2.2.3.

2.2.6. Urine Sample Preparation Prior to GC-MS

Urine samples were prepared according to a previously published procedure, with minor modifications [16,17]. Samples were divided into two aliquots of 2.5 mL, one aliquot was mixed with 0.1 mL of internal standard solution (0.1 mg/mL diazepam-d_5 in MeOH), and 1 mL of HCl (37%) was added. This mixture was hydrolyzed for 15 min under reflux and 2 mL of ammonium sulfate solution (30%) and 1 mL of NaOH solution (10 M) were added (final pH 8–9). Then, the second aliquot of 2.5 mL of untreated urine was added. This mixture was extracted using 5 mL of ethyl acetate/DCM/isopropanol (3:1:1, *v*/*v*/*v*), followed by manually shaking for 20 s and centrifugation for two minutes at 3000× *g*. The organic layer was transferred into a glass flask and evaporated to dryness at 60 °C. Samples were then derivatized using a mixture of acetic aldehyde and pyridine (3:2, *v*/*v*), coupled with microwave irradiation for 5 min at 580 W. After the acetylation, extracts were evaporated to dryness at 60 °C, reconstituted with 100 µL MeOH, and 1 µL was injected onto the GC-MS system.

2.3. Human In Vitro Incubations for the Conformation of In Vivo Rat Phase I Metabolites

According to previously published procedures, with minor modifications [18,19], deschloroketamine derivatives with a final concentration of 25 µM were incubated with pHLMs (1 mg microsomal protein/mL). Additionally, the reaction mixture contained 90 mM phosphate buffer (pH 7.4), superoxide dismutase (200 U/mL), isocitrate (5 mM), $MgCl_2$ (5 mM), isocitrate dehydrogenase (0.5 U/mL), and $NADP^+$ (1.2 mM). Incubations were performed at 37 °C for 60 min and terminated by adding 50 µL of ice-cold ACN containing 0.1 mg/mL of diazepam-d_5. The mixtures were then centrifuged at 18,407× *g* for 5 min, before 70 µL of the supernatants was transferred into autosampler vials and 10 µL was injected onto the LC-HRMS/MS system. Negative controls without pHLMs, for every deschloroketamine derivative, and blank incubations containing pHLMs without the deschloroketamine derivatives, were prepared to identify the compounds without a metabolic origin. All incubations were performed in duplicate.

2.4. LOI

2.4.1. LOI for Analysis by LC-HRMS/MS

LOI, by LC-HRMS/MS (n = 3), were determined as follows. A volume of 90 µL of blank human urine was fortified with 10 µL of an analyte mixture in MeOH at concentrations of 1000 µg/L, 100 µg/L, 10 µg/L, and 1 µg/L. All given concentrations are the final urine concentrations. Spiked urine samples were extracted via urine precipitations, as described in Section 2.2.4, which corresponds to the LC-HRMS/MS SUSA sample preparation. A 10 µL aliquot each was injected onto the LC-HRMS/MS system. The criteria for the LOI were fulfilled when the S/N was at least 3 and the compound could be identified via its MS^2 spectra using an extended version of the Maurer/Meyer/Helfer/Weber 2018 library [20].

2.4.2. LOI for Analysis by GC-MS

LOI, for GC-MS (n = 3), were determined as follows. A volume of 4.5 mL of blank human urine was fortified with 500 µL of an analyte mixture in MeOH at concentrations of 20 mg/L, 10 mg/L, 1 mg/L, and 0.1 mg/L. All given concentrations are the final urine concentrations. Spiked urine samples were extracted by LLE after partial urine hydrolysis followed by acetylation (UHyAc), as described in Section 2.2.6, which corresponds to the GC-MS SUSA sample preparation. A 1 µL aliquot of each sample was injected onto the GC-MS system. The criteria for the LOI were fulfilled when the compound could

be identified via the automated mass spectral deconvolution and identification system (AMDIS, https://chemdata.nist.gov/dokuwiki/doku.php?id=chemdata:amdis (accessed on 2 January 2024)) in simple mode. Further AMDIS settings were previously described [21] and were used with the following modifications: width, 20; adjacent peak subtraction, 2; resolution, high; sensitivity, very high; shape requirement, low; minimal match factor, 50.

2.5. Detectability in Rat Urine Using SUSA

SUSAs were performed as described earlier [14,22,23], including urine precipitation (UPP) in combination with LC-HRMS/MS or UHyAc in combination with GC-MS. Furthermore, the metabolites of the deschloroketamine derivatives detected via both SUSAs were compared to the ketamine metabolites detected in human urine samples submitted to the authors' clinical toxicology laboratory for regular toxicological analyses.

2.6. Human Urine Sample

A urine sample was submitted to the authors' laboratory for toxicological analysis after a suspected intake of 2-oxo-PCMe. The urine sample was prepared and analyzed following both GC-MS SUSA and LC-HRMS/MS SUSA.

2.7. LC-HRMS/MS Apparatus

According to a previous study, with minor changes [24], 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) was used. 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: a TF Accucore Phenyl Hexyl column (100 × 2.1 mm ID, 2.6 µm particle size); gradient elution using eluents A and B. The flow rate was set as follows: 0–11.5 min at 0.500 mL/min and 11.5–13.5 min at 0.800 mL/min. The following gradient settings were used: 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; sheath gas, 60 arbitrary units (AU); auxiliary gas, 10 AU; spray voltage, 4.00 kV; and S lens RF level, 50.0. Mass spectrometry experiments for the identification of metabolites in rat urine were performed using a full scan mode and data-dependent acquisition (DDA) with an inclusion list containing the masses of the expected metabolites. For full data scans, 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 using SUSA conditions were the same as for the identification of metabolites, with one difference: the ionization mode was set to positive and negative. The mass spectrometry settings using SUSA conditions for a full data scan 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 130–930. The settings for the DDA without the inclusion list 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; and stepped normalized collision energy, 17.5, 35, 52.5%. ChemSketch 2018 2.1 (ACD/Labs, Toronto, Canada) was used to draw the structures of the hypothetical metabolites and for the calculations of their exact masses. Xcalibur Qual Browser version 4.1.31.9 (TF, Dreieich, Germany) was used for data handling.

2.8. GC-MS Apparatus

According to a previously published procedure, with minor modifications [14], a Hewlett Packard (HP, Agilent Technologies, Waldbronn, Germany) 5890 II gas chromatograph combined with a HP 5972 MSD mass spectrometer and a HP MS ChemStation (DOS series), with HP G1034C software version C03.00, was used for systematic urine screening. The condition of the GC system were as follows: Macherey Nagel (Düren, Germany) capillary dimethylpolysiloxane columns (Optima 1 MS; 12 m × 0.2 mm I. D.; film thickness, 0.35 µm); splitless injection mode; column and injector port temperature, 280 °C; carrier gas, helium; flow rate, 1 mL/min; column temperature, programmed from 90 to 310 °C at 30 °C/min—initial time 2 min, final time 5 min. The MS conditions were as follows: full scan mode, m/z 50–550; electron ionization (EI) mode; ionization energy, 10 eV; ion source temperature, 150 °C; capillary direct interface, heated to 280 °C. Data Analysis standalone (G1034C Version C.03.00, Hewlett Packard) was used for the data evaluation of the samples analyzed for the detection of metabolites in rat urine. Data obtained by GC-MS using SUSA conditions were evaluated using AMDIS; the settings were as described in Section 2.4.2. An extended version of the Maurer/Meyer/Pfleger/Weber 2023 library was used as the target library [14].

3. Results

3.1. Identification of In Vivo Phase I Metabolites Using LC-HRMS/MS

All phase I metabolites of 2-oxo-PCcP, 2-oxo-PCE, 2-oxo-PCiP, 2-oxo-PCMe, and 2-oxo-PCPr identified in rat urine after their oral administration are listed in Tables S1–S5, and their mass spectra are shown in Figures S1–S5 in the electronic Supplementary Materials (ESMs). The metabolite ID, masses of precursor ions (PIs) and characteristic fragment ions (FIs) in the MS^2 spectra, calculated exact masses, elemental compositions, deviation of the measured masses from the calculated masses in parts per million (ppm), and retention times (RTs) are included in these tables and/or figures. Three phase I metabolites of 2-oxo-PCcP, four of 2-oxo-PCE, seven of 2-oxo-PCiP, six of 2-oxo-PCMe, and nine of 2-oxo-PCPr were tentatively identified. The masses of their PIs and FIs described below are calculated exact masses.

3.1.1. Higher-Energy Collisional Dissociation (HCD) Fragmentation Patterns of 2-Oxo-PCcP and Its Phase I Metabolites

The MS^2 of 2-oxo-PCcP (PI at m/z 230.1539) showed an initial loss of water (−18.0106 u) leading to FI at m/z 212.1434 or a loss of cyclopropylamine (−57.0578 u) forming FI at m/z 173.0961, which corresponds to the 2-oxo-phenylcyclohexyl core structure. This core structure was fragmented by several neutral losses: CO, resulting in an FI at m/z 145.1012; ethanone (FI at m/z 129.0699); propanone (FI at m/z 117.0699); and pentanone (FI at m/z 91.0542). This is in accordance with the fragmentation pattern of 2-oxo-PCE described by Larabi et al. [11]. Furthermore, the FI at m/z 58.0651 was detected as corresponding to the mass of protonated cyclopropylamine. CM1 (*N*-dealkylation, PI at m/z 190.1226) showed an initial neutral loss of ammonia (−17.0265 u) leading to an FI at m/z 173.0961. Further FIs at m/z 145.1012, 129.0699, 117.0699, and 91.0542 (the most abundant) corresponded with the fragmentation pattern of the parent compound. The FI at m/z 67.0542 resulted from the cleavage of the cyclohexanone ring, followed by a neutral loss of CO and the formation of a cyclopentene cation. Two metabolites with PIs at m/z 246.1489 (hydroxylation isomers 1 and 2) were detected as well, showing mass shifts of +15.9949 u compared to their parent compound. Hydroxylation isomer 1 (CM3) showed an initial loss of cyclopropylamine (−57.0578 u), which led to an FI at m/z 189.0910. The hydroxy group was localized at the cyclohexanone ring due to the mass shift of −2.0157 u of the FI at m/z 171.0804 compared to the FI at m/z 173.0961 in the MS^2 of the parent compound, as well as the mass shift of +18.0106 u compared to the FI at m/z 189.0910. Moreover, the FI at m/z 91.0542 indicated that there was no modification at the benzene ring. CM4's hydroxy group was localized at the benzene ring due to the mass shift of +15.9949 u between the FIs at m/z 161.0961 in the

spectrum of CM4 and 145.1012 in the parent spectrum, and the FIs at m/z 107.0491 (CM4) and 91.0542 (parent compound).

3.1.2. HCD Fragmentation Patterns of 2-Oxo-PCE and Its Phase I Metabolites

The MS^2 of 2-oxo-PCE (PI at m/z 218.1539) showed an initial loss of ethylamine (−45.0578 u), forming an FI at m/z 173.0961, which corresponds to the 2-oxo-phenylcyclohexyl core structure, or a loss of water resulting in an FI at m/z 200.1434. The core structure fragmented as described for 2-oxo-PCcP, including an FI at m/z 67.0542 (cyclopentene cation). The metabolite EM2 was tentatively identified as hydroxylation at the cyclohexanone moiety, with further oxidation to a ketone function, with a PI at m/z 232.1332. The initial fragmentation step was a neutral loss of ethylamine (FI at m/z 187.0754) followed by a loss of CO leading to an FI at m/z 159.0804. Both FIs showed a mass shift of +13.9792 u compared to the corresponding FI in the parent compound's spectrum; this was achieved by the addition of oxygen (+15.9949 u) and a formal dehydration of the ketone function (−2.0157 u). Furthermore, EM3 and EM4 were identified as isomers of the hydroxylation at the cyclohexanone ring, with almost identical MS^2 spectra but different retention times. The FIs at m/z 161.0961 and 189.0910 showed mass shifts of +15.9949 u compared to the corresponding FI in the spectrum of 2-oxo-PCE and +2.0157 u compared to the FI in the spectrum of EM2. The presence of an FI at m/z 91.0542 in the spectra of EM3 and EM4 ruled out changes at the benzene ring. Moreover, a mass shift of 18.0106 u between the PI at m/z 234.1489 and the FI at m/z 216.1383 in both spectra indicated a neutral loss of water.

3.1.3. HCD Fragmentation Patterns of 2-Oxo-PCiP and Its Phase I Metabolites

The MS^2 of 2-oxo-PCiP (PI at m/z 232.1696) showed initial losses of either isopropylamine (−59.0735 u), followed by the FI described for 2-oxo-PCcP, corresponding to the fragmentation pattern of the 2-oxo-phenylcyclohexyl core structure, or water, leading to an FI at m/z 214.1590. Furthermore, a protonated isopropylamine (FI at m/z 60.0808) was detected. IM2 (*N*-dealkylation + hydroxylation) with a PI at m/z 206.1176 was identified as follows: the mass shift between the PI and FI at m/z 189.0910 corresponded to a loss of ammonia (−17.0265 u) and showed a mass shift of +15.9949 u compared to the deamino FI at m/z 173.0961 in the 2-oxo-PCiP's MS^2 spectrum. There were three metabolites (IM4–6) with a PI at m/z 248.1645 that were identified as products of hydroxylation. Hydroxylation isomer 1 (IM4) was identified as hydroxylation at the cyclohexanone part, as described for EM3 and 4. IM5 was identified as follows: the FI at m/z 189.0910 showed a hydroxylation at the 2-oxo-phenylcyclohexyl core due to a mass shift of +15.9949, compared to the FI at m/z 173.0961 in the parent compound's MS^2 spectrum; the loss of CO led to an FI at m/z 161.0961. Furthermore, the FI at m/z 107.0491 indicated a hydroxylation of the phenyl part, however, the exact position could not be determined. The hydroxyl group of IM6 was located at the isopropyl structure. The spectrum of IM6 showed an initial loss of water (−18.0106 u) leading to an FI at m/z 230.1539, followed by a loss of 2-propenamine (FI at m/z 173.0961) and a loss of CO (FI at m/z 145.1012) or ethanone (FI at m/z 129.0699). Furthermore, the FI at 91.0542 showed that the hydroxylation was not located at the phenyl part, the whole FIs at m/z 76.0757 (C_3H_9NO) and 58.0651 (C_3H_7N) indicated that the hydroxyl group's location was in the isopropyl part.

3.1.4. HCD Fragmentation Patterns of 2-Oxo-PCMe and Its Phase I Metabolites

The MS^2 of 2-oxo-PCMe (PI at m/z 204.1383) showed an initial loss of methylamine (−32.0422 u, FI at m/z 173.0961) or water (−18.0106 u, FI at m/z 186.1277), followed by the fragmentation pattern of the 2-oxo-phenylcyclohexyl core structure described for 2-oxo-PCcP. Metabolite MM3 was identified as a *N*-dealkylation followed by hydroxylation to a hydroxylamine, with a retention time of 5.61 min compared to 5.0 min (*N*-dealkylated metabolite MM1). The initial fragmentation step was a loss of water (−18.0106 u, FI at m/z 188.1070), followed by the fragmentation pattern of the 2-oxo-phenylcyclohexyl core structure, including the following FIs at m/z 173.0961, 145.1012, 129.0699, and 91.0542. Two

hydroxylation metabolites were detected with PIs at m/z 220.1332: MM5 was identified as a hydroxylation at the cyclohexanone part, and MM6 as a hydroxylation at the phenyl part. Their identification was performed as described above. In brief: MM5 showed an initial loss of water leading to an FI at m/z 202.1226 and an initial loss of methylamine (FI at m/z 189.0910), followed by a further loss of water (FI at m/z 171.0804) and CO (FI at m/z 143.0855). In the MS^2 of MM6, the FI at m/z 189.0910 resulted from an initial loss of methylamine, followed by a loss of CO (FI at m/z 161.0961), with further fragmentation to an FI at m/z 107.0491 (C_7H_7O) and an FI at m/z 67.0542.

3.1.5. HCD Fragmentation Patterns of 2-Oxo-PCPr and Its Phase I Metabolites

The MS^2 of 2-oxo-PCPr (PI at m/z 232.1696) showed an initial loss of water (−18.0106 u, FI at m/z 214.1590) or propylamine (−59.0735 u, FI at m/z 173.0961), followed by the fragmentation pattern of the 2-oxo-phenylcyclohexyl core structure described for 2-oxo-PCcP. There were two metabolites with PIs at m/z 262.1438: PM6 was identified as a metabolite obtained via dihydroxylation and the further oxidation of one hydroxy group to a ketone. PM6 showed initial neutral loss of water (−18.0106 u) resulting in an FI at m/z 244.1332. A second initial fragmentation step starting at the PI was the loss of 3-hydroxypropylamine (−75.0684 u) leading to an FI at m/z 187.0754; the further loss of CO resulted in an FI at m/z 159.0804. Both FIs were also detected in the spectrum of the ketone metabolite EM2 of 2-oxo-PCE. Finally, an FI at m/z 76.0757 ($C_3H_{10}ON$) was also detected in this spectrum and identified as the protonated 3-hydroxypropylamine group. PM7 was identified as a hydroxylation with further oxidation to a carboxylic acid, also showing an initial loss of water (FI at m/z 244.1332). The loss of 3-aminopropanoic acid led to an FI at m/z 173.0961 (2-oxo-phenylcyclohexyl core) and fragmentation as already described before. Furthermore, there were two metabolites with PIs at m/z 264.1594, identified as dihydroxylation isomers (PM8, PM9). PM8 was identified as follows: two initial losses of water (−18.0106 u each), with FIs at m/z 246.1489 and 228.1383 and an FI at m/z 189.0910 resulting from the neutral loss of 3-hydroxypropylamine (−75.0684 u) as a further possible initial fragmentation step. The FIs at m/z 189.0910, 171.0804, and 143.0855 showed that one hydroxy group was located at the cyclohexanone ring, while the second hydroxy group was identified at the propyl group, due to an FI at m/z 76.0757 ($C_3H_{10}ON$). In the MS^2 of PM9 there was also a mass shift of −18.0106 u between the PI and FI at m/z 246.1489, indicating a neutral loss of water. Furthermore, an FI at m/z 76.0757 was detected, which meant that one hydroxy group was located at the propyl chain. The FIs at m/z 189.0910, 161.0961, and 107.0491 allowed for the localization of the second hydroxy group at the phenyl ring.

3.2. Identification of In Vivo Phase II Metabolites Using LC-HRMS/MS

The phase II metabolites of 2-oxo-PCcP, 2-oxo-PCE, 2-oxo-PCiP, 2-oxo-PCMe, and 2-oxo-PCPr identified in rat urine after their oral administration are listed in Tables S1–S5, and their mass spectra are shown in Figures S1–S5 in the ESMs. The metabolite ID, masses of PIs and characteristic FIs in MS^2, calculated exact masses, elemental compositions, deviation of the measured from the calculated masses in ppm, and RTs are included in these tables and/or figures. Two phase II metabolites of 2-oxo-PCcP, one of 2-oxo-PCE, two of 2-oxo-PCiP, three of 2-oxo-PCMe, and two of 2-oxo-PCPr were tentatively identified. The masses of PIs and FIs described below are calculated exact masses.

3.2.1. HCD Fragmentation Patterns of Common Phase II Metabolites

A *N*-dealkylation and glucuronidation was detected as the phase II metabolite of all five deschloroketamine derivatives (CM5, EM5, IM8, MM8, and PM10), with a PI at m/z 366.1547. It showed a mass shift of +176.0321 u compared to the corresponding phase I metabolite with a PI at m/z 190.1226. The initial fragmentation steps were two consecutive losses of water (−18.0106 u), leading to FIs at m/z 348.1442 and 330.1336. Furthermore, FIs at m/z 173.0961, 145.1012, 129.0699, and 91.0542 were also present in the MS^2 spectrum of

its corresponding phase I metabolite. *N*-Dealkylation followed by acetylation was detected as the phase II metabolite of 2-oxo-PCcP (CM2) and 2-oxo-PCMe (MM7), with the PI at m/z 232.1332 showing a mass shift of +42.0106 u compared to the corresponding phase I metabolite. The MS^2 spectrum included the characteristic FIs also detected in the MS^2 spectrum of the phase I metabolite, including FIs at m/z 173.0961, 145.1012, 129.0699, and 91.0542.

3.2.2. HCD Fragmentation Patterns of 2-Oxo-PCiP Phase II Metabolites

IM9 was identified as a glucuronide of the hydroxy metabolite isomer 3 of 2-oxo-PCiP, with a PI at m/z 424.1966 and a mass shift of +176.0321 u compared to the corresponding phase I metabolite (IM6). Characteristic FIs, detected in the MS^2 spectrum of the phase I metabolite, were detected in the MS^2 spectrum of the phase II metabolite as well, including FIs at m/z 230.1539, 173.0961, 145.1012, 129.0699, 91.0542, and 76.0757.

3.2.3. HCD Fragmentation Patterns of 2-Oxo-PCMe Phase II Metabolites

A further phase II metabolite of 2-oxo-PCMe was formed after *N*-dealkylation, combined with hydroxylation and further glucuronidation (MM9), with a PI at m/z 382.1469. It showed a mass shift of +176.0321 u compared to the corresponding phase I metabolite and its MS^2 spectrum contained the characteristic FIs which were also detected in the phase I metabolite, including FIs at m/z 171.0804, 161.0961, 143.0855, and 91.0542. Furthermore, the spectrum showed an initial loss of water −18.0106 u, leading to an FI at m/z 364.1391.

3.2.4. HCD Fragmentation Patterns of 2-Oxo-PCPr Phase II Metabolites

PM11 (PI at m/z 424.1966) was identified as a glucuronide of the hydroxylation metabolite isomer 2 of 2-oxo-PCPr (PM4). Its identification was performed as follows: the cleavage of glucuronic acid led to an FI at m/z 248.1645, which corresponds to the PI of the phase I metabolite. This was followed by either a loss of water (FI at m/z 230.1539) or cleavage of 3-hydroxypropylamine (FI at m/z 173.0961) and further fragmentation of the 2-oxo-phenylcyclohexyl core, as described in the phase I metabolite section. Additionally, an FI at m/z 76.0757 was detected.

3.3. Identification of Metabolites Using GC-MS

The parent compounds could not be detected in rat urine using a GC-MS analysis, but the acetylated *N*-dealkylation metabolite of each deschloroketamine derivative was found. The GC-MS spectra are depicted in Figure S6 (ESMs) and more details can be found in Table S6 (ESMs).

EI Fragmentation Patterns of Deschloroketamine Derivative and Their Metabolites

The mass spectrum of 2-oxo-PCcP showed an initial loss of CHO^+, shown by a loss of 29 u between the PI at m/z 229 and FI at m/z 200, and the additional cleavage of cyclopropylamine led to an FI at m/z 145. The combined cleavage of the cyclopropylamine and loss of water led to an FI at m/z 172. The further loss of C_3H_5 resulted in an FI at m/z 104, while the FI at m/z 91 corresponded to a benzyl cation. 2-Oxo-PCE displayed a PI at m/z 217. Cleavage CO led to an FI at m/z 189 and a subsequent loss of the ethyl group led to an FI at m/z 160. Further cleavage of the amine led to an FI at m/z 146, and the cleavage of carbons led to FIs at m/z 132, 117, 104, and 91. 2-Oxo-PCiP was identified as follows: an initial loss of CO led to a mass shift of −28 u between the PI at m/z 231 and FI at m/z 203. An FI at m/z 174 resulted from the cleavage of isopropylamine, while a loss of CO in combination with isopropyl chain cleavage led to an FI at m/z 160. The further loss of carbons led to FIs at m/z 132, 117, 104, and 91. Only 2-oxo-PCMe AC, with a PI at m/z 245, could be identified. An initial loss of CO led to an FI at m/z 217, the initial loss of the amine group resulted in an FI at m/z 174, with further fragmentation of the cyclohexanone part leading to FIs at m/z 144, 132, 118, 104, and 91. 2-Oxo-PCPr showed a similar mass spectrum compared to 2-oxo-PCiP, with a PI at m/z 231 and the initial cleavage of CO

resulting in an FI at m/z 203. Further FIs at m/z 174, 160, 144, 132, 104, and 91 were also detected. Furthermore, 2-oxo-PCPr AC could be identified, with a PI at m/z 273. The initial fragmentation step was a loss of C_2H_4, leading to an FI at m/z 245. FIs at m/z 203, 174. 160, 132, 117, 104, and 91 were detected as described for 2-oxo-PCPr. Only the acetylated *N*-dealkylation metabolite could be detected for all five deschloroketamine derivatives. Its identification was performed as follows: a mass shift of −43 u between the PI at m/z 231 and FI at m/z 188 indicated the loss of an acetyl group, the FI at m/z 174 corresponded to a cleavage of the amine function, a further loss of CH_2O (−30 u) at the cyclohexanone core led to an FI at m/z 144, a loss of C_4H_6O to an FI at m/z 104, and a loss of CH_2 to an FI at m/z 91. The FI at m/z 132 resulted from the cleavage of the amine group combined with the subsequent loss of C_2H_2O.

3.4. Confirmation of In Vivo Phase I Metabolites Using Human In Vitro Incubations

In vitro pHLM incubations confirmed 19 of the 29 phase I metabolites identified in vivo. The metabolic systems in which the metabolites were identified are given in Tables S1–S5 (ESMs). Monohydroxylation and *N*-dealkylation metabolites, as well as a metabolite formed by a combination of both metabolic reactions, were identified in vivo and in vitro. Further oxidated metabolites such as ketones, dihydroxylations, and carboxylic acid were not detected after human in vitro incubations.

3.5. Proposed In Vivo Metabolic Pathways

Based on the identified metabolites in rat urine after oral administrations, the proposed in vivo metabolic pathways of 2-oxo-PCcP, 2-oxo-PCiP, and 2-oxo-PCPr are given in Figures 2–4, the pathways of 2-oxo-PCE and 2-oxo-PCMe are given in Figures S7 and S8 (ESMs), and their IDs are given in Tables S1–S5 (ESM).

3.5.1. 2-Oxo-PCcP

N-dealkylation led to the formation of CM1. Two different hydroxylation isomers were detected (CM3 and CM4). CM3 was formed by the hydroxylation of the cyclohexanone part, CM4 via the hydroxylation of the phenyl ring. Furthermore, two phase II metabolites were identified, namely, *N*-dealkylation in combination with *N*-acetylation (CM2) or glucuronidation (CM5).

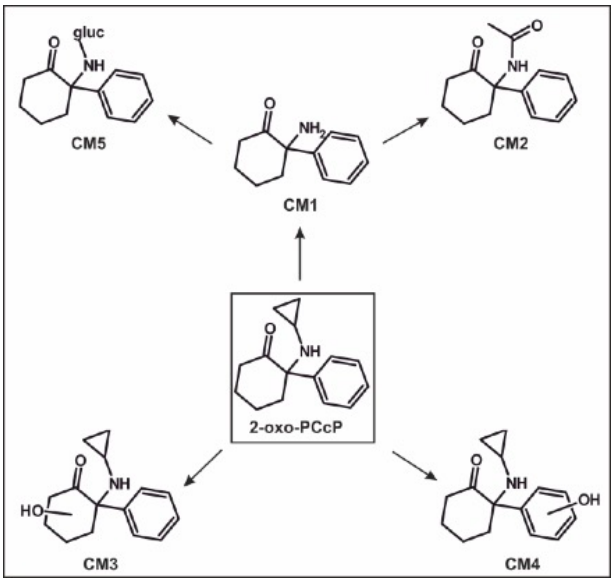

Figure 2. In vivo metabolic pathways of 2-oxo-PCcP. ID corresponding to Table S1. CM, 2-oxo-PCcP metabolite; →, metabolized to.

3.5.2. 2-Oxo-PCE

EM1 was formed by *N*-dealkylation. Furthermore, there were two hydroxylation metabolites (EM3 and EM4), both formed via the hydroxylation of the cyclohexanone part. As the exact position of the hydroxy groups could not be determined, both isomers could only be differentiated by their RTs (4.11 min for EM3 and 4.62 for EM4). Further oxidation of the hydroxy group to a ketone led to EM2. There was also one phase II metabolite identified as a *N*-dealkylation, followed by a glucuronidation (EM5).

3.5.3. 2-Oxo-PCiP

The *N*-Dealkylation of the parent compound led to IM1. This was followed by either hydroxylation at the cyclohexanone part (IM2) or glucuronidation (IM8). Three hydroxylation isomers were identified, with IM4 hydroxylated at the cyclohexanone part, IM5 at the phenyl ring, and IM6 at the isopropyl part. The further oxidation of IM4 to the ketone that formed IM3, while a combination of IM4 and IM6 formed dihydroxy metabolite IM7. The glucuronidation of IM6 formed phase II metabolite IM9.

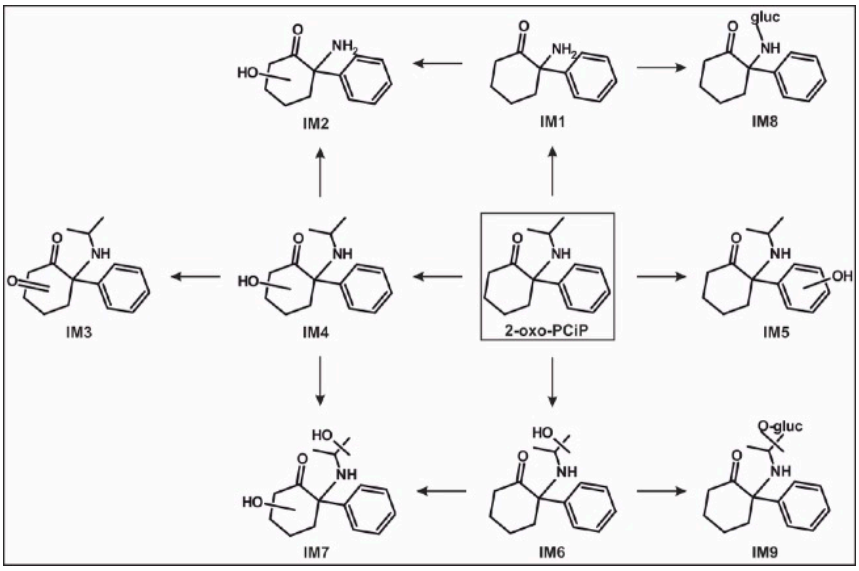

Figure 3. In vivo metabolic pathways of 2-oxo-PCiP. ID corresponding to Table S3. IM, 2-oxo-PCiP metabolite; →, metabolized to.

3.5.4. 2-Oxo-PCMe

MM1 was formed by *N*-dealkylation. The further hydroxylation of MM1 led to two isomers, MM2 and MM3, with MM2 hydroxylated at the cyclohexanone part and MM3 formed by *N*-hydroxylation and also showing a higher RT compared to MM1. There were also two hydroxylated metabolites of the parent compound MM5 (hydroxylation at the cyclohexanone part) and MM6 (hydroxylation at the phenyl ring). The further oxidation of MM5 to a ketone resulted in MM4. Their phase II metabolites included the combination of *N*-dealkylation with either *N*-acetylation (MM7) or glucuronidation (MM8) and the glucuronidated *N*-dealkylation + hydroxylation metabolite (MM9).

3.5.5. 2-Oxo-PCPr

The *N*-Dealkylation of 2-oxo-PCPr led to PM1. Hydroxylation of the cyclohexanone part led to PM3, hydroxylation of the propyl chain led to PM4, and *N*-hydroxylation led to the formation of a hydroxylamine (PM5). The further oxidation of PM3 led to PM2 (ketone). PM7 was identified as product of hydroxylation at the propyl chain (PM4) followed by oxidation to a carboxylic acid. Hence, the hydroxy group of PM4 should be located at the terminal carbon in the chain. Furthermore, there were two dihydroxylation isomers (PM8 and PM9), both with a hydroxylation at the propyl chain. PM8 was further hydroxylated at

the cyclohexanone ring and PM9 at the phenyl ring. The oxidation of the hydroxy group at the cyclohexanone ring to a ketone of PM8 led to PM6. There were two phase II metabolites identified for 2-oxo-PCPr. PM10 was formed by the glucuronidation of PM1 and PM11 by the glucuronidation of PM4.

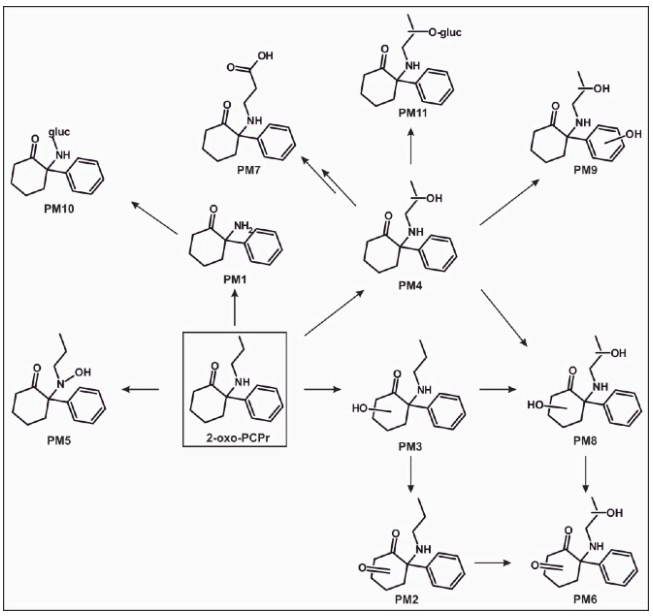

Figure 4. In vivo metabolic pathways of 2-oxo-PCPr. ID corresponding to Table S5. PM, 2-oxo-PCPr metabolite; →, metabolized to.

3.6. LOI in Spiked Urine

The analysis of spiked human urines showed LOI at levels of 10 µg/L after urine precipitation, followed by their analysis via LC-HRMS/MS. Using UHyAc in combination with GC-MS, the LOI were determined to be at 1 mg/L for all parent compounds except 2-oxo-PCMe (acetylated) and 2-oxo-PCcP (LOI at 10 mg/L).

3.7. Comparison of Different Sample Preparations and Their Detectability in Rat Urine

Tables S7–S11 (ESM) show the metabolites detected using LC-HRMS/MS and GC-MS in combination with different sample preparations. The analysis using SPE in combination with LC-HRMS/MS enabled the detection of all parent compounds and all metabolites, except two (IM5 and IM6). The UGLUC SPE in combination with LC-HRMS/MS led to the detection of 32 out of the 44 possible metabolites and parent compounds; 31 hits were achieved using UGLUC LLE, and 29 using UPP. The least number of metabolites was detectable via GC-MS in combination with UHyAc, where only the *N*-dealkylation metabolites could be identified.

3.8. Toxicological Detectability Using SUSA

Table 1 shows the parent compounds and metabolites detected in rat urine using the LC-HRMS/MS SUSA. 2-Oxo-PCE, 2-oxo-PCMe, 2-oxo-PCPr, *N*-dealkylation metabolites (CM1, EM1, IM1, MM1, and PM1), and N-dealkylations in combination with glucuronidation (CM5, EM5, IM9, MM8, and PM10) were detectable. Furthermore, hydroxylation metabolites (EM3, PM8, and PM9) and further oxidized metabolites (EM2, IM3, PM2, PM6, and PM7) were detected. The comparison of their absolute peak areas showed that CM5, EM5, IM8, MM8 (each *N*-dealkylation + glucuronidation metabolites), and PM7 (hydroxylation isomer 2 + oxidation to carboxylic acid) were the most abundant metabolites. Out of these five metabolites, only PM7 was a compound-specific metabolite. Further compound-specific metabolites with high absolute peak areas were EM2 and IM3. Regarding 2-oxo-PCMe, the parent compound itself showed the highest absolute peak area

when using the LC-HRMS/MS SUSA. No compound-specific metabolites could be detected in these experiments for 2-oxo-PCcP. Table 2 shows the metabolites detected in rat urine using the GC-MS SUSA. Here, only acetylated *N*-dealkylation metabolites were detectable. Metabolic yields were calculated using the respective metabolites' absolute peak areas.

Table 1. Proposed analytical targets for the LC-HRMS/MS SUSA. PI, precursor ion; CM, 2-oxo-PCcP metabolite; EM, 2-oxo-PCE metabolite; IM, 2-oxo-PCiP metabolite; MM, 2-oxo-PCMe metabolite; PM, 2-oxo-PCPr metabolite; Yield, metabolic yield in %.

Metabolite ID	Metabolic Reaction	Exact Mass of PI, *m*/*z*	RT, min	Absolute Peak Area	Yield, %
CM1	*N*-Dealkylation	230.1539	5.70	4.88×10^8	34
CM5	*N*-Dealkylation + glucuronidation	366.1547	5.35	9.38×10^8	66
2-Oxo-PCE	Parent compound	218.1539	5.44	1.31×10^8	-
EM1	*N*-Dealkylation	190.1226	5.04	6.47×10^8	34
EM2	Hydroxylation + oxidation to a ketone	232.1332	4.75	3.06×10^8	16
EM3	Hydroxylation isomer 1	234.1489	4.11	1.41×10^8	7
EM5	*N*-Dealkylation + glucuronidation	366.1547	5.20	8.21×10^8	43
IM1	*N*-Dealkylation	190.1226	5.03	3.13×10^7	4
IM3	Hydroxylation isomer 1 + oxidation to a ketone	246.1489	5.22	1.92×10^8	27
IM8	*N*-Dealkylation + glucuronidation	366.1547	5.22	4.32×10^8	60
IM9	Hydroxylation isomer 3 + glucuronidation	424.1966	5.24	6.51×10^7	9
2-Oxo-PCMe	Parent compound	204.1483	5.10	1.39×10^8	-
MM1	*N*-Dealkylation	190.1226	5.00	6.86×10^8	36
MM3	*N*-Dealkylation + hydroxylamine	206.1176	5.61	9.25×10^6	1
MM8	*N*-Dealkylation + glucuronidation	366.1547	5.25	1.17×10^9	63
2-Oxo-PCPr	Parent compound	232.1969	5.96	1.90×10^7	-
PM1	*N*-Dealkylation	190.1226	5.05	2.05×10^8	9
PM2	Hydroxylation isomer 1 + oxidation to a ketone	246.1489	5.37	3.68×10^8	14
PM6	Dihydroxylation isomer 1 + oxidation to a ketone	262.1438	4.85	5.67×10^7	2
PM7	Hydroxylation isomer 2 + oxidation to carboxylic acid	262.1438	5.31	8.91×10^8	35
PM8	Dihydroxylation isomer 1	264.1594	4.61	2.65×10^7	1
PM9	Dihydroxylation isomer 2	264.1594	5.09	1.50×10^8	6
PM10	*N*-Dealkylation + glucuronidation	366.1547	5.29	2.33×10^8	9
PM11	Hydroxylation + glucuronidation	424.1966	5.24	6.23×10^8	24

Table 2. Proposed analytical targets of five deschloroketamine derivatives for the GC-MS SUSA, including the masses of precursor ions (PIs) and the elemental composition and masses of characteristic fragment ions (FIs). AC, acetylated; RI, retention index.

Metabolite	PI Mass, *m*/*z*	RI	Elemental Composition	Characteristic FI
2-Oxo-PCcP *N*-dealkyl AC	231	1874	$C_{14}H_{17}NO_2$	188, 174, 144, 132, 104, 91
2-Oxo-PCE *N*-dealkyl AC	231	1874	$C_{14}H_{17}NO_2$	188, 174, 144, 132, 104, 91
2-Oxo-PCiP *N*-dealkyl AC	231	1874	$C_{14}H_{17}NO_2$	188, 174, 144, 132, 104, 91
2-Oxo-PCMe *N*-dealkyl AC	231	1874	$C_{14}H_{17}NO_2$	188, 174, 144, 132, 104, 91
2-Oxo-PCPr *N*-dealkyl AC	231	1874	$C_{14}H_{17}NO_2$	188, 174, 144, 132, 104, 91

3.9. Analysis of a Human Urine Sample

The analysis of a human urine sample after UPP in combination with LC-HRMS/MS allowed for the identification of 2-oxo-PCMe and several phase I metabolites, including a *N*-dealkylation (MM1), reduction (not identified in rat urine in this study), hydroxylation (MM5 and MM6), and hydroxylation with further oxidation to a ketone (MM4), while no phase II metabolites could be detected. A screening using GC-MS after UHyAc only allowed for the detection of MM1. The most abundant analytical targets were MM1 and 2-oxo-PCMe.

4. Discussion

Larabi et al. investigated the in vitro metabolism of 2-oxo-PCE after an HLM incubation and identified 12 phase I and 3 phase II metabolites [11]. This included *N*-dealkylation, hydroxylation, reduction, oxidative deamination, and dehydration metabolites in phase I and *N*- as well as *O*-glucuronides as phase II metabolites. A comparison with its metabolism in rat showed predominant differences concerning reduction and dehydration. Furthermore, no *O*-glucuronide metabolites of 2-oxo-PCE could be detected in rat urine, whereas two hydroxylation isomers were detected in this study, compared to one detected by Larabi et al. Differences in metabolite detection might be explained due to use of different systems and species (in vivo rat vs. in vitro HLM) and different concentrations of parent compounds. The metabolism of 2-oxo-PCMe in rats (urine, serum, and brain tissue) after the subcutaneous administration of 30 or 10 mg/kg BW was investigated by Hájková et al. [10]. The authors identified 17 phase I and 9 phase II metabolites, including *N*-dealkylation, hydroxylation, and reduction of the cyclohexanone metabolites, as well as combinations of these transformations in phase I and glucuronides in phase II metabolism. The differences in the number and type of metabolites detected compared to the study by Hájková et al. might reflect differences in our doses and/or route of administration, as well as the different biosamples obtained (serum and brain tissue in addition to urine samples).

The differences between the metabolites detected in the in vivo and in vitro experiments were related to further oxidized metabolites. This could be due to the 24 h time period of the rat experiments (compared to the 1 h for the pHLM incubations) allowing for further metabolic steps, which is a known challenge when comparing in vivo with in vitro models [25,26].

The analysis of a human urine sample after a suspected intake of an unknown amount of 2-oxo-PCMe showed similar results for the phase I metabolites as the metabolites detected in rat urine, while no phase II metabolites could be detected in the human urine sample. A noticeable difference was in the detection of a reduced metabolite in human urine; in HLMs by Larabi et al.; and in rat urine, serum, and brain tissue by Hájková et al. This difference might be due to species and/or dosage differences, but also due to different routes of administration compared to the present study. Users of 2-oxo-PCMe reported, in an online forum, doses between 0.3 and 1.5 mg/kg BW and varying routes of administration such as oral, sublingual, intramuscular, or rectal, as well as vaporization or insufflation [27]. So far, no metabolism data on 2-oxo-PCcP, 2-oxo-PCiP or 2-oxo-PCPr are available, nor have reports of their use in online forums, or seizures, been reported by relevant authorities. However, the 3-methoxy-analogs of 2-oxo-PCPr (MXPr) and 2-oxo-PCiP (MxiPr) have been reported as SPDs [28,29].

Using GC-MS, only the acetylated *N*-dealkylation metabolite was detected. Due to the acetylation step during sample preparation, those were largely derived from the phase I metabolites CM1, EM1, IM1, MM1, and PM1, instead of being phase II metabolites. This assumption is supported by the fact that no acetylated *N*-dealkylation metabolites of 2-oxo-PCE, 2-oxo-PCiP, or 2-oxo-PCPr could be detected using the LC-HRMS/MS-based analysis.

In human urine samples collected following a ketamine administration, ketamine, as well as its *N*-dealkylation, hydroxylation, and *N*-dealkylation metabolites, in combination with hydroxylation metabolites, was also frequently detected using the GC-MS-based SUSA [14,15,20]. The detection of the parent compound might be explained by the comparable high doses used for anesthesia (1–4.5 mg/kg BW i.v., 6.5–13 mg/kg BW i.m.) [30].

The current data showed similar metabolic pathways amongst the five deschloroketamine derivatives. *N*-Dealkylation metabolites were the most abundant metabolites detected in rat urine, but they were not compound-specific as they can be formed via the *N*-dealkylation of any derivative with an *N*-substituent. Based on the absolute peak areas of the parent compounds and metabolites detected in the LC-HRMS/MS SUSA EM2, IM3, 2-oxo-PCMe itself, and PM7, might be recommended as specific screening targets. As for 2-oxo-PCcP, no specific metabolites could be detected in the SUSA; only the unspecific

metabolites CM1 and CM5 might be used as targets. However, the detection of unspecific metabolites may at least indicate the ingestion of a deschloroketamine derivative. Subsequent analysis using more sensitive approaches, such as SPE prior to an LC-HRMS/MS or GC-MS analysis, might be used for substance-specific detection. Sample preparation by SPE would concentrate the samples, enabling the detection of low-concentrated analytes which were not detectable by both SUSA approaches.

Comparing the different sample preparations and MS approaches used in this study, SPE in combination with LC-HRMS/MS and an inclusion list led to the detection of most metabolites. UGLUC followed by LLE or SPE was used for the identification of phase I metabolites, since the cleavage of phase II metabolites should enhance the detectability of their corresponding phase I metabolites. Extraction via SPE should enhance detectability even more by concentrating samples. SPE without prior conjugate cleavage and UPP were used for the identification of phase II metabolites, as SPE could enhance detectability of low-concentration metabolites as described above. Furthermore, UPP was used as it is a fast and simple sample preparation, and one also used in the LC-HRMS/MS SUSA. For detectability studies, the laboratories' SUSAs were used (UPP followed by LC-HRMS/MS analysis and UHyAc followed by GC-MS analysis) as they are fast and simple workflows well established in the context of clinical toxicology [14,20]. Of the two screening (SUSA) methods, the LC-HRMS/MS SUSA showed lower LOI compared to the GC-MS SUSA and enabled the detection of substance-specific metabolites, including the metabolites and parent compounds also detected after pHLM incubations. Concerning the LOI, Gomila et al. reported limits for the structurally related 3-methoxy-phenylcyclohexylpiperidine (3-MeO-PCP) using a GC-MS-based approach at concentrations of 1 µg/L in blood and urine [31]. Differences in sensitivity might be explained by different sample preparations, as Gomila et al. used an SPE-based extraction method for their study, in contrast to the LLE in our study. However, the data obtained using GC-MS also allowed for the detection of a deschloroketamine metabolite, as well as of parent compounds in concentrations of at least 1 mg/L.

5. Conclusions

In total, 39 metabolites derived from five deschloroketamine derivatives were tentatively identified in rat urine, after their oral administration, by means of LC-HRMS/MS. Human in vitro incubations using pHLMs confirmed 19 phase I metabolites. The most abundant metabolites were formed by *N*-dealkylation, which did not allow for differentiation between the deschloroketamine derivatives only differing in their *N*-substitution. The comparison of different sample preparation strategies showed that UPP followed by an LC-HRMS/MS analysis is sufficient for the detection of specific metabolites for every NPS included in this study, except for 2-oxo-PCcP. In the samples analyzed using GC-MS, only the acetylated *N*-dealkylation metabolites were detected. The LOI were determined at levels of 10 µg/L using UPP prior to LC-HRMS/MS and at 1 mg/L using UHyAc prior to GC-MS, except for 2-oxo-PCcP (10 mg/L). Using the LC-HRMS/MS SUSA, EM2, IM3, 2-oxo-PCMe itself, and PM7 were identified as specific screening targets. Since only the *N*-dealkylated metabolites of 2-oxo-PCiP could be detected in rat urine using the SUSA conditions, no substance-specific screening targets could be suggested for this analyte. A human urine sample after a suspected intake of 2-oxo-PCMe was analyzed as well, and the results were in accordance with those obtained after the analysis of rat urine. The results of this study showed the importance of identifying specific screening targets for structurally similar compounds. Furthermore, all compounds can be detected using different sample preparation procedures combined with LC-HRMS/MS or GC-MS. The data from this study will thus help us to detect deschloroketamine derivatives in the context of forensic and clinical toxicology and for doping control.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/metabo14050270/s1, Table S1: List of phase I and II metabolites of 2-oxo-PCcP; Table S2: List of phase I and II metabolites of 2-oxo-PCE; Table S3: List of phase I and II metabolites of 2-oxo-PCiP; Table S4: List of phase I and II metabolites of 2-oxo-PCMe; Table S5: List of phase I and II metabolites of 2-oxo-PCPr; Table S6: Parent compounds and metabolites of five deschloroketamine derivatives detected by GC-MS; Table S7: 2-Oxo-PCcP and its metabolites, detected in rat urine after its oral administration using different sample preparations in combination with LC-HRMS/MS or GC-MS; Table S8: 2-Oxo-PCE and its metabolites, detected in rat urine after its oral administration using different sample preparations in combination with LC-HRMS/MS or GC-MS; Table S9: 2-Oxo-PCiP and its metabolites, detected in rat urine after its oral administration using different sample preparations in combination with LC-HRMS/MS or GC-MS; Table S10: 2-Oxo-PCMe and its metabolites, detected in rat urine after its oral administration using different sample preparations in combination with LC-HRMS/MS or GC-MS; Table S11: 2-Oxo-PCPr and its metabolites, detected in rat urine after its oral administration using different sample preparations in combination with LC-HRMS/MS or GC-MS; Figure S1: $HRMS^2$ spectra of 2-oxo-PCcP and its metabolites, detected in rat urine after its oral administration; Figure S2: $HRMS^2$ spectra of 2-oxo-PCE and its metabolites, detected in rat urine after its oral administration; Figure S3: $HRMS^2$ spectra of 2-oxo-PCiP and its metabolites, detected in rat urine after its oral administration; Figure S4: $HRMS^2$ spectra of 2-oxo-PCMe and its metabolites, detected in rat urine after its oral administration; Figure S5: $HRMS^2$ spectra of 2-oxo-PCPr and its metabolites, detected in rat urine after its oral administration; Figure S6: EI-MS spectra of 2-oxo-PCcP, 2-oxo-PCE, 2-oxo-PCiP, 2-oxo-PCMe AC, 2-oxo-PCPr, and acetylated *N*-dealkylation metabolites. Figure S7: In vivo metabolic pathways of 2-oxo-PCE; Figure S8: In vivo metabolic pathways of 2-oxo-PCE.

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List of Abbreviations

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; ACN, acetonitrile; AGC, automatic gain control; AUs, arbitrary units; BW, body weight; DCM, dichloromethane; DDA, data-dependent acquisition; EI, electron ionization; EMCDDA, European Monitoring Centre for Drugs and Drug Addiction; ESM, electronic Supplementary Materials; FI, fragment ion; GC-MS, gas chromatography coupled with mass spectrometry; HCl, hydrochloric acid; HCD, higher-energy collisional dissociation; HESI-II, heated electrospray ionization II source; HLMs, human liver microsomes; HP, Hewlett Packard; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; IT, injection time; LC-HRMS/MS, liquid chromatography high-resolution tandem mass spectrometry; LLE, liquid–liquid extraction; LOI, limit(s) of identification; NADP+, nicotinamide adenine dinucleotide phosphate; NaOH, sodium hydroxide;

NMR, nuclear magnetic resonance; NPSs, new psychoactive substances; MeOH, methanol; MgCl2, magnesium chloride; MXiPr, methoxisopropamine; MXPr, methoxpropamine; pHLMs, pooled human liver microsomes; PI, precursor ion; ppm, parts per million; RT, retention time; SPE, solid-phase extraction; SUSA, standard urine screening approach; TF, Thermo Fisher Scientific; UGLUC, cleaved urine; UHyAc, partial urine hydrolysis followed by acetylation; UPP, urine precipitation.

References

1. EMCDDA. *European Drug Report 2022*; European Union: Lisbon, Portugal, 2022.
2. EMCDDA. *Other Drugs—The Current Situation in Europe*; European Union: Lisbon, Portugal, 2023.
3. WHO. 22nd Model List of Essential Medicines. 2021. Available online: https://www.who.int/publications/i/item/WHO-MHP-HPS-EML-2021.02 (accessed on 2 January 2024).
4. Wallach, J.; Brandt, S.D. Phencyclidine-Based New Psychoactive Substances. *Handb. Exp. Pharmacol.* **2018**, *252*, 261–303. [PubMed]
5. Marguilho, M.; Figueiredo, I.; Castro-Rodrigues, P. A Unified Model of Ketamine's Dissociative and Psychedelic Properties. *J. Psychopharmacol.* **2023**, *37*, 14–32. [CrossRef]
6. Pelletier, R.; Le Daré, B.; Le Bouëdec, D.; Kernalléguen, A.; Ferron, P.J.; Morel, I.; Gicquel, T. Arylcyclohexylamine Derivatives: Pharmacokinetic, Pharmacodynamic, Clinical and Forensic Aspects. *Int. J. Mol. Sci.* **2022**, *23*, 15574. [CrossRef] [PubMed]
7. EMCDDA. Europol 2015 Annual Report on the Implementation of Council Decision 2005/387/Jha. 2015. Available online: https://www.emcdda.europa.eu/publications/implementation-reports/2015_en (accessed on 2 January 2024).
8. EMCDDA. Europol 2016 Annual Report on the Implementation of Council Decision 2005/387/Jha. 2016. Available online: https://www.emcdda.europa.eu/system/files/publications/4724/TDAN17001ENN_PDFWEB.pdf (accessed on 2 January 2024).
9. Stevens, L.C. Amino Ketones and Methods for Their Production. U.S. Patent 3,254,124, 31 May 1966.
10. Hájková, K.; Jurásek, B.; Čejka, J.; Štefková, K.; Páleníček, T.; Sýkora, D.; Kuchař, M. 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 Test. Anal.* **2020**, *12*, 343–360. [CrossRef] [PubMed]
11. Larabi, I.A.; Zerizer, F.; Ameline, A.; Etting, I.; Joseph, D.; Kintz, P.; Alvarez, J.C. 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 Test. Anal.* **2021**, *13*, 1108–1117. [CrossRef] [PubMed]
12. Tang, M.H.; Chong, Y.; Chan, C.Y.; Ching, C.; Lai, C.; Li, Y.; Mak, T.W. Cluster of Acute Poisonings Associated with an Emerging Ketamine Analogue, 2-Oxo-Pce. *Forensic Sci. Int.* **2018**, *290*, 238–243. [CrossRef] [PubMed]
13. Welter, J.; Meyer, M.R.; Wolf, E.; Weinmann, W.; Kavanagh, P.; Maurer, H.H. 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. *Anal. Bioanal. Chem.* **2013**, *405*, 3125–3135. [CrossRef] [PubMed]
14. Maurer, H.H.; Wissenbach, D.K.; Weber, A.A. *Gc-Ms Library of Drugs, Poisons, and Their Metabolites*, 6th ed.; Mass Spectral Library of Drugs, Poisons, Pesticides, Pollutants, and Their Metabolites 5th Edition Upgrade; Wiley-VCH: Weinheim, Germany, 2023.
15. Wissenbach, D.K.; Meyer, M.R.; Remane, D.; Weber, A.A.; Maurer, H.H. Development of the First Metabolite-Based Lc-Ms(N) Urine Drug Screening Procedure-Exemplified for Antidepressants. *Anal. Bioanal. Chem.* **2011**, *400*, 79–88. [CrossRef] [PubMed]
16. Meyer, M.R.; Du, P.; Schuster, F.; Maurer, H.H. Studies on the Metabolism of the Alpha-Pyrrolidinophenone Designer Drug Methylenedioxy-Pyrovalerone (Mdpv) in Rat and Human Urine and Human Liver Microsomes Using Gc-Ms and Lc-High-Resolution Ms and Its Detectability in Urine by Gc-Ms. *J. Mass Spectrom.* **2010**, *45*, 1426–1442. [CrossRef]
17. Vollmer, A.C.; Wagmann, L.; Meyer, M.R. Toxic Plants-Detection of Colchicine in a Fast Systematic Clinical Toxicology Screening Using Liquid Chromatography-Mass Spectrometry. *Drug Test. Anal.* **2021**, *14*, 377–381. [CrossRef]
18. Wagmann, L.; Frankenfeld, F.; Park, Y.M.; Herrmann, J.; Fischmann, S.; Westphal, F.; Müller, R.; Flockerzi, V.; Meyer, M.R. How to Study the Metabolism of New Psychoactive Substances for the Purpose of Toxicological Screenings-a Follow-up Study Comparing Pooled Human Liver S9, Heparg Cells, and Zebrafish Larvae. *Front. Chem.* **2020**, *8*, 539. [CrossRef]
19. Wagmann, L.; Meyer, M.R.; Maurer, H.H. What Is the Contribution of Human FMO_3 in the N-Oxygenation of Selected Therapeutic Drugs and Drugs of Abuse? *Toxicol. Lett.* **2016**, *258*, 55–70. [CrossRef]
20. Maurer, H.H.; Meyer, M.R.; Helfer AGWeber, A.A. *Maurer/Meyer/Helfer/Weber Mmhw Lc-Hr-Ms/Ms Library of Drugs, Poisons, and Their Metabolites*; Wiley-VCH: Weinheim, Germany, 2018.
21. Meyer, M.R.; Peters, F.T.; Maurer, H.H. Automated Mass Spectral Deconvolution and Identification System for Gc-Ms Screening for Drugs, Poisons, and Metabolites in Urine. *Clin. Chem.* **2010**, *56*, 575–584. [CrossRef] [PubMed]
22. Wagmann, L.; Hempel, N.; Richter, L.H.; Brandt, S.D.; Stratford, A.; Meyer, M.R. Phenethylamine-Derived New Psychoactive Substances 2c-E-Fly, 2c-Ef-Fly, and 2c-T-7-Fly: Investigations on Their Metabolic Fate Including Isoenzyme Activities and Their Toxicological Detectability in Urine Screenings. *Drug Test. Anal.* **2019**, *11*, 1507–1521. [CrossRef] [PubMed]
23. Helfer, A.G.; Michely, J.A.; Weber, A.A.; Meyer, M.R.; Maurer, H.H. Orbitrap Technology for Comprehensive Metabolite-Based Liquid Chromatographic-High Resolution-Tandem Mass Spectrometric Urine Drug Screening—Exemplified for Cardiovascular Drugs. *Anal. Chim. Acta* **2015**, *891*, 221–233. [CrossRef]

24. Richter, M.J.; Wagmann, L.; Gampfer, T.M.; Brandt, S.D.; Meyer, M.R. In Vitro Metabolic Fate of the Synthetic Cannabinoid Receptor Agonists Qmpsb and Qmpcb (Sgt-11) Including Isozyme Mapping and Esterase Activity. *Metabolites* **2021**, *11*, 509. [CrossRef]
25. Caspar, A.T.; Gaab, J.B.; Michely, J.A.; Brandt, S.D.; Meyer, M.R.; Maurer, H.H. Metabolism of the Tryptamine-Derived New Psychoactive Substances 5-Meo-2-Me-Dalt, 5-Meo-2-Me-Alcht, and 5-Meo-2-Me-Dipt and Their Detectability in Urine Studied by Gc-Ms, Lc-Ms(N), and Lc-Hr-Ms/Ms. *Drug Test. Anal.* **2018**, *10*, 184–195. [CrossRef] [PubMed]
26. Carmo, H.; Hengstler, J.G.; de Boer, D.; Ringel, M.; Remião, F.; Carvalho, F.; Fernandes, E.; dos Reys, L.A.; Oesch, F.; Bastos, M.D.L. Metabolic Pathways of 4-Bromo-2,5-Dimethoxyphenethylamine (2c-B): Analysis of Phase I Metabolism with Hepatocytes of Six Species Including Human. *Toxicology* **2005**, *206*, 75–89. [CrossRef]
27. Erowid. Erowid Experience Vaults. Available online: https://erowid.org/experiences/ (accessed on 2 January 2024).
28. Tanaka, R.; Kawamura, M.; Mizutani, S.; Hakamatsuka, T.; Kikura-Hanajiri, R. Identification of Three Arylcyclohexylamines (Mxpr, Mxipr, and Dmxe) in Illegal Products. *Yakugaku Zasshi J. Pharm. Soc. Jpn.* **2022**, *142*, 675–681. [CrossRef]
29. Goncalves, R.; Castaing, N.; Richeval, C.; Ducint, D.; Titier, K.; Morvan, E.; Grélard, A.; Loquet, A.; Molimard, M. Methoxpropamine (Mxpr) in Powder, Urine and Hair Samples: Analytical Characterization and Metabolite Identification of a New Threat. *Forensic Sci. Int.* **2022**, *333*, 111215. [CrossRef]
30. Baselt, R.C. *Disposition of Toxic Drugs and Chemicals in Man*, 11th ed.; Biomedical Publications: Seal Beach, CA, USA, 2017.
31. Gomila, I.; Leciñena, M.Á.; Elorza, M.Á.; Pastor, Y.; Sahuquillo, L.; Servera, M.; Puiguriguer, J.; Barcelo, B. Detectability of Dissociative Psychoactive Substances in Urine by Five Commercial Phencyclidine Immunoassays. *J. Anal. Toxicol.* **2019**, *43*, 497–503. [CrossRef] [PubMed]

In Vivo and *in Vitro* Metabolic Fate and Urinary Detectability of Five Deschloroketamine Derivatives Studied by Means of Hyphenated Mass Spectrometry

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Table S1. List of *in vivo* phase I and II metabolites of 2-oxo-PCcP, identified in rat urine samples and *in vitro* phase I metabolites identified in incubations using pooled human liver microsomes (pHLM), including the respective metabolite ID, metabolic reaction, masses of the precursor ion (PI) and characteristic fragment ions (FI) detected in MS^2, calculated exact masses, elemental composition, calculated mass errors in parts per million (ppm), retention times (RT) in minutes, and system in which metabolites were identified. The metabolites are sorted by their mass and RT. pHLM, identified in pHLM incubations; rat, identified in rat urine samples

Metabolite ID	Metabolic Reaction	Characteristic Ions Measured Accurate Masses	Calculated Exact Masses, *m/z*	Elemental Composition	Error, ppm	RT, min	Identified in
2-Oxo-PCcP	Parent compound	PI at *m/z* 230.1537	230.1539	$C_{15}H_{20}ON$	-0.91	5.70	pHLM and rat
		FI at *m/z* 212.1430	212.1434	$C_{15}H_{18}N$	-1.68		
		FI at *m/z* 173.0958	173.0961	$C_{12}H_{13}O$	-1.63		
		FI at *m/z* 155.0849	155.0855	$C_{12}H_{11}$	-4.04		
		FI at *m/z* 145.1009	145.1012	$C_{11}H_{13}$	-1.76		
		FI at *m/z* 129.0697	129.0699	$C_{10}H_9$	-1.12		
		FI at *m/z* 117.0698	117.0699	C_9H_9	-0.78		
		FI at *m/z* 91.0546	91.0542	C_7H_7	3.72		
		FI at *m/z* 58.0659	58.0651	C_3H_8N	13.0		
CM1	*N*-Dealkylation	PI at *m/z* 190.1222	190.1226	$C_{12}H_{16}ON$	-2.52	5.06	pHLM and rat
		FI at *m/z* 173.0961	173.0961	$C_{12}H_{13}O$	-0.05		
		FI at *m/z* 155.0854	155.0855	$C_{12}H_{11}$	-0.94		
		FI at *m/z* 145.1011	145.1012	$C_{11}H_{13}$	-0.37		
		FI at *m/z* 129.0700	129.0699	$C_{10}H_9$	0.89		
		FI at *m/z* 117.0702	117.0699	C_9H_9	2.74		
		FI at *m/z* 91.0548	91.0542	C_7H_7	6.10		
		FI at *m/z* 67.0550	67.0542	C_5H_7	12.0		
CM2	*N*-Dealkylation + acetylation	PI at *m/z* 232.1330	232.1332	$C_{14}H_{18}O_2N$	-0.90	6.81	rat
		FI at *m/z* 173.0959	173.0961	$C_{12}H_{13}O$	-1.19		
		FI at *m/z* 145.1015	145.1012	$C_{11}H_{13}$	2.34		
		FI at *m/z* 129.0968	129.0699	$C_{10}H_9$	-0.41		
		FI at *m/z* 91.0547	91.0542	C_7H_7	5.47		
		FI at *m/z* 67.0549	67.0542	C_5H_7	10.6		
CM3	Hydroxylation isomer 1	PI at *m/z* 246.1486	246.1489	$C_{15}H_{20}O_2N$	-0.95	4.56	pHLM and rat
		FI at *m/z* 189.0907	189.0910	$C_{12}H_{13}O_2$	-1.55		
		FI at *m/z* 171.0804	171.0804	$C_{12}H_{11}O$	-0.43		
		FI at *m/z* 143.0856	143.0855	$C_{11}H_{11}$	0.31		
		FI at *m/z* 91.0547	91.0542	C_7H_7	4.89		
		FI at *m/z* 58.0660	58.0651	C_3H_8N	15.0		
CM4	Hydroxylation isomer 2	PI at *m/z* 246.1490	246.1489	$C_{15}H_{20}O_2N$	0.78	4.85	pHLM and rat
		FI at *m/z* 189.0912	189.0910	$C_{12}H_{13}O_2$	0.95		
		FI at *m/z* 161.0962	161.0961	$C_{11}H_{13}O$	0.43		
		FI at *m/z* 107.0496	107.0491	C_7H_7O	3.98		
		FI at *m/z* 58.0660	58.6051	C_3H_8N	15.2		
CM5	*N*-Dealkylation + glucuronidation	PI at *m/z* 366.1552	366.1547	$C_{18}H_{24}O_7N$	1.15	5.35	rat
		FI at *m/z* 348.1449	348.1442	$C_{18}H_{22}O_6N$	-1.73		
		FI at *m/z* 330.1336	330.1336	$C_{18}H_{20}O_5N$	-0.77		
		FI at *m/z* 173.0964	173.0961	$C_{12}H_{13}O$	1.72		
		FI at *m/z* 145.1014	145.1012	$C_{11}H_{13}$	1.29		
		FI at *m/z* 129.0701	129.0699	$C_{10}H_9$	1.60		
		FI at *m/z* 91.0548	91.0542	C_7H_7	6.73		
		FI at *m/z* 67.0551	67.0542	C_5H_7	13.4		

Table S2. List of *in vivo* phase I and II metabolites of 2-oxo-PCE, identified in rat urine samples and *in vitro* phase I metabolites identified in incubations using pooled human liver microsomes (pHLM), including the respective metabolite ID, metabolic reaction, masses of the precursor ion (PI) and characteristic fragment ions (FI) detected in MS^2, calculated exact masses, elemental composition, calculated mass errors in parts per million (ppm), retention times (RT) in minutes, and system in which metabolites were identified. The metabolites are sorted by their mass and RT. pHLM, identified in pHLM incubations; rat, identified in rat urine samples

Metabolite ID	Metabolic Reaction	Characteristic Ions Measured Accurate Masses	Calculated Exact Masses, *m/z*	Elemental Composition	Error, ppm	RT, min	Identified in
2-Oxo-PCE	Parent compound	PI at *m/z* 218.1540	218.1539	$C_{14}H_{20}ON$	-2.21	5.44	pHLM and rat
		FI at *m/z* 200.1429	200.1434	$C_{14}H_{18}N$	-2.54		
		FI at *m/z* 173.0957	173.0961	$C_{12}H_{13}O$	-1.98		
		FI at *m/z* 155.0851	155.0855	$C_{12}H_{11}$	-3.06		
		FI at *m/z* 145.1009	145.1012	$C_{11}H_{13}$	-2.18		
		FI at *m/z* 129.0697	129.0699	$C_{10}H_9$	-1.12		
		FI at *m/z* 117.0699	117.0699	C_9H_9	-0.12		
		FI at *m/z* 91.0545	91.0542	C_7H_7	3.38		
		FI at *m/z* 67.0549	67.0542	C_5H_7	9.37		
EM1	*N*-Dealkylation	PI at *m/z* 190.1225	190.1226	$C_{12}H_{16}ON$	-0.78	5.04	pHLM and rat
		FI at *m/z* 173.0961	173.0961	$C_{12}H_{13}O$	0.14		
		FI at *m/z* 155.0854	155.0855	$C_{12}H_{11}$	-0.60		
		FI at *m/z* 145.1012	145.1012	$C_{11}H_{13}$	0.03		
		FI at *m/z* 129.0700	129.0699	$C_{10}H_9$	0.89		
		FI at *m/z* 117.0701	117.0699	C_9H_9	2.29		
		FI at *m/z* 91.0547	91.0542	C_7H_7	5.65		
		FI at *m/z* 67.0550	67.0542	C_5H_7	11.6		
EM2	Hydroxylation + oxidation to a ketone	PI at *m/z* 232.1333	232.1332	$C_{14}H_{18}O_2N$	0.61	4.75	rat
		FI at *m/z* 187.0754	187.0754	$C_{12}H_{11}O_2$	0.28		
		FI at *m/z* 159.0805	159.0804	$C_{11}H_{11}O$	0.11		
		FI at *m/z* 91.0547	91.0542	C_7H_7	5.48		
EM3	Hydroxylation isomer 1	PI at *m/z* 234.1491	234.1489	$C_{14}H_{20}O_2N$	1.21	4.11	pHLM and rat
		FI at *m/z* 216.1389	216.1383	$C_{14}H_{18}ON$	2.96		
		FI at *m/z* 189.0910	189.0910	$C_{12}H_{13}O_2$	-0.01		
		FI at *m/z* 171.0805	171.0804	$C_{12}H_{11}O$	0.37		
		FI at *m/z* 161.0963	161.0961	$C_{11}H_{13}O$	1.57		
		FI at *m/z* 143.0856	143.0855	$C_{11}H_{11}$	0.84		
		FI at *m/z* 129.0701	129.0699	$C_{10}H_9$	1.84		
		FI at *m/z* 91.0548	91.0542	C_7H_7	6.23		
EM4	Hydroxylation isomer 2	PI at *m/z* 234.1490	234.1489	$C_{14}H_{20}O_2N$	0.63	4.62	pHLM and rat
		FI at *m/z* 216.1381	216.1383	$C_{14}H_{18}ON$	-0.84		
		FI at *m/z* 189.0912	189.0910	$C_{12}H_{13}O_2$	1..12		
		FI at *m/z* 171.0805	171.0804	$C_{12}H_{11}O$	0.28		
		FI at *m/z* 161.0961	161.0961	$C_{11}H_{13}O$	-0.23		
		FI at *m/z* 143.0856	143.0855	$C_{11}H_{11}$	0.84		
		FI at *m/z* 129.0701	129.0699	$C_{10}H_9$	1.47		
		FI at *m/z* 91.0548	91.0542	C_7H_7	6.57		
EM5	*N*-Dealkylation + glucuronidation	PI at *m/z* 366.1546	366.1547	$C_{18}H_{24}O_7N$	-0.26	5.20	rat
		FI at *m/z* 348.1444	348.1442	$C_{18}H_{22}O_6N$	0.61		
		FI at *m/z* 330.1329	330.1336	$C_{18}H_{20}O_5N$	-2.01		
		FI at *m/z* 173.0963	173.0961	$C_{12}H_{13}O$	0.93		
		FI at *m/z* 145.1012	145.1012	$C_{11}H_{13}$	0.45		
		FI at *m/z* 129.0703	129.0699	$C_{10}H_9$	3.14		
		FI at *m/z* 91.0548	91.0542	C_7H_7	6.32		
		FI at *m/z* 67.0550	67.0542	C_5H_7	11.9		

Table S3. List of *in vivo* phase I and II metabolites of 2-oxo-PCiP, identified in rat urine samples and *in vitro* phase I metabolites identified in incubations using pooled human liver microsomes (pHLM), including the respective metabolite ID, metabolic reaction, masses of the precursor ion (PI) and characteristic fragment ions (FI) detected in MS^2, calculated exact masses, elemental composition, calculated mass errors in parts per million (ppm), retention times (RT) in minutes, and system in which metabolites were identified. The metabolites are sorted by their mass and RT. pHLM, identified in pHLM incubations; rat, identified in rat urine samples

Metabolite ID	Metabolic Reaction	Characteristic Ions Measured Accurate Masses	Calculated Exact Masses, *m/z*	Elemental Composition	Error, ppm	RT, min	Identified in
2-Oxo-PCiP	Parent compound	PI at *m/z* 232.1690	232.1696	$C_{15}H_{22}ON$	-2.71	5.95	pHLM and rat
		FI at m/z 214.1596	214.1590	$C_{15}H_{20}N$	2.50		
		FI at *m/z* 173.0958	173.0961	$C_{12}H_{13}O$	-1.54		
		FI at *m/z* 155.0852	155.0855	$C_{12}H_{11}$	-2.17		
		FI at *m/z* 145.1009	145.1012	$C_{11}H_{13}$	-2.08		
		FI at *m/z* 129.0697	129.0699	$C_{10}H_{9}$	-1.00		
		FI at *m/z* 117.0700	117.0699	$C_{9}H_{9}$	0.66		
		FI at *m/z* 91.0546	91.0542	$C_{7}H_{7}$	3.72		
		FI at *m/z* 60.0815	60.0808	$C_{3}H_{10}N$	12.1		
IM1	*N*-Dealkylation	PI at *m/z* 190.1229	190.1226	$C_{12}H_{16}ON$	1.49	5.03	pHLM and rat
		FI at *m/z* 173.0962	173.0961	$C_{12}H_{13}O$	0.66		
		FI at *m/z* 155.0856	155.0855	$C_{12}H_{11}$	0.29		
		FI at *m/z* 145.1012	145.1012	$C_{11}H_{13}$	0.45		
		FI at *m/z* 129.0701	129.0699	$C_{10}H_{9}$	1.48		
		FI at *m/z* 117.0702	117.0699	$C_{9}H_{9}$	2.42		
		FI at *m/z* 91.0548	91.0542	$C_{7}H_{7}$	6.07		
		FI at *m/z* 67.0550	67.0542	$C_{5}H_{7}$	12.1		
IM2	*N*-Dealkylation + hydroxylation	PI at *m/z* 206.1175	206.1176	$C_{12}H_{16}O_2N$	-0.38	4.78	pHLM and rat
		FI at *m/z* 189.0910	189.0910	$C_{12}H_{13}O_2$	-0.01		
		FI at *m/z* 171.0804	171.0804	$C_{12}H_{11}O$	0.02		
		FI at *m/z* 161.0961	161.0961	$C_{11}H_{13}O$	-0.04		
		FI at *m/z* 143.0856	143.0855	$C_{11}H_{11}$	0.31		
		FI at *m/z* 129.0701	129.0699	$C_{10}H_{9}$	1.37		
		FI at *m/z* 91.0547	91.0542	$C_{7}H_{7}$	5.64		
IM3	Hydroxylation isomer 1 + oxidation to a ketone	PI at *m/z* 246.1490	246.1489	$C_{15}H_{20}O_2N$	0.66	5.22	rat
		FI at *m/z* 187.0755	187.0754	$C_{12}H_{11}O_2$	0.77		
		FI at *m/z* 159.0805	159.0804	$C_{11}H_{11}O$	0.40		
		FI at *m/z* 91.0547	91.0542	$C_{7}H_{7}$	5.48		
		FI at *m/z* 60.0817	60.0808	$C_{3}H_{10}N$	14.6		
IM4	Hydroxylation isomer 1	PI at *m/z* 248.1644	248.1645	$C_{12}H_{22}O_2N$	-0.61	4.75	pHLM and rat
		FI at *m/z* 230.1541	230.1539	$C_{12}H_{20}ON$	0.81		
		FI at *m/z* 189.0912	189.0910	$C_{12}H_{13}O_2$	0.87		
		FI at *m/z* 171.0805	171.0804	$C_{12}H_{11}O$	0.28		
		FI at *m/z* 143.0856	143.0855	$C_{11}H_{11}$	0.84		
		FI at *m/z* 91.0548	91.0542	C7H7	6.15		
		FI at *m/z* 60.0817	60.0808	$C_{3}H_{10}N$	15.1		
IM5	Hydroxylation isomer 2	PI at *m/z* 248.1645	248.1645	$C_{15}H_{22}O_2N$	0.23	5.04	pHLM and rat
		FI at *m/z* 189.0910	189.0910	$C_{12}H_{13}O$	-0.18		
		FI at *m/z* 161.0961	161.0961	$C_{11}H_{13}O$	-0.09		
		FI at *m/z* 107.0496	107.0491	$C_{7}H_{7}O$	4.30		
		FI at *m/z* 60.0816	60.0808	$C_{3}H_{10}N$	14.2		

IM6	Hydroxylation isomer 3	PI at m/z 248.1646	248.1645	$C_{15}H_{22}O_2N$	0.55	5.58	pHLM and rat
		FI at m/z 230.1541	230.1539	$C_{15}H_{20}ON$	0.69		
		FI at m/z 173.0963	173.0961	$C_{12}H_{13}O$	1.02		
		FI at m/z 145.1013	145.1012	$C_{11}H_{13}$	0.66		
		FI at m/z 129.0700	129.0699	$C_{10}H_9$	1.13		
		FI at m/z 91.0548	91.0542	C_7H_7	6.23		
		FI at m/z 76.0764	76.0757	$C_3H_{10}ON$	9.24		
		FI at m/z 58.0660	58.0651	C_3H_8N	15.4		
IM7	Dihydroxylation	PI at m/z 264.1593	264.1645	$C_{15}H_{22}O_3N$	-0.45	4.61	rat
		FI at m/z 246.1490	246.1489	$C_{15}H_{20}O_2N$	0.66		
		FI at m/z 228.1383	228.1383	$C_{15}H_{18}ON$	-0.07		
		FI at m/z 189.0908	189.0910	$C_{12}H_{13}O_2$	-1.14		
		FI at m/z 171.0805	171.0804	$C_{12}H_{11}O$	0.37		
		FI at m/z 143.0856	143.0855	$C_{11}H_{11}$	0.63		
		FI at m/z 76.0764	76.0757	$C_3H_{10}ON$	9.94		
		FI at m/z 58.0660	58.0651	C_3H_8N	15.4		
IM8	*N*-Dealkylation + glucuronidation	PI at m/z 366.1543	366.1547	$C_{18}H_{24}O_7N$	-1.18	5.22	rat
		FI at m/z 348.1441	348.1442	$C_{18}H_{22}O_6N$	-0.08		
		FI at m/z 330.1340	330.1336	$C_{18}H_{20}O_5N$	1.17		
		FI at m/z 173.0962	173.0961	$C_{12}H_{13}O$	0.47		
		FI at m/z 145.1012	145.1012	$C_{11}H_{13}$	0.14		
		FI at m/z 129.0701	129.0699	$C_{10}H_9$	1.58		
		FI at m/z 91.0548	91.0542	C_7H_7	6.58		
IM9	Hydroxylation isomer 3 + glucuronidation	PI at m/z 424.1966	424.1966	$C_{21}H_{30}O_8N$	0.00	5.24	rat
		FI at m/z 230.1537	230.1539	$C_{15}H_{20}ON$	-0.85		
		FI at m/z 173.0963	173.0961	$C_{12}H_{13}O$	1.28		
		FI at m/z 145.1013	145.1012	$C_{12}H_{13}O$	0.72		
		FI at m/z 129.0703	129.0699	$C_{10}H_9$	3.06		
		FI at m/z 91.0548	91.0542	C_7H_7	6.62		
		FI at m/z 76.0764	76.0757	$C_3H_{10}ON$	8.90		

Table S4. List of *in vivo* phase I and II metabolites of 2-oxo-PCMe, identified in rat urine samples and *in vitro* phase I metabolites identified in incubations using pooled human liver microsomes (pHLM), including the respective metabolite ID, metabolic reaction, masses of the precursor ion (PI) and characteristic fragment ions (FI) detected in MS^2, calculated exact masses, elemental composition, calculated mass errors in parts per million (ppm), retention times (RT) in minutes, and system in which metabolites were identified. The metabolites are sorted by their mass and RT. pHLM, identified in pHLM incubations; rat, identified in rat urine samples

Metabolite ID	Metabolic Reaction	Characteristic Ions Measured Accurate Masses	Calculated Exact Masses, *m/z*	Elemental Composition	Error, ppm	RT, min	Identified in
2-Oxo-PCMe	Parent compound	PI at m/z 204.1383	204.1383	$C_{13}H_{18}ON$	0.15	5.10	pHLM and rat
		FI at m/z 186.1277	186.1277	$C_{13}H_{16}N$	0.03		
		FI at m/z 173.0962	173.0961	$C_{12}H_{13}O$	0.75		
		FI at m/z 155.0855	155.0855	$C_{12}H_{11}$	-0.01		
		FI at m/z 145.1012	145.1012	$C_{11}H_{13}$	0.45		
		FI at m/z 129.0700	129.0699	$C_{10}H_9$	1.13		
		FI at m/z 117.0702	117.0699	C_9H_9	2.87		
		FI at m/z 91.0548	91.0542	C_7H_7	6.07		
		FI at m/z 67.0551	67.0542	C_5H_7	12.3		
MM1	*N*-Dealkylation	PI at m/z 190.1222	190.1226	$C_{12}H_{16}ON$	-2.28	5.00	pHLM and rat
		FI at m/z 173.0962	173.0961	$C_{12}H_{13}O$	0.84		
		FI at m/z 155.0856	155.0855	$C_{12}H_{11}$	0.48		
		FI at m/z 145.1013	145.1012	$C_{11}H_{13}$	0.69		
		FI at m/z 129.0701	129.0699	$C_{10}H_9$	1.72		
		FI at m/z 117.0703	117.0699	C_9H_9	3.33		
		FI at m/z 91.0548	91.0542	C_7H_7	6.23		
		FI at m/z 67.0551	67.0542	C_5H_7	12.5		
MM2	*N*-Dealkylation + hydroxylation	PI at m/z 206.1181	206.1176	$C_{12}H_{16}O_2N$	2.73	4.82	pHLM and rat
		FI at m/z 189.0912	189.0910	$C_{12}H_{13}O_2$	0.87		
		FI at m/z 171.0805	171.0804	$C_{12}H_{11}O$	0.55		
		FI at m/z 161.0962	161.0961	$C_{11}H_{13}O$	0.71		
		FI at m/z 143.0857	143.0855	$C_{11}H_{11}$	0.95		
		FI at m/z 129.0701	129.0699	$C_{10}H_9$	1.48		
		FI at m/z 117.0702	117.0699	C_9H_9	2.87		
		FI at m/z 91.0548	91.0542	C_7H_7	6.32		
		FI at m/z 67.0548	67.0542	C_5H_7	8.69		
MM3	*N*-Dealkylation + hydroxylamine	PI at m/z 206.1177	206.1176	$C_{12}H_{16}O_2N$	0.88	5.61	rat
		FI at m/z 188.1071	188.1070	$C_{12}H_{14}ON$	0.51		
		FI at m/z 173.0962	173.0961	$C_{12}H_{13}O$	0.58		
		FI at m/z 145.1012	145.1012	$C_{11}H_{13}$	0.13		
		FI at m/z 129.0701	129.0699	$C_{10}H_9$	1.37		
		FI at m/z 117.0702	117.0699	C_9H_9	2.35		
		FI at m/z 91.0548	91.0542	C_7H_7	5.81		
		FI at m/z 67.0550	67.0542	C_5H_7	12.0		
MM4	Hydroxylation isomer 1 + oxidation to a ketone	PI at m/z 218.1172	218.1176	$C_{13}H_{16}O_2N$	-1.48	4.61	rat
		FI at m/z 187.0751	187.0754	$C_{12}H_{11}O_2$	-1.27		
		FI at m/z 159.0802	159.0804	$C_{11}H_{11}O$	-1.23		
		FI at m/z 91.0546	91.0542	C_7H_7	4.56		
MM5	Hydroxylation isomer 1	PI at m/z 220.1330	220.1332	$C_{13}H_{18}O_2N$	-0.88	4.49	pHLM and rat
		FI at m/z 202.1223	202.1226	$C_{13}H_{16}ON$	-1.61		
		FI at m/z 189.0910	189.0910	$C_{12}H_{13}O_2$	-0.01		
		FI at m/z 171.0804	171.0804	$C_{12}H_{11}O$	-0.07		
		FI at m/z 143.0856	143.0855	$C_{11}H_{11}$	0.52		
		FI at m/z 91.0548	91.0542	C_7H_7	5.98		

MM6	Hydroxylation isomer 2	PI at m/z 220.1335	220.1332	$C_{13}H_{18}O_2N$	1.27	4.70	pHLM and rat
		FI at m/z 189.0911	189.0910	$C_{12}H_{13}O_2$	0.55		
		FI at m/z 161.0961	161.0961	$C_{11}H_{13}O$	0.34		
		FI at m/z 107.0496	107.0491	C_7H_7O	4.13		
		FI at m/z 67.0551	67.0542	C_5H_7	12.4		
MM7	*N*-Dealkylation + acetylation	PI at m/z 232.1341	232.1332	$C_{14}H_{18}O_2N$	3.83	6.90	rat
		FI at m/z 190.1231	190.1226	$C_{12}H_{16}ON$	2.62		
		FI at m/z 173.0962	173.0961	$C_{12}H_{13}O$	0.75		
		FI at m/z 155.0853	155.0855	$C_{12}H_{11}$	-1.58		
		FI at m/z 145.1013	145.1012	$C_{11}H_{13}$	0.66		
		FI at m/z 129.0697	129.0699	$C_{10}H_9$	-1.00		
		FI at m/z 91.0548	91.0542	C_7H_7	6.23		
		FI at m/z 67.0550	67.0542	C_5H_7	12.0		
MM8	*N*-Dealkylation + glucuronidation	PI at m/z 366.1552	366.1547	$C_{18}H_{24}O_7N$	1.15	5.25	rat
		FI at m/z 348.1444	348.1442	$C_{18}H_{22}O_6N$	0.70		
		FI at m/z 330.1332	330.1336	$C_{18}H_{20}O_5N$	-1.28		
		FI at m/z 173.0963	173.0961	$C_{12}H_{13}O$	1.11		
		FI at m/z 155.0856	155.0855	$C_{12}H_{11}$	0.19		
		FI at m/z 145.1013	145.1012	$C_{11}H_{13}$	0.76		
		FI at m/z 129.0701	129.0699	$C_{10}H_9$	1.37		
		FI at m/z 91.0548	91.0542	C_7H_7	6.57		
		FI at m/z 67.0551	67.0542	C_5H_7	12.9		
MM9	*N*-Dealkylation + hydroxylation glucuronidation	PI at m/z 382.1491	382.1469	$C_{18}H_{24}O_8N$	-1.52	4.37	rat
		FI at m/z 364.1381	364.1391	$C_{18}H_{22}O_7N$	-2.59		
		FI at m/z 171.0803	171.0804	$C_{12}H_{11}O$	-0.91		
		FI at m/z 161.0960	161.0961	$C_{11}H_{13}O$	-0.79		
		FI at m/z 143.0856	143.0855	$C_{11}H_{11}$	0.29		
		FI at m/z 91.0553	91.0542	C_7H_7	11.5		

Table S5. List of *in vivo* phase I and II metabolites of 2-oxo-PCPr, identified in rat urine samples and *in vitro* phase I metabolites identified in incubations using pooled human liver microsomes (pHLM), including the respective metabolite ID, metabolic reaction, masses of the precursor ion (PI) and characteristic fragment ions (FI) detected in MS^2, calculated exact masses, elemental composition, calculated mass errors in parts per million (ppm), retention times (RT) in minutes, and system in which metabolites were identified. The metabolites are sorted by their mass and RT. pHLM, identified in pHLM incubations; rat, identified in rat urine samples

Metabolite ID	Metabolic Reaction	Characteristic Ions Measured Accurate Masses	Calculated Exact Masses, *m/z*	Elemental Composition	Error, ppm	RT, min	Identified in
2-Oxo-PCPr	Parent compound	PI at m/z 232.1690	232.1696	$C_{15}H_{22}ON$	-2.39	5.96	pHLM and rat
		FI at m/z 214.1587	214.1590	$C_{15}H_{20}N$	-1.71		
		FI at m/z 173.0958	173.0961	$C_{12}H_{13}O$	-1.54		
		FI at m/z 155.0851	155.0855	$C_{12}H_{11}$	-2.47		
		FI at m/z 145.1009	145.1012	$C_{11}H_{13}$	-1.87		
		FI at m/z 129.0697	129.0699	$C_{10}H_{9}$	-1.71		
		FI at m/z 117.0700	117.0699	$C_{9}H_{9}$	1.31		
		FI at m/z 91.0546	91.0542	$C_{7}H_{7}$	3.89		
		FI at m/z 60.0815	60.0808	$C_{3}H_{10}N$	12.4		
PM1	*N*-Dealkylation	PI at m/z 190.1223	190.1226	$C_{12}H_{16}ON$	-1.82	5.05	pHLM and rat
		FI at m/z 173.0962	173.0961	$C_{12}H_{13}O$	0.63		
		FI at m/z 155.0854	155.0855	$C_{12}H_{11}$	-1.05		
		FI at m/z 145.1012	145.1012	$C_{11}H_{13}$	0.17		
		FI at m/z 129.0700	129.0699	$C_{10}H_{9}$	0.80		
		FI at m/z 117.0702	117.0699	$C_{9}H_{9}$	2.35		
		FI at m/z 91.0548	91.0542	$C_{7}H_{7}$	5.99		
		FI at m/z 67.0550	67.0542	$C_{5}H_{7}$	11.5		
PM2	Hydroxylation isomer 1 + oxidation to a ketone	PI at m/z 246.1490	246.1489	$C_{15}H_{20}O_2N$	0.66	5.37	rat
		FI at m/z 228.1380	228.1383	$C_{15}H_{18}ON$	-1.07		
		FI at m/z 187.0753	187.0754	$C_{12}H_{11}O_2$	-0.53		
		FI at m/z 159.0804	159.0804	$C_{11}H_{11}O$	-0.46		
		FI at m/z 141.0698	141.0699	$C_{11}H_{9}$	-0.48		
		FI at m/z 91.0546	91.0542	$C_{7}H_{7}$	4.14		
		FI at m/z 60.0816	60.0808	$C_{3}H_{10}N$	14.2		
PM3	Hydroxylation isomer 1	PI at m/z 248.1639	248.1645	$C_{15}H_{22}O_2N$	-2.52	4.75	pHLM and rat
		FI at m/z 230.1540	230.1539	$C_{15}H_{20}ON$	0.15		
		FI at m/z 189.0912	189.0910	$C_{12}H_{13}O_2$	0.87		
		FI at m/z 171.0805	171.0804	$C_{12}H_{11}O$	0.28		
		FI at m/z 143.0857	143.0855	$C_{11}H_{11}$	0.95		
		FI at m/z 60.0817	60.0808	$C_{3}H_{10}N$	17.8		
PM4	Hydroxylation isomer 2	PI at m/z 248.1649	248.1645	$C_{15}H_{22}O_2N$	1.41	5.31	pHLM and rat
		FI at m/z 230.1537	230.1539	$C_{15}H_{20}ON$	-0.91		
		FI at m/z 173.0963	173.0961	$C_{12}H_{13}O$	1.02		
		FI at m/z 155.0854	155.0855	$C_{12}H_{11}$	-0.50		
		FI at m/z 145.1012	145.1012	$C_{11}H_{13}$	0.34		
		FI at m/z 129.0701	129.0699	$C_{10}H_{9}$	1.96		
		FI at m/z 117.0702	117.0699	$C_{9}H_{9}$	2.94		
		FI at m/z 91.0548	91.0542	$C_{7}H_{7}$	6.07		
		FI at m/z 76.0764	76.0757	$C_{3}H_{10}ON$	9.44		
		FI at m/z 67.0550	67.0542	$C_{5}H_{7}$	12.2		

PM5	Hydroxylamine	PI at m/z 248.1648	248.1645	$C_{15}H_{22}O_2N$	1.29	6.21	pHLM and rat
		FI at m/z 230.1541	230.1539	$C_{15}H_{20}ON$	0.55		
		FI at m/z 173.0962	173.0961	$C_{12}H_{13}O$	0.40		
		FI at m/z 155.0860	155.0855	$C_{12}H_{11}$	3.04		
		FI at m/z 145.1013	145.1012	$C_{11}H_{13}$	0.69		
		FI at m/z 129.0701	129.0699	$C_{10}H_9$	1.72		
		FI at m/z 91.0548	91.0542	C_7H_7	6.65		
		FI at m/z 67.0550	67.0542	C_5H_7	11.3		
PM6	Dihydroxylation isomer 1 + monooxoidation to a ketone	PI at m/z 262.1438	262.1438	$C_{15}H_{20}O_3N$	0.11	4.85	rat
		FI at m/z 244.1321	244.1332	$C_{15}H_{18}O_2N$	-4.61		
		FI at m/z 187.0754	187.0754	$C_{12}H_{11}O_2$	0.28		
		FI at m/z 159.0805	159.0804	$C_{11}H_{11}O$	0.21		
		FI at m/z 76.0764	76.0757	$C_3H_{10}ON$	9.64		
PM7	Hydroxylation isomer 2 + oxidation to carboxylic acid	PI at m/z 262.1440	262.1438	$C_{15}H_{20}O_3N$	0.69	5.31	rat
		FI at m/z 244.1338	244.1332	$C_{15}H_{18}O_2N$	2.33		
		FI at m/z 173.0962	173.0961	$C_{12}H_{13}O$	0.58		
		FI at m/z 155.0855	155.0855	$C_{12}H_{11}$	-0.11		
		FI at m/z 145.1012	145.1012	$C_{11}H_{13}$	0.45		
		FI at m/z 129.0700	129.0699	$C_{10}H_9$	0.77		
		FI at m/z 117.0702	117.0699	C_9H_9	2.87		
		FI at m/z 91.0548	91.0542	C_7H_7	5.98		
		FI at m/z 67.0660	67.0542	C_5H_7	12.2		
PM8	Dihydroxylation isomer 1	PI at m/z 264.1596	264.1594	$C_{15}H_{22}O_3N$	0.82	4.61	rat
		FI at m/z 246.1490	246.1489	$C_{15}H_{20}O_2N$	0.72		
		FI at m/z 228.1382	228.1383	$C_{15}H_{18}ON$	-0.60		
		FI at m/z 189.0911	189.0910	$C_{12}H_{13}O_2$	0.31		
		FI at m/z 171.0805	171.0804	$C_{12}H_{11}O$	0.55		
		FI at m/z 143.0857	143.0855	$C_{11}H_{11}$	1.06		
		FI at m/z 76.0764	76.0757	$C_3H_{10}ON$	9.94		
PM9	Dihydroxylation isomer 2	PI at m/z 264.1595	264.1594	$C_{15}H_{22}O_3N$	0.36	5.09	rat
		FI at m/z 246.1490	246.1489	$C_{15}H_{20}O_2N$	0.72		
		FI at m/z 189.0910	189.0910	$C_{12}H_{13}O_2$	-0.01		
		FI at m/z 161.0961	161.0961	$C_{11}H_{13}O$	0.15		
		FI at m/z 107.0495	107.0491	C_7H_7O	3.77		
		FI at m/z 76.0764	76.0757	$C_3H_{10}ON$	9.45		
PM10	*N*-Dealkylation + glucuronidation	PI at m/z 366.1547	366.1547	$C_{18}H_{24}O_7N$	-0.01	5.29	rat
		FI at m/z 348.1437	348.1442	$C_{18}H_{22}O_6N$	-1.22		
		FI at m/z 330.1341	330.1336	$C_{18}H_{20}O_5N$	1.50		
		FI at m/z 173.0962	173.0961	$C_{12}H_{13}O$	0.66		
		FI at m/z 145.1013	145.1012	$C_{11}H_{13}$	0.66		
		FI at m/z 129.0701	129.0699	$C_{10}H_9$	1.72		
		FI at m/z 91.0548	91.0542	C_7H_7	6.23		
PM11	Hydroxylation isomer 2 + glucuronidation	PI at m/z 424.1964	424.1966	$C_{21}H_{30}O_8N$	-0.36	5.24	rat
		FI at m/z 248.1640	248.1645	$C_{15}H_{22}O_2N$	-1.85		
		FI at m/z 230.1539	230.1539	$C_{15}H_{20}ON$	-0.31		
		FI at m/z 173.0965	173.0961	$C_{12}H_{13}O$	2.52		
		FI at m/z 145.1012	145.1012	$C_{11}H_{13}$	0.34		
		FI at m/z 129.0702	129.0699	$C_{10}H_9$	2.19		
		FI at m/z 91.0548	91.0542	C_7H_7	6.23		
		FI at m/z 76.0764	76.0757	$C_3H_{10}ON$	9.73		

Table S6. Parent compounds and metabolites of five deschloroketamine derivatives detected by GC-MS, including masses of precursor ions (PI), elemental composition, and masses of characteristic fragment ions (FI). AC, acetylated.

Parent compound or metabolite	PI mass, *m/z*	RI	Elemental composition	Characteristic FI
2-Oxo-PCcP	229	1760	$C_{15}H_{19}NO$	200, 172, 145, 104, 91
2-Oxo-PCE	217	1635	$C_{14}H_{19}NO$	189, 160, 146, 132, 117, 104, 91
2-Oxo-PCiP	231	1648	$C_{15}H_{21}NO$	203, 174, 160, 132, 117, 104, 91
2-Oxo-PCMe AC	245	1990	$C_{15}H_{19}NO_2$	217, 174, 160, 144, 132, 118, 104, 91
2-Oxo-PCPr	231	1747	$C_{15}H_{21}NO$	203, 174, 160, 132, 117, 104, 91
2-Oxo-PCPr AC	273	2038	$C_{15}H_{21}NO$	245, 203, 174, 160, 144, 132, 117, 104, 91
N-Dealkylation AC	231	1874	$C_{14}H_{17}NO_2$	188, 174, 144, 132, 104, 91

Table S7. 2-Oxo-PCcP and its metabolites detected in rat urine after oral administration using different sample preparations in combination with LC-HRMS/MS (#) or GC-MS (*). Metabolite IDs correspond to Tables S1. CM, 2-oxo-PCcP metabolite; UPP, urine precipitation; UGLUC, urine after glucuronidase/arylsulfatase cleavage; LLE, liquid-liquid extraction; SPE, solid-phase extraction; UHyAc, partial urine hydrolysis followed by LLE and acetylation

Metabolite ID	Sample preparation performed				
	UPP#	UGLUC LLE#	SPE#	UGLUC SPE#	UHyAC*
2-oxo-PCcP	not detected	not detected	detected	detected	not detected
CM1	detected	detected	detected	detected	detected
CM2	not detected	not detected	detected	not detected	not detected
CM3	not detected	detected	detected	detected	not detected
CM4	not detected	detected	detected	detected	not detected
CM5	detected	not detected	detected	not detected	not detected
Summary	2 of 6	3 of 6	6 of 6	4 of 6	1 of 6

Table S8. 2-Oxo-PCE and its metabolites detected in rat urine after oral administration using different sample preparations in combination with LC-HRMS/MS (#) or GC-MS (*). Metabolite IDs correspond to Table S2. EM, 2-oxo-PCE metabolite; UPP, urine precipitation; UGLUC, urine after glucuronidase/arylsulfatase cleavage; LLE, liquid-liquid extraction; SPE, solid-phase extraction; UHyAc, partial urine hydrolysis followed by LLE and acetylation

Metabolite ID	Sample preparation performed				
	UPP#	UGLUC LLE#	SPE#	UGLUC SPE#	UHyAC*
2-Oxo-PCE	detected	detected	detected	detected	not detected
EM1	detected	detected	detected	detected	detected
EM2	detected	detected	detected	detected	not detected
EM3	detected	detected	detected	detected	not detected
EM4	detected	detected	detected	detected	not detected
EM5	detected	not detected	detected	not detected	not detected
Summary	6 of 6	5 of 6	6 of 6	5 of 6	1 of 6

Table S9. 2-Oxo-PCiP and its metabolites detected in rat urine after oral administration using different sample preparations in combination with LC-HRMS/MS (#) or GC-MS (*). Metabolite IDs correspond to Table S3. IM, 2-oxo-PCiP metabolite; UPP, urine precipitation; UGLUC, urine after glucuronidase/arylsulfatase cleavage; LLE, liquid-liquid extraction; SPE, solid-phase extraction; UHyAc, partial urine hydrolysis followed by LLE and acetylation

Metabolite ID	Sample preparation performed				
	UPP#	UGLUC LLE#	SPE#	UGLUC SPE#	UHyAC*
2-Oxo-PCiP	not detected	detected	detected	detected	not detected
IM1	detected	detected	detected	detected	detected
IM2	not detected	detected	detected	detected	not detected
IM3	not detected	not detected	detected	not detected	not detected
IM4	detected	detected	detected	detected	not detected
IM5	not detected	detected	not detected	detected	not detected
IM6	not detected	detected	not detected	detected	not detected
IM7	not detected	not detected	detected	not detected	not detected
IM8	detected	not detected	detected	not detected	not detected
IM9	detected	not detected	detected	not detected	not detected
Summary	4 of 10	6 of 10	8 of 10	6 of 10	1 of 10

Table S10. 2-Oxo-PCMe and its metabolites detected in rat urine after oral administration using different sample preparations in combination with LC-HRMS/MS (#) or GC-MS (*). Metabolite IDs correspond to Table S4. MM, 2-oxo-PCMe metabolite; UPP, urine precipitation; UGLUC, urine after glucuronidase/arylsulfatase cleavage; LLE, liquid-liquid extraction; SPE, solid-phase extraction; UHyAc, partial urine hydrolysis followed by LLE and acetylation

Metabolite ID	Sample preparation performed				
	UPP#	UGLUC LLE#	SPE#	UGLUC SPE#	UHyAC*
2-Oxo-PCMe	detected	detected	detected	detected	not detected
MM1	detected	detected	detected	detected	detected
MM2	detected	detected	detected	detected	not detected
MM3	detected	detected	detected	detected	not detected
MM4	detected	detected	detected	detected	not detected
MM5	detected	detected	detected	detected	not detected
MM6	not detected	detected	detected	detected	not detected
MM7	not detected	not detected	detected	not detected	not detected
MM8	detected	not detected	detected	not detected	not detected
MM9	not detected	not detected	detected	not detected	not detected
Summary	7 of 10	7 of 10	10 of 10	7 of 10	1 of 10

Table S11. 2-Oxo-PCPr and its metabolites detected in rat urine after oral administration using different sample preparations in combination with LC-HRMS/MS (#) or GC-MS (*). Metabolite IDs correspond to Table S5. PM, 2-oxo-PCPr metabolite; UPP, urine precipitation; UGLUC, urine after glucuronidase/arylsulfatase cleavage; LLE, liquid-liquid extraction; SPE, solid-phase extraction; UHyAc, partial urine hydrolysis followed by LLE and acetylation

Metabolite ID	**Sample preparation performed**				
	UPP#	**UGLUC LLE#**	**SPE#**	**UGLUC SPE#**	**UHyAC***
2-Oxo-PCPr	detected	detected	detected	detected	not detected
PM1	detected	detected	detected	detected	detected
PM2	detected	detected	detected	detected	not detected
PM3	detected	detected	detected	detected	not detected
PM4	detected	detected	detected	detected	not detected
PM5	detected	detected	detected	detected	not detected
PM6	detected	detected	detected	detected	not detected
PM7	detected	detected	detected	detected	not detected
PM8	not detected	detected	detected	detected	not detected
PM9	detected	detected	detected	detected	not detected
PM10	detected	not detected	detected	not detected	not detected
PM11	not detected	not detected	detected	not detected	not detected
Summary	10 of 12	10 of 12	12 of 12	10 of 12	1 of 12

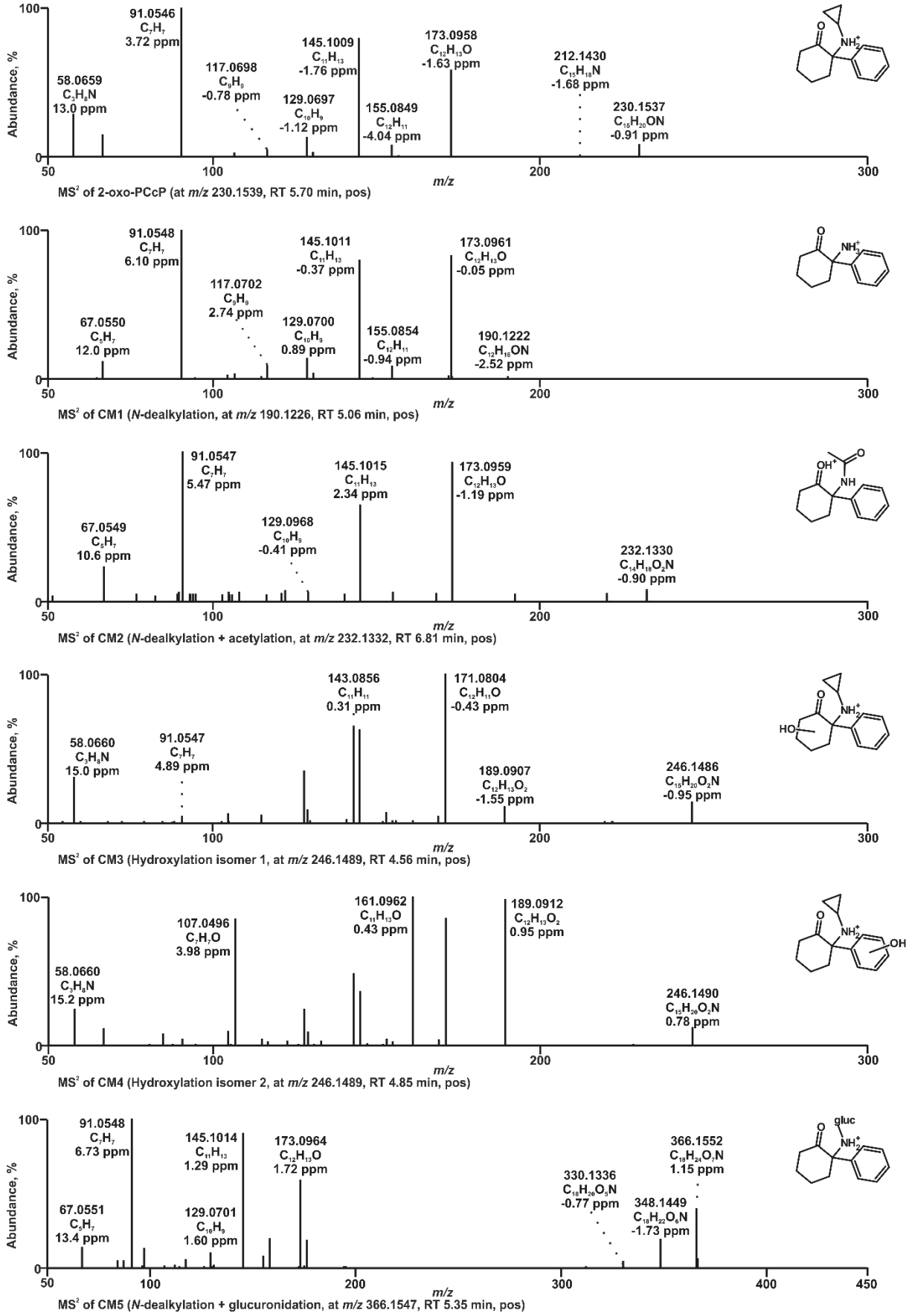

Figure S1. HRMS^2 spectra of 2-oxo-PCcP and its metabolites detected in rat urine after oral administration. Metabolite IDs correspond to Table S1. CM, 2-oxo-PCcP metabolite; RT, retention time

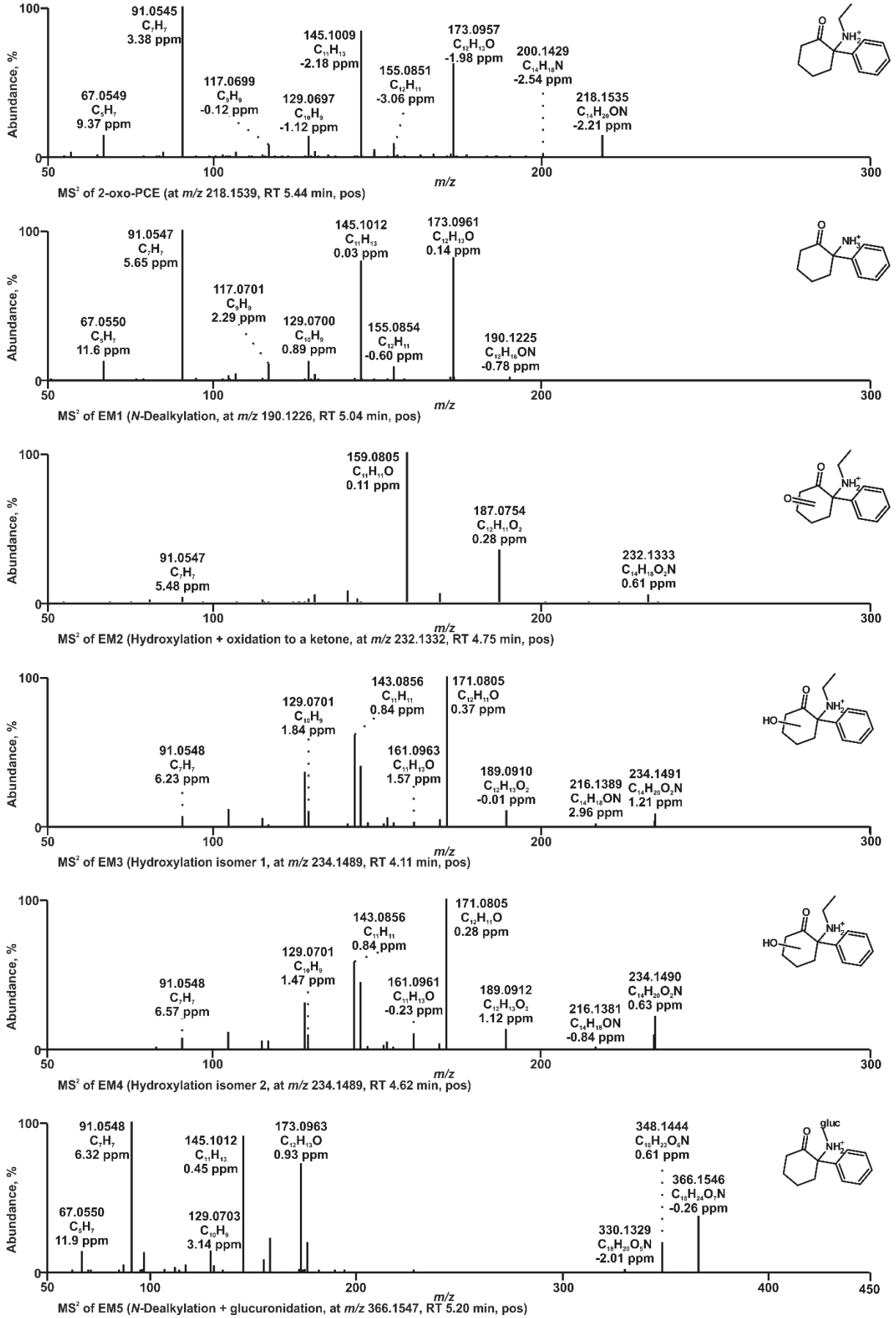

Figure S2. HRMS^2 spectra of 2-oxo-PCE and its metabolites detected in rat urine after oral administration. Metabolite IDs correspond to Table S2. EM, 2-oxo-PCE metabolite; RT, retention time

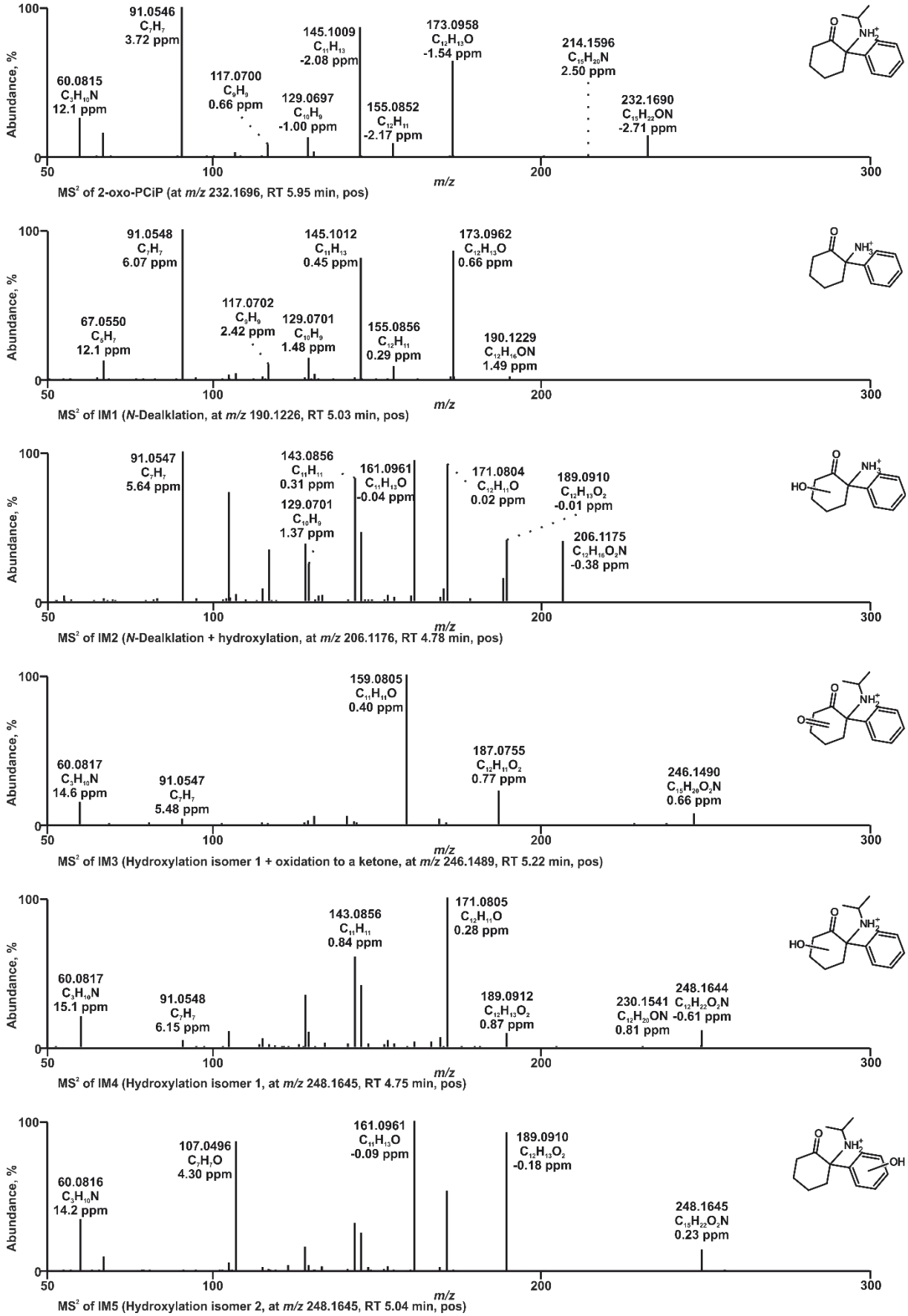

Figure S3. HRMS^2 spectra of 2-oxo-PCiP and its metabolites detected in rat urine after oral administration. Metabolite IDs correspond to Table S3. IM, 2-oxo-PCiP metabolite; RT, retention time

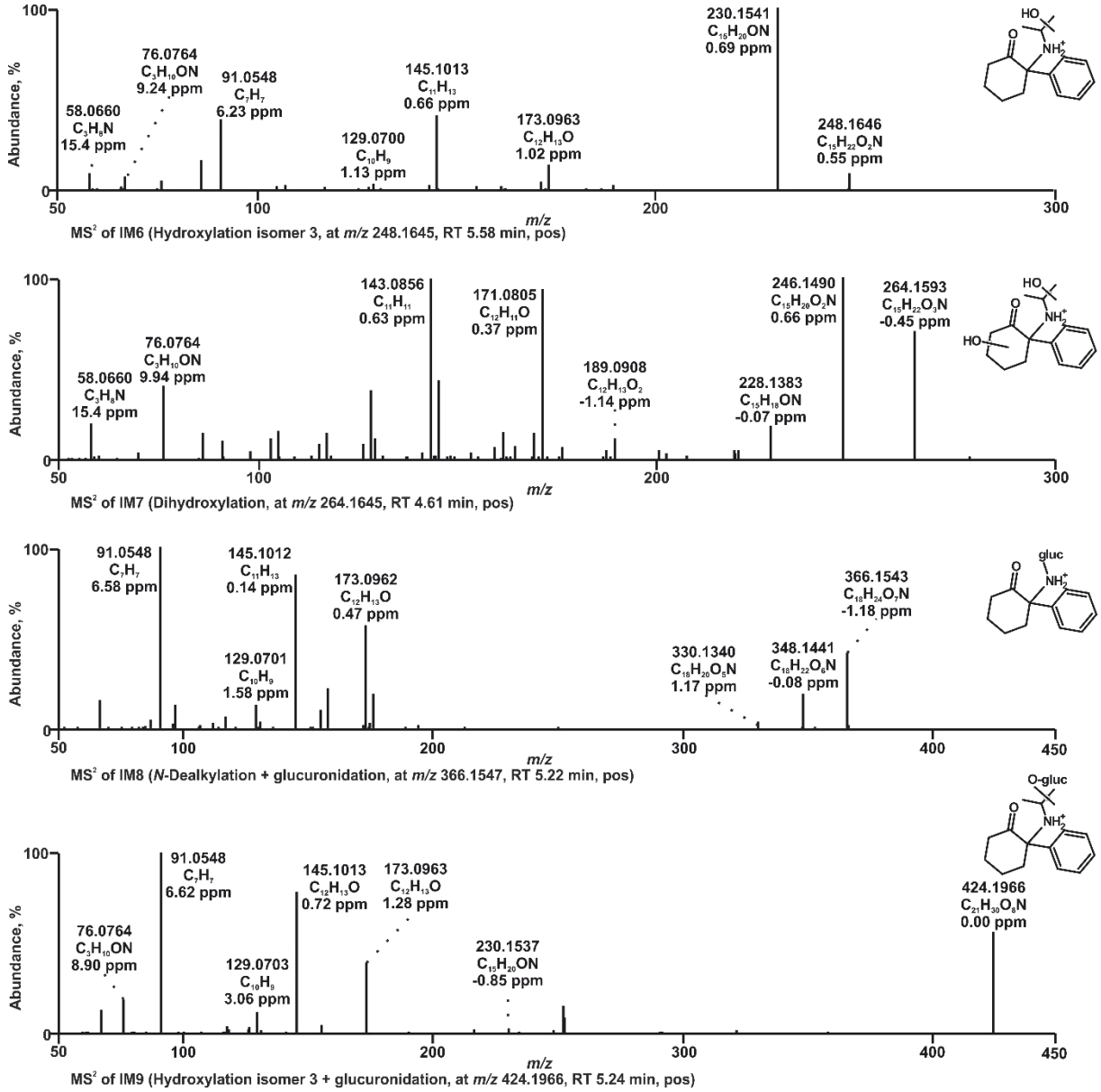

Figure S3. continued

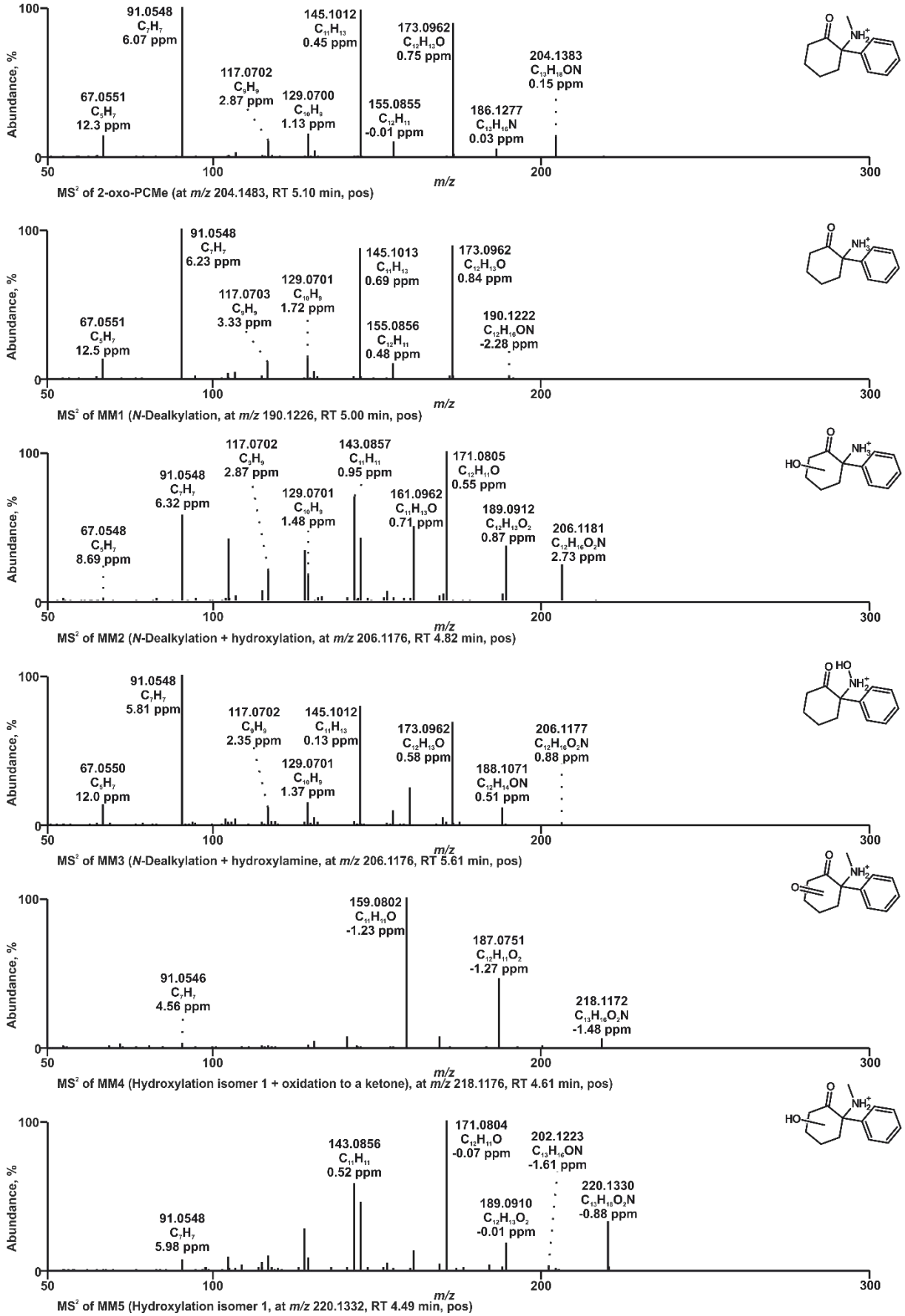

Figure S4. HRMS^2 spectra of 2-oxo-PCMe and its metabolites detected in rat urine after oral administration. Metabolite IDs correspond to Table S4. MM, 2-oxo-PCMe metabolite; RT, retention time

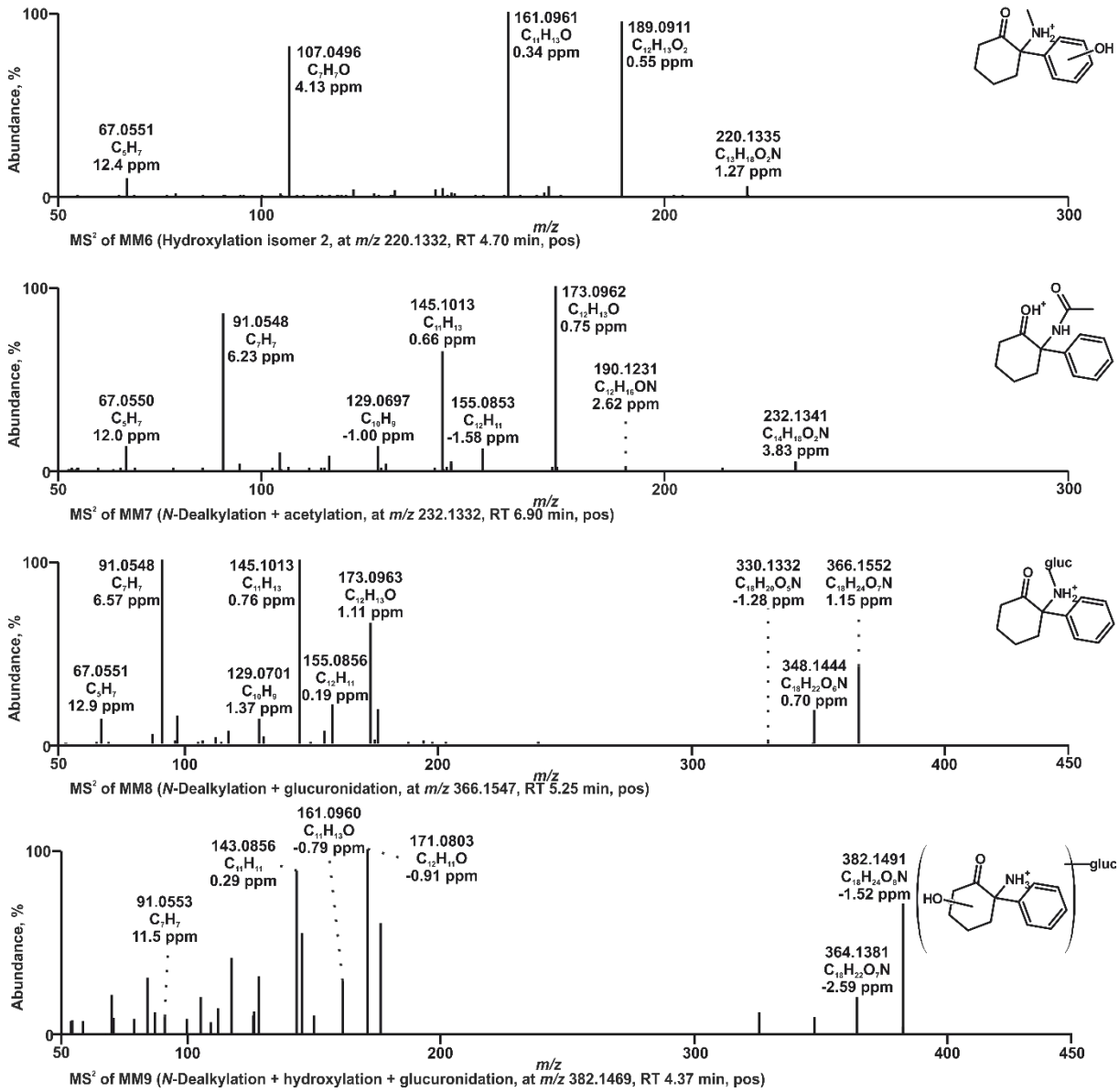

Figure S4. continued

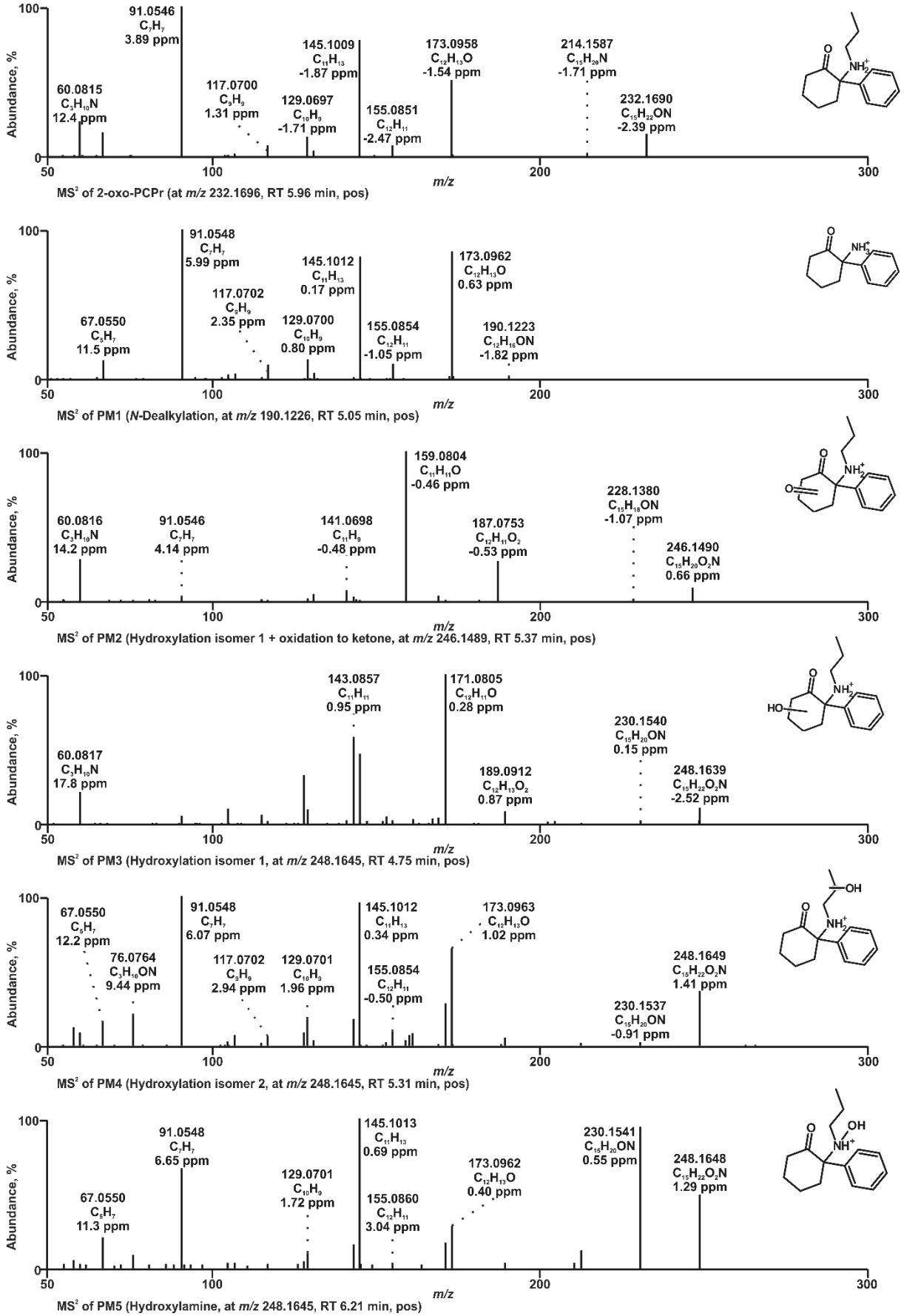

Figure S5. HRMS² spectra of 2-oxo-PCPr and its metabolites detected in rat urine after oral administration. Metabolite IDs correspond to Table S5. PM, 2-oxo-PCPr metabolite; RT, retention time

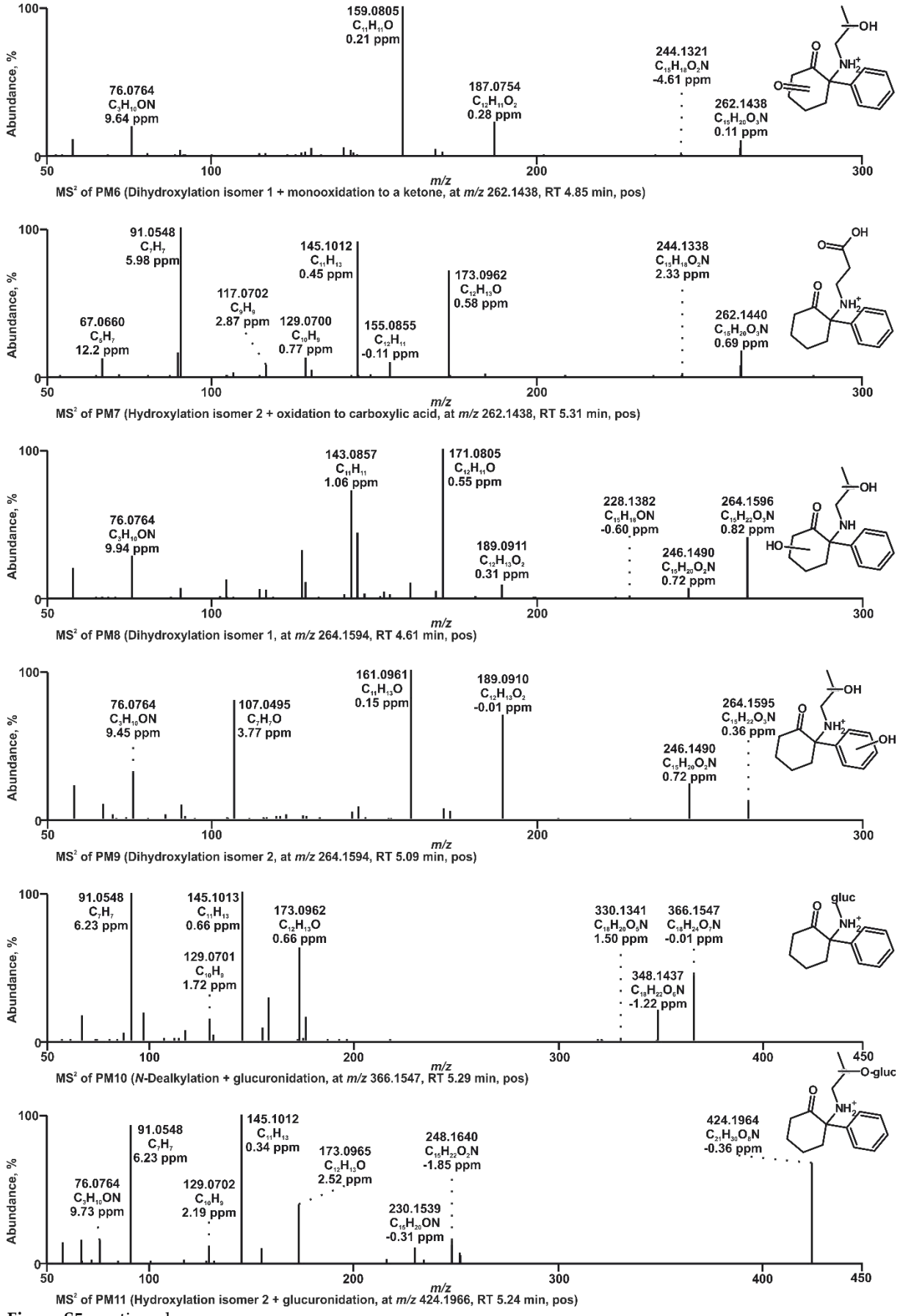

Figure S5. continued

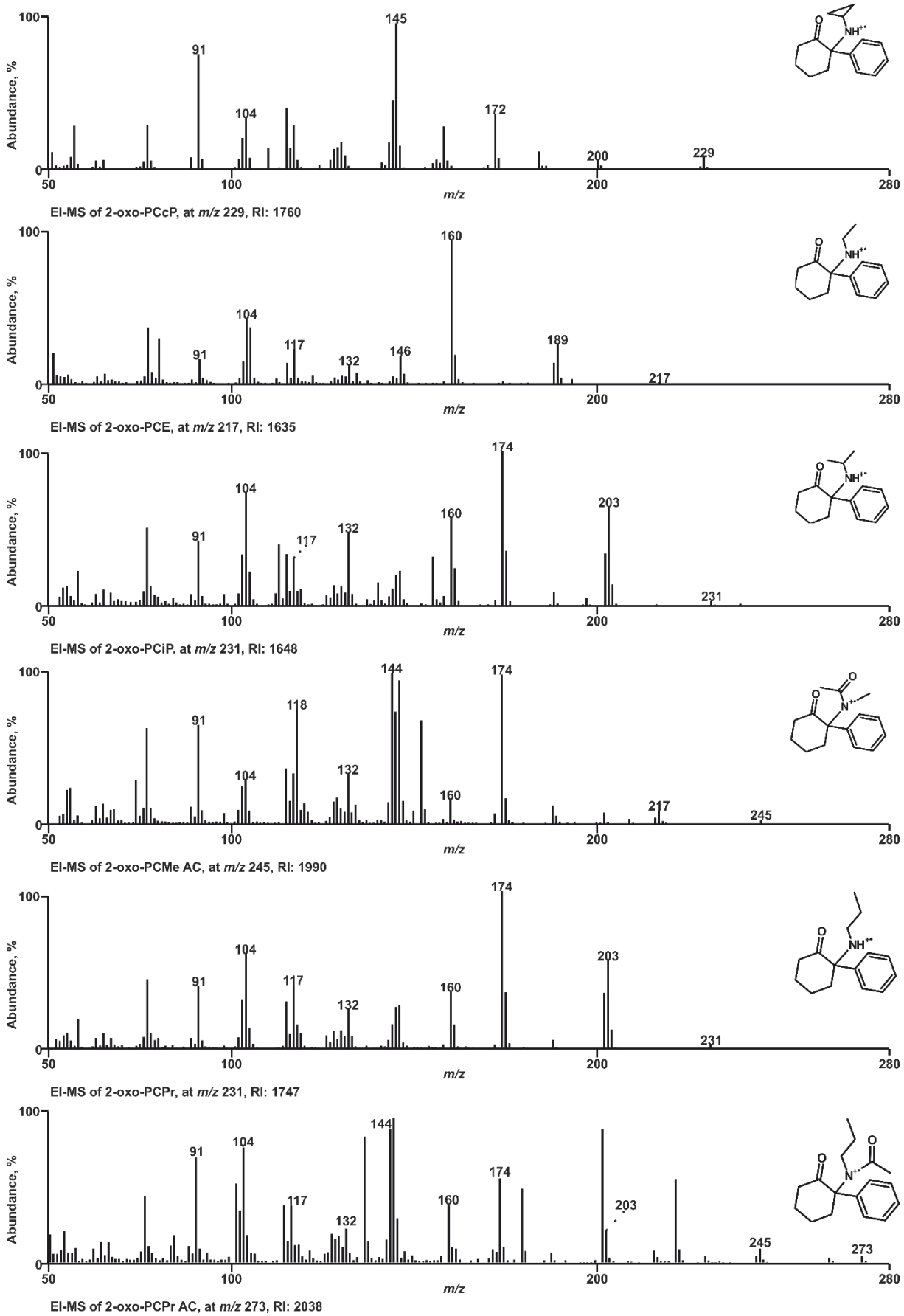

Figure S6. EI-MS spectra of 2-oxo-PCcP, 2-oxo-PCE, 2-oxo-PCiP, 2-oxo-PCMe AC, 2-oxo-PCPr, and acetylated *N*-dealkylation metabolites. Metabolite IDs correspond to Tables S1-S5. RI: retention index.

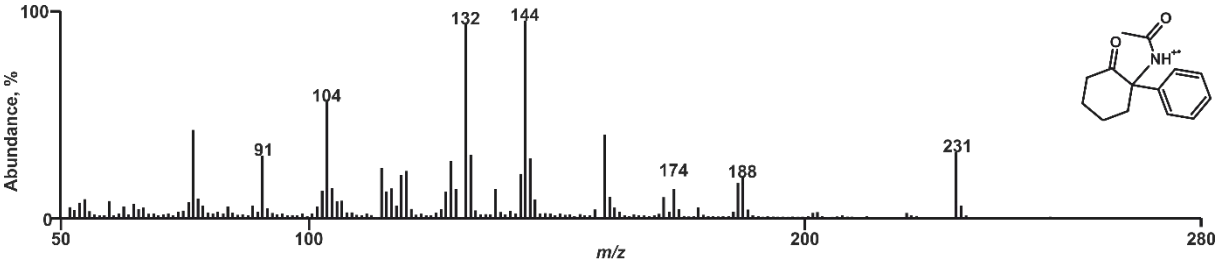

Figure S6. continued

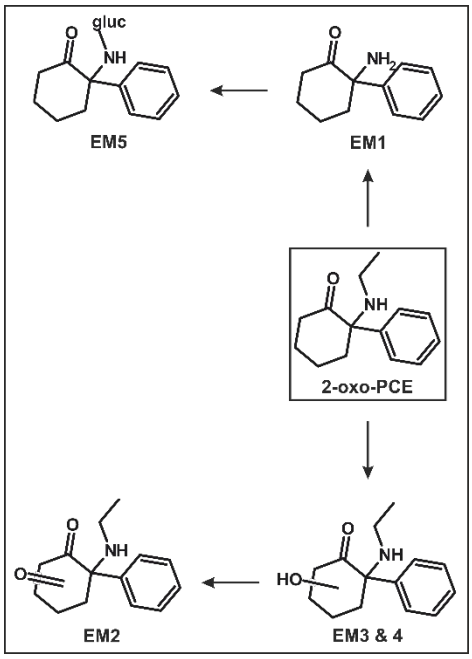

Figure S7. *In vivo* metabolic pathways of 2-oxo-PCE, ID corresponding to Table S2. EM, 2-oxo-PCE metabolite.

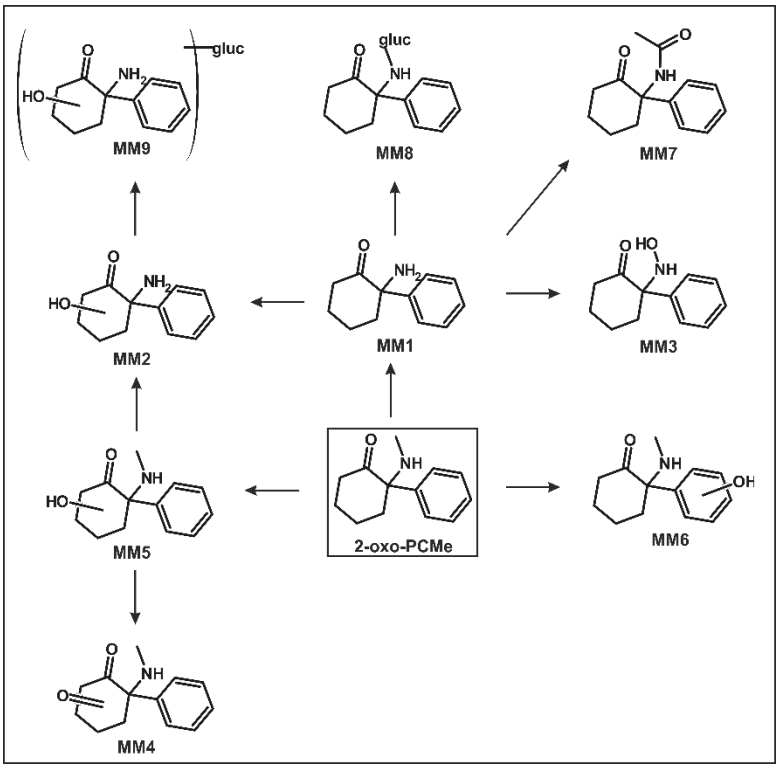

Figure S8. *In vivo* metabolic pathways of 2-oxo-PCMe, ID corresponding to Table S4. MM, 2-oxo-PCMe metabolite.

4.4. Studies on the Stability and Microbial Biotransformation of Five Deschloroketamine Derivatives in Wastewater by Means of Hyphenated Mass Spectrometry [68]

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Author contribution

F.F.: formal analysis, investigation, data curation, writing-original draft preparation, writing-review and editing, visualization; L.W.: conceptualization, writing-review and editing; M.R.M.: conceptualization, resources, writing-review and editing, supervision, project administration.

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

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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.

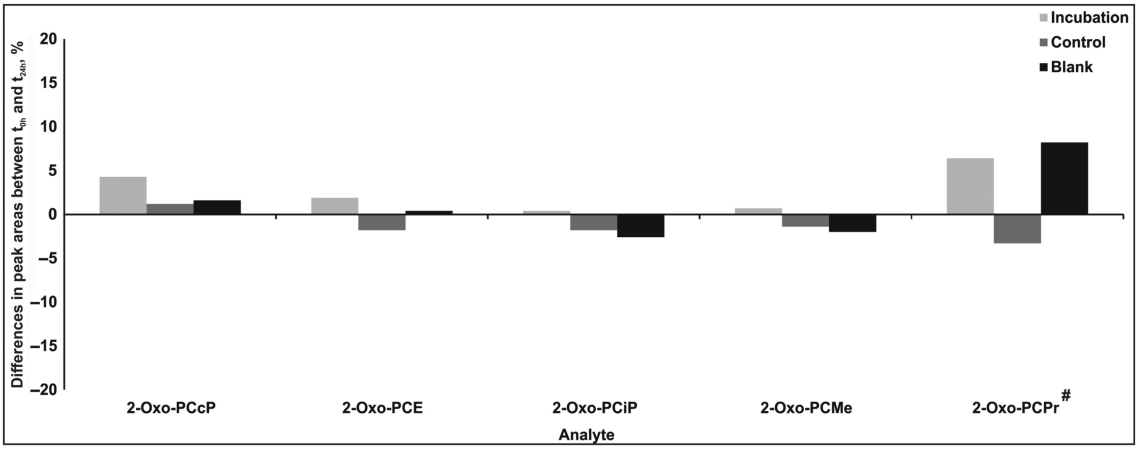

FIGURE 1 | Stability of deschloroketamine derivatives, as pure substances, in wastewater, control, and blank incubations over 24 h. Stability values are given as differences in peak areas between t_{0h} and t_{24h}, negative values indicating a decrease in peak area, positive values indicating an increase 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.

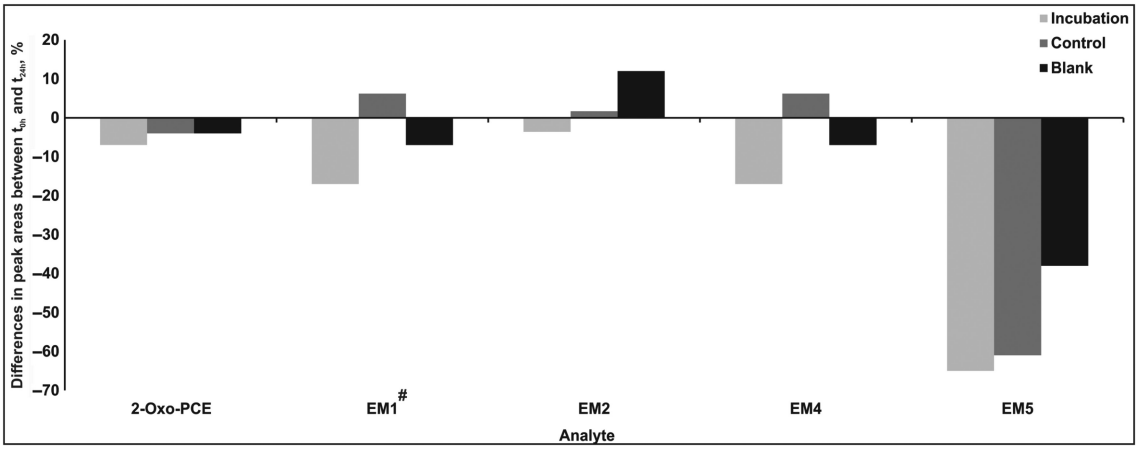

FIGURE 2 | Stability of deschloroketamine derivatives, and their metabolites obtained from rat urine, after wastewater, control, and blank incubations over 24 h, exemplified for 2-Oxo-PCE. Stability values are given as differences in peak areas between t_{0h} and t_{24h}, negative values indicating a 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,

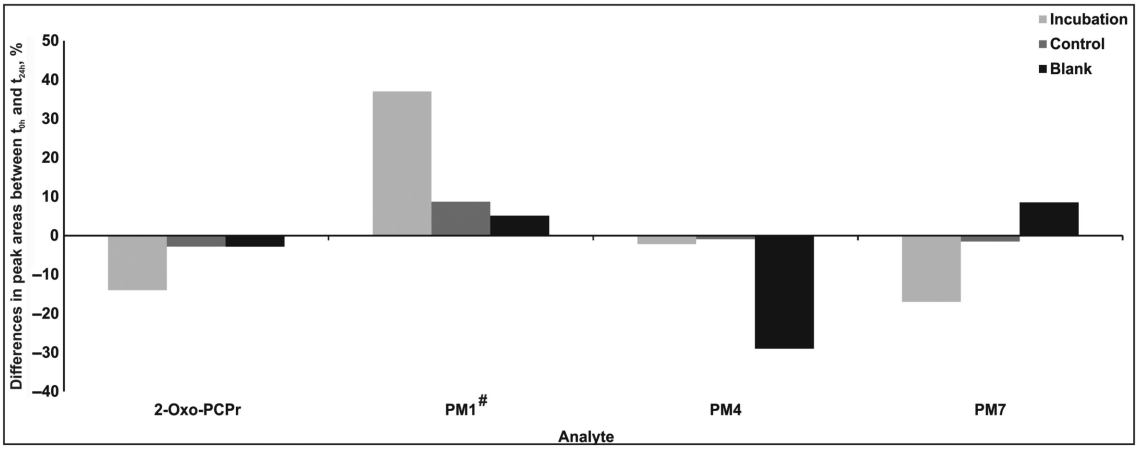

FIGURE 3 | Stability of deschloroketamine derivatives, and their metabolites obtained from rat feces, after wastewater, control, and blank incubations over 24 h, exemplified for 2-Oxo-PCPr. Stability values are given as differences in peak areas between t_{0h} and t_{24h}, negative values indicating 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.

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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.

Studies on the Stability and Microbial Biotransformation of Five Deschloroketamine Derivatives in Wastewater by Means of Hyphenated Mass Spectrometry

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Table S1. Difference in peak areas between t_{0h} and t_{24h}, after blank incubations in purified water. 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; CV, coefficient of variation; IS, internal standard.

Analyte	Difference in peak areas between t_{0h} and t_{24h}, % (CV, %)			
	Without IS-normalization *(n=10)*	With IS-normalization *(n=10)*	Without IS-normalization *(n=3)*	With IS-normalization *(n=3)*
2-Oxo-PCcP	– 4.6 (8.3)	20 (30)	– 1.1 (2.6)	5 (29)
2-Oxo-PCE	3.5 (5.5)	25 (30)	3.6 (7.9)	15 (27)
2-Oxo-PCiP	2.8 (5.3)	54 (33)	7.2 (3.4)	54 (50)
2-Oxo-PCMe	0.9 (3.6)	45 (36)	1.1 (3.7)	34 (14)
2-Oxo-PCPr	1.9 (4.5)	50 (27)	5.1 (4.3)	54 (5)

Table S2. Difference in peak areas between t_{0h} and t_{24h}, after wastewater incubations of five deschloroketamine derivatives. 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; CV, coefficient of variation; #, $p < 0.05$ in the two-sided t-test (TOST) comparing incubation and control.

Analyte	Difference in peak areas between t_{0h} and t_{24h}, % (CV, %)		
	Incubation	Control	Blank
2-Oxo-PCcP	4.3 (3.0)	1.2 (1.7)	1.6 (8.8)
2-Oxo-PCE	1.9 (8.6)	– 1.8 (4.6)	0.4 (2.3)
2-Oxo-PCiP	0.4 (3.2)	– 1.8 (4.9)	– 2.6 (3.4)
2-Oxo-PCMe	0.7 (2.8)	– 1.4 (4.0)	– 2.0 (4.4)
2-Oxo-PCPr$^{\#}$	6.4 (1.9)	– 3.3 (3.3)	8.2 (3.4)

Table S3. Difference in peak areas between t_{0h} and t_{24h}, after wastewater incubations of rat urines after oral administration of five deschloroketamine derivatives. 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.CM, 2-Oxo-PCcP metabolite; CV, coefficient of variation; EM, 2-Oxo-PCE metabolite; IM, 2-Oxo-PciP metabolite; MM, 2-Oxo-PCMe metabolite; PM, 2-Oxo-PCPr metabolite; #, $p < 0.05$ in the two sided t-test (TOST) comparing incubation and control.

Analyte	Difference in peak areas between t_{0h} and t_{24h}, % (CV, %)		
	Incubation	Control	Blank
2-Oxo-PCcP	55 (23)	7.0 (4.2)	– 0.8 (20)
CM1[#] (*N*-dealkylation)	– 33 (13)	18 (13)	– 6.0 (17)
CM2 (*N*-dealkylation + acetylation)	– 11 (7.0)	17 (15)	– 17 (7.9)
CM4[#] (hydroxylation isomer 2)	600 (14)	520 (13)	– 14 (2.0)
CM5[#] (*N*-dealkylation + glucuronidation)	– 89 (16)	– 76 (10)	– 36 (22)
2-Oxo-PCE	– 7.0 (2.5)	– 4.0 (6.0)	– 4.0 (4.0)
EM1[#] (*N*-dealkylation)	– 17 (12)	6.2 (4)	– 7.0 (8.9)
EM2 (hydroxylation + oxidation to a ketone)	– 3.6 (7.5)	1.7 (8.7)	12 (7.9)
EM4 (hydroxylation isomer 2)	3.0 (4.0)	16 (6.6)	– 7.0 (4.6)
EM5 (*N*-dealkylation + glucuronidation)	– 65 (10)	– 61 (32)	– 38 (15)
2-Oxo-PCiP[#]	– 3.6 (10)	– 22 (4.3)	– 0.5 (15)
IM1[#] (*N*-dealkylation)	– 35 (19)	5.5 (7.1)	17 (6.8)
IM6[#] (hydroxylation isomer 3)	320 (7.7)	93 (20)	14 (18)
IM8 (*N*-dealkylation + glucuronidation)	– 71 (10)	– 71 (18)	– 20 (29)
IM9[#] (hydroxylation isomer 3 + glucuronidation)	– 100 (7.8)	– 71 (26)	– 9.9 (25)
2-Oxo-PCMe[#]	21 (3.0)	2.9 (2.1)	– 0.7 (7.2)
MM1 (*N*-dealkylation)	7.5 (15)	0.93 (17)	1.4 (8.6)
MM2 (*N*-dealkylation + hydroxylation)	– 7.5 (13)	– 4.0 (6.3)	– 3.4 (4.9)
MM4[#] (hydroxylation isomer 1+ oxidation to a ketone)	– 28 (12)	– 6.6 (2.2)	– 9.1 (4.3)
MM6[#] (hydroxylation isomer 2)	20 (5.2)	– 0.5 (8.9)	-2.9 (6.3)
MM7 (*N*-dealkylation + acetylation)	–1.6 (22)	1.8 (2.9)	– 3.3 (10)
MM8[#] (*N*-dealkylation + glucuronidation)	– 80 (5.2)	– 48 (13)	– 44 (1.7)

Table S3. Continued.

Analyte	Difference in peak areas between t_{0h} and t_{24h}, % (CV, %)		
	Incubation	Control	Blank
2-Oxo-PCPr[#]	– 9.3 (0.8)	1.9 (4.6)	– 0.7 (7.2)
PM1[#] (*N*-dealkylation)	– 27 (11)	5.2 (18)	1.7 (20)
PM2 (hydroxylation isomer 1 + oxidation to a ketone)	– 11 (12)	11 (19)	1.0 (15)
PM4[#] (hydroxylation isomer 2)	1200 (8.3)	790 (20)	– 17 (21)
PM6 (dihydroxylation + monooxidation to a ketone)	– 10 (12)	– 2.0 (11)	– 2.7 (10)
PM7 (hydroxylation isomer 2 + oxidation to carboxylic acid)	– 15 (13)	4.9 (5.8)	– 8.1 (17)
PM9 (dihydroxylation isomer 2)	170 (3.7)	210 (19)	– 0.89 (11)
PM10 (*N*-dealkylation + glucuronidation)	– 61 (5.2)	– 61 (8.6)	– 30 (20)
PM11 (hydroxylation isomer 2 + glucuronidation)	– 100 (73)	– 81 (91)	– 18 (28)

Table S4. Parent compounds and metabolites detected in rat feces after oral administration of five deschloroketamine derivatives and difference in peak areas between t_{0h} and t_{24h}, after wastewater incubations of rat feces after oral administration of five deschloroketamine derivatives. 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.CM, 2-Oxo-PCcP metabolite; CV, coefficient of variation; EM, 2-Oxo-PCE metabolite; IM, 2-Oxo-PciP metabolite; MM, 2-Oxo-PCMe metabolite; PM, 2-Oxo-PCPr metabolite; #, $p < 0.05$ in the two sided t-test (TOST) comparing incubation and control.

Analyte	Difference in peak areas between t_{0h} and t_{24h}, % (CV, %)		
	Incubation	Control	Blank
2-Oxo-PCcP	5.3 (5.3)	– 7.0 (7.4)	– 2.1 (3.4)
CM1 (*N*-dealkylation)	140 (20)	94 (22)	100 (8.0)
2-Oxo-PCE	20 (27)	– 4.5 (2.7)	1.5 (5.1)
EM1 (*N*-dealkylation)	130 (23)	63 (20)	52 (5.6)
2-Oxo-PCiP	– 7.8 (21)	– 3.8 (5.8)	1.1 (19)
IM1 (*N*-dealkylation)	– 3.0 (8.1)	5.7 (7.4)	3.7 (10)
2-Oxo-PCMe[#]	5.9 (1.8)	– 7.9 (4.3)	– 0.8 (3.9)
MM1 (*N*-dealkylation)	3.1 (8.5)	– 5.3 (15)	– 3.4 (6.1)
MM8 (*N*-dealkylation + glucuronidation)	– 17 (5.2)	– 18 (3.3)	– 18 (8.8)
2-Oxo-PCPr	– 14 (17)	– 2.8 (5.4)	– 2.8 (12)
PM1[#] (*N*-dealkylation)	37 (14)	8.7 (3.6)	5.1 (11)
PM4 (hydroxylation isomer 2)	– 2.2 (23)	– 0.9 (20)	– 29 (9.1)
PM7 (hydroxylation isomer 2 + oxidation to carboxylic acid)	– 17 (20)	– 1.5 (3.1)	8.5 (8.7)

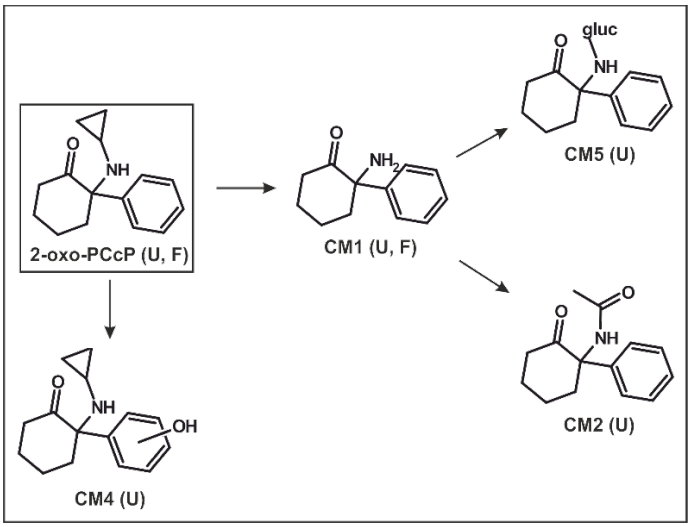

Figure S1. *In vivo* metabolic pathway of deschloro-*N*-cyclopropyl-ketamine (2-Oxo-PCcP), including only metabolites detected after wastewater (WW) incubations. CM; 2-Oxo-PCcP metabolite; F, metabolite detected after WW incubations of rat feces samples; U, metabolite detected after WW incubations of rat urine samples. Pathway modified according to [Frankenfeld et al., Metabolites, 2024 PMID: 38786747].

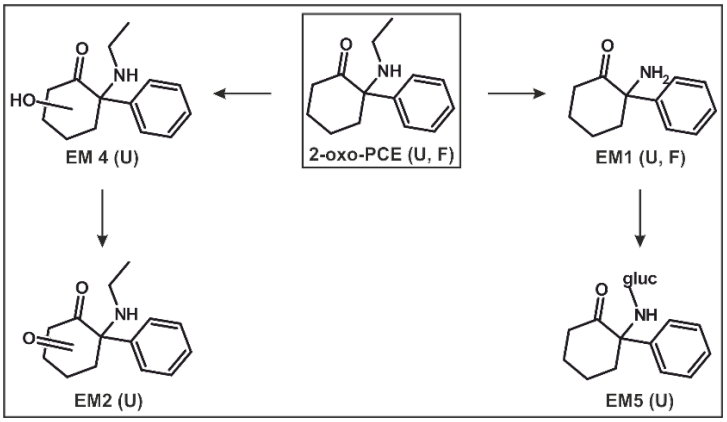

Figure S2. *In vivo* metabolic pathway of deschloro-*N*-ethyl-ketamine (2-Oxo-PCE), including only metabolites detected after wastewater (WW) incubations. EM; 2-Oxo-PCE metabolite; F, metabolite detected after WW incubations of rat feces samples; U, metabolite detected after WW incubations of rat urine samples. Pathway modified according to [Frankenfeld et al., Metabolites, 2024 PMID: 38786747].

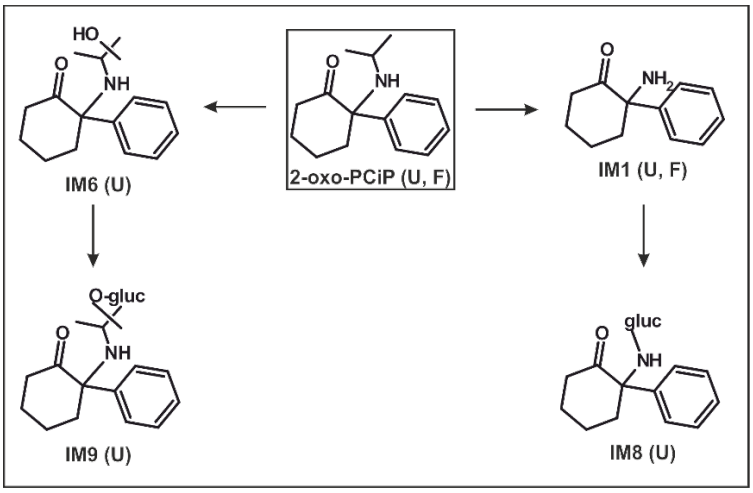

Figure S3. *In vivo* metabolic pathway of deschloro-*N*-isopropyl-ketamine (2-Oxo-PCiP), including only metabolites detected after wastewater (WW) incubations. IM; 2-Oxo-PCiP metabolite; F, metabolite detected after WW incubations of rat feces samples; U, metabolite detected after WW incubations of rat urine samples. Pathway modified according to [Frankenfeld et al., Metabolites, 2024 PMID: 38786747].

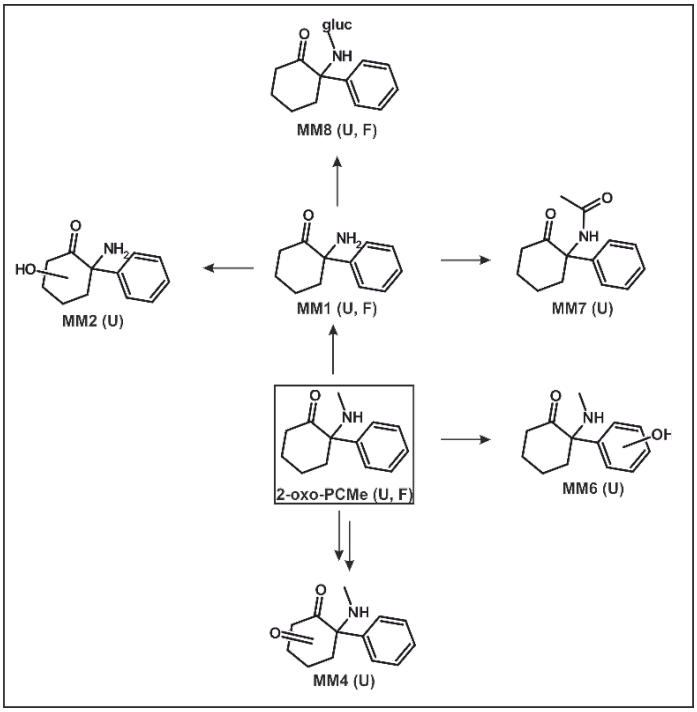

Figure S4. *In vivo* metabolic pathway of deschloro-*N*-methyl-ketamine (2-Oxo-PCMe), including only metabolites detected after wastewater (WW) incubations. MM; 2-Oxo-PCMe metabolite; F, metabolite detected after WW incubations of rat feces samples; U, metabolite detected after WW incubations of rat urine samples. Pathway modified according to [Frankenfeld et al., Metabolites, 2024 PMID: 38786747].

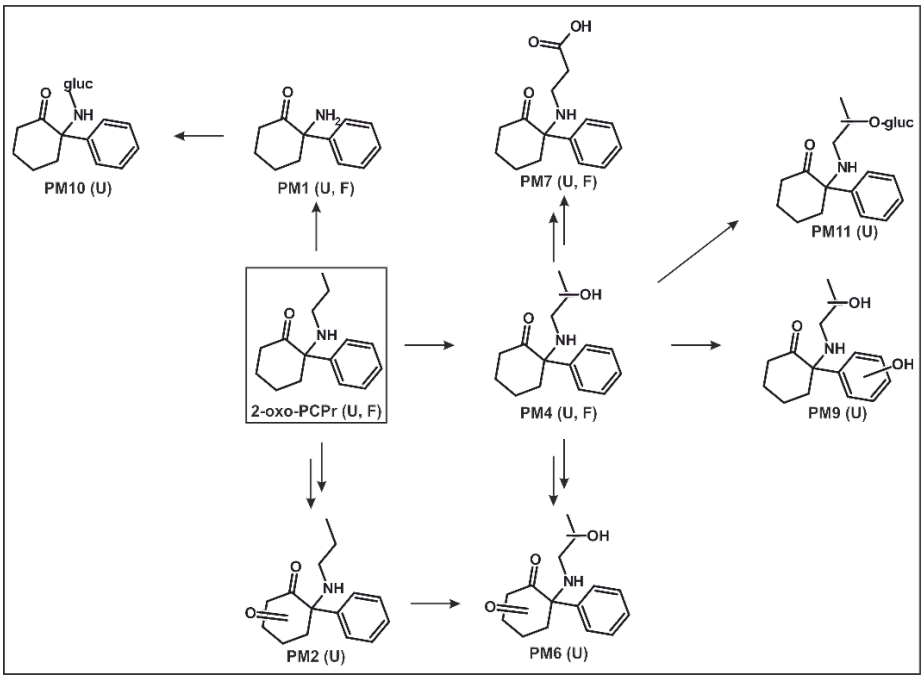

Figure S5. *In vivo* metabolic pathway of deschloro-*N*-propyl-ketamine (2-Oxo-PCPr), including only metabolites detected after wastewater (WW) incubations. PM; 2-Oxo-PCPr metabolite; F, metabolite detected after WW incubations of rat feces samples; U, metabolite detected after WW incubations of rat urine samples. Pathway modified according to [Frankenfeld et al., Metabolites, 2024 PMID: 38786747].

5. DISCUSSION

The presented studies contribute to the expansion of knowledge in both WBE and clinical toxicology, by elucidating consumptive patterns of DOA and cognitive enhancers in WW, as well as the *in vivo* and *in vitro* metabolism, microbial biotransformation, and WW stability of five DCKD.

In the first study, two complementary LC-HRMS/MS-based methods for the qualitative and quantitative analysis of four DOA, a cognitive enhancer, and three of their biomarkers in WW were successfully developed and validated. Validation experiments were performed according to the ICH M10 guideline [69]. Both methods differ as two different columns were investigated for the chromatographic separation of the target analytes. While an RP C_{18} column was implemented into the first method, for the second method a HILIC column was used. This difference in stationary phase had a significant impact on retention behavior and overall performance of the respective method. A common sample preparation via SPE enabled concentration of analytes, only differing in the respective reconstitution mixtures. Both methods enabled a high throughput of samples due to short run times (C_{18}, 10 min; HILIC, 15 min), which is crucial for large studies. Validation results showed advantages of the C_{18} method, as only two analytes (methamphetamine; METH and 4-hydroxy-3-methoxymethamphetamine; HMMA) did not fulfill all validation criteria, set by the ICH M10 guideline [69]. Using the HILIC method, four analytes (AMPH, BZE, ethyl sulfate, and HMMA) did not fulfill all validation criteria [69]. Thus, HMMA was the only compound of interest which could not be reliably analyzed using either method. Furthermore, ethyl sulfate-d_5, used as an internal standard for the quantification of ethyl sulfate, was not extractable via SPE, although it was detectable in stock solutions. Hence, analysis of ethyl sulfate was not possible, as none of the other IS could be used to achieve reliable quantification results. However, in literature, dilute-and-shoot approaches were described for the analysis of ethyl sulfate [70, 71]. Based on the results obtained after method validation, both columns are necessary for the comprehensive analysis of the selected compounds. Moreover, analysis of proof-of-concept samples showed the method's applicability for qualitative and quantitative analysis of multiple analytes in WW grab samples. Hence, this approach including both

validated methods is suitable to monitor trends in consumption of selected DOA and one cognitive enhancer in WW samples.

In the second study, influent WW 24 h composite samples were obtained from a local WWTP and WW grab samples were obtained from two different sampling sites in the same city. Analysis was performed using both methods developed and validated in the first study of this thesis. For each of the included analytes, either the C_{18} or the HILIC method was used, depending on which method showed the best results during method validation. Evaluation of grab samples showed that ritalinic acid, AMPH, BZE, and COC were most frequently identified and quantified. Frequent detection of ritalinic acid may be explained by high prescription rates of its parent compound MPH as ADHD medication or its abuse as a cognitive enhancer [44, 72]. Concerning AMPH, BZE, and COC, high detection rates may be explained by the common use of AMPH and COC as recreational drugs, as reported in the European Drug report and shown, also for (Western) German cities, in a Europe-wide WBE study [22, 29]. Loads quantified in composite 24 h WW samples were compared to a previous study, where sampling was performed at the same WWTP, revealing an increase in loads of AMPH and METH [3]. This observed rise in DOA loads was in line with trends published for German cities in a study by the SCORE group in 2024 [29]. Although METH could be quantified in this study, its loads in WW were lower compared to AMPH. These differences in loads may be explained by the sampling site's location relative to the European AMPH/METH distribution border in Germany [3]. Moreover, loads calculated for COC and MDMA were also in accordance with data published for several German cities in a Europewide study [29]. Results obtained after analysis of grab samples were compared to those of the 24 h composite WW samples, which showed more consistent results using composite samples. This consistency may be explained due to the continuous sampling process when acquiring composite samples.

The third study focused on the *in vivo* metabolic fate of five DCKD in rat urine after oral administration, followed by confirmation of phase I metabolites after incubations using pHLM. In total 39 phase I and II metabolites were tentatively identified by means of LC-HRMS/MS analysis. Nineteen of those phase I metabolites were confirmed using pHLM incubations. The most abundant metabolites (*N*-dealkylation) could not be used for differentiation between the five parent compounds. Therefore, substance specific

metabolites with an intact *N*-substitute were used as screening targets. Furthermore, different sample preparation strategies were compared, highlighting UPP using ACN followed by LC-HRMS/MS analysis as a sufficient strategy for the detection of specific metabolites of all DCKD except 2-oxo-PCcP. An additional approach using UHyAc and GC-MS analysis only led to tentative identification of the *N*-acetylated parent compounds. Detectability experiments showed limits of identification ranging between 10 µg/L using the UPP approach and 10 mg/L after the UHyAc procedure. This further underlines the advantages of the UPP approach, which also requires a lower sample volume compared to the UHyAc [73, 74]. Via the laboratories LC-HRMS/MS-based standard urine screening approach (SUSA), one parent compound and several specific metabolites were defined as screening targets for the use in clinical toxicology routine screenings. These targets included hydroxylation + oxidation metabolites of 2-oxo-PCE and 2-oxo-PCiP, 2-oxo-PCMe itself, and the carboxylic acid metabolite of 2-oxo-PCP. Only for 2-oxo-PCcP no compound specific screening target could be proposed based on the SUSA results. Additionally, one human urine sample after suspected 2-oxo-PCMe intake was included in this study. Screening results were in accordance with the findings in rat urine samples.

The fourth study focused on the microbial biotransformation and WW stability of the previously investigated DCKD, and their metabolites. Parent compound solutions, rat urine, and feces samples, containing the five DCKD and their metabolites were incubated in raw, influent, WW samples for 24 h. Of all parent compounds only 2-oxo-PCcP showed a relevant decrease in peak areas after WW incubation. Thus, the remaining parent compounds were suggested as potential screening targets in WW samples. However, including only parent compounds into analytical methods used for WBE does not allow for differentiation between use and disposal of the tested compounds. Hence, compound specific metabolites were also investigated as potential consumption biomarkers. As most phase I metabolites showed only little differences in peak areas after WW incubations, those metabolites were assumed to be stable under the tested conditions. Since only metabolites with an intact *N*-substitute allow for differentiation between the five structurally similar DCKD, particular focus was placed on compound specific metabolites. Hydroxylation and hydroxylation + oxidation metabolites may be used as WW biomarkers in future studies, since they showed only low differences in peak areas after WW incubations. Nevertheless, it must be

considered that cleavage of their corresponding phase II metabolites could lead to a potentially high increase in peak areas. Although qualitative analysis would not be negatively affected by this, quantitative results might be heavily biased. The observed instability of glucuronides in WW was already described in literature and excludes them from being used as a biomarker in WBE [75, 76]. This study also shows the difficulties when implementing NPS into WBE methods. Even though parent compounds were available as pure substances, metabolites of DCKD could only be obtained after extraction of rat urine and feces samples after prior *in vivo* experiments. In order to establish the investigated metabolites as reliable biomarkers for WBE, future studies should focus on assessing the adhesion of proposed biomarkers to materials used for sewer-lines, particles in WW, or filter materials used for sample pretreatment.

The four studies showed the potential applications of WBE beyond environmental monitoring and public health surveillance. WBE is a valuable tool for clinical toxicologists by enabling the monitoring of consumptive trends even in small populations, which are not included in large (e.g., Europe-wide) studies. This emphasizes the importance of (quick) recognition of patterns in illicit drug consumption, which may be also beneficial for health or law enforcement authorities or forensic toxicologists. However, limitations need to be discussed as well. Data obtained via the WBE approach lacks individual specificity, as it only shows consumptive trends on an anonymized population level. Hence, the direct impact of the obtained loads may be hard to interpret for clinical toxicologists. Furthermore, implementation of new compounds, such as NPS, to already or even new established methods is challenging. Since not only human metabolism of new drugs needs to be elucidated prior to find suitable biomarkers, but also microbial and WW stability are of importance. As for most NPS, no metabolites are available as reference standards, researchers rely on biosamples containing metabolites e.g., rat urines or feces, are required to conduct these studies. Such biosamples are mostly limited in their amount and may vary in metabolite concentrations, depending on the system used to produce metabolites or intraindividual fluctuations when conducting *in vivo* metabolism experiments for their acquisition. Furthermore, metabolites could be obtained via synthesis, however, this approach is also time and resource consuming. The limited availability of those compounds may also affect method development procedures and even the studies executed after method development and validation. While WBE may provide

population level data on (illicit) drug consumption to clinical toxicologists, it should be used as a complementary tool rather than a standalone resource.

6. CONCLUSION

The presented studies demonstrated the use of WBE for the qualitative and quantitative analysis of (illicit) drugs and their metabolites in WW. The WBE approach allowed for a population level surveillance of compounds of interest and reflected their consumptive trends in a smaller community. However, WBE is not able to provide individual diagnostic information and is limited by the extent to which existing methods can be extended to include NPS and their metabolites. Hence, while WBE offers valuable data for clinical toxicologists, it remains a complementary tool.

7. REFERENCES

1. Mardal, M., et al., *Microbial biotransformation of five pyrrolidinophenone-type psychoactive substances in wastewater and a wastewater isolated Pseudomonas putida strain.* Drug Test Anal, 2017. **9**(10): p. 1522-1536.
2. Gracia-Lor, E., et al., *Measuring biomarkers in wastewater as a new source of epidemiological information: Current state and future perspectives.* Environ Int, 2017. **99**: p. 131-150.
3. Meyer, M.R., 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 Alcohol Depend, 2015. **156**: p. 311-314.
4. Chen, C., et al., *Towards finding a population biomarker for wastewater epidemiology studies.* Sci Total Environ, 2014. **487**: p. 621-8.
5. Ort, C., et al., *Sampling for pharmaceuticals and personal care products (PPCPs) and illicit drugs in wastewater systems: are your conclusions valid? A critical review.* Environ Sci Technol, 2010. **44**(16): p. 6024-35.
6. de Oliveira, A.F.B., A. de Melo Vieira, and J.M. Santos, *Trends and challenges in analytical chemistry for multi-analysis of illicit drugs employing wastewater-based epidemiology.* Analytical and Bioanalytical Chemistry, 2023. **415**(18): p. 3749-3758.
7. Baker, D.R. and B. Kasprzyk-Hordern, *Critical evaluation of methodology commonly used in sample collection, storage and preparation for the analysis of pharmaceuticals and illicit drugs in surface water and wastewater by solid phase extraction and liquid chromatography-mass spectrometry.* J Chromatogr A, 2011. **1218**(44): p. 8036-59.
8. Castiglioni, S., et al., *Identification of cocaine and its metabolites in urban wastewater and comparison with the human excretion profile in urine.* Water Res, 2011. **45**(16): p. 5141-50.
9. van Nuijs, A.L., et al., *Sewage epidemiology--a real-time approach to estimate the consumption of illicit drugs in Brussels, Belgium.* Environ Int, 2011. **37**(3): p. 612-21.
10. Ort, C., et al., *Spatial differences and temporal changes in illicit drug use in Europe quantified by wastewater analysis.* Addiction, 2014. **109**(8): p. 1338-52.
11. van Nuijs, A.L., et al., *Analysis of drugs of abuse in wastewater by hydrophilic interaction liquid chromatography-tandem mass spectrometry.* Anal Bioanal Chem, 2009. **395**(3): p. 819-28.
12. Plewa, S., et al., *A Review of Wastewater-Based Epidemiology Studies for the Assessment of Over-the-Counter Medicines Used as Recreational Drugs: The Example of Dextromethorphan.* Med Sci Monit, 2024. **30**: p. e944120.
13. Ostman, M., et al., *A snapshot of illicit drug use in Sweden acquired through sewage water analysis.* Sci Total Environ, 2014. **472**: p. 862-71.
14. Gheorghe, A., et al., *Analysis of cocaine and its principal metabolites in waste and surface water using solid-phase extraction and liquid chromatography-ion trap tandem mass spectrometry.* Anal Bioanal Chem, 2008. **391**(4): p. 1309-19.
15. Mari, F., et al., *Cocaine and heroin in waste water plants: a 1-year study in the city of Florence, Italy.* Forensic Sci Int, 2009. **189**(1-3): p. 88-92.

16. Crocq, M.A., *Historical and cultural aspects of man's relationship with addictive drugs.* Dialogues Clin Neurosci, 2007. **9**(4): p. 355-61.
17. Sneader, W., *The discovery of heroin.* Lancet, 1998. **352**(9141): p. 1697-9.
18. Rasmussen, N., *Amphetamine-Type Stimulants: The Early History of Their Medical and Non-Medical Uses.* Int Rev Neurobiol, 2015. **120**: p. 9-25.
19. Böllinger, L., *Drug Law and Policy in Germany and the European Community: Recent Developments.* Journal of Drug Issues, 2004. **34**(3): p. 491-510.
20. EMCDDA, *European Drug Report 2022.* Publications of of the European Union, 2022.
21. EUDA. *European Drug Report 2023: Trends and Developments*. 2023; 2023:[Available from: https://www.euda.europa.eu/publications/european-drug-report/2023_en.
22. EUDA. *European Drug Report 2024*. 2024; 2024:[Available from: https://www.euda.europa.eu/publications/european-drug-report/2024_en#pdf.
23. UNODC, *World Drug Report 2023.* 2024.
24. Daughton, C.G., *Illicit Drugs in Municipal Sewage*, in *Pharmaceuticals and Care Products in the Environment*, C.G. Daughton and T.L. Jones-Lepp, Editors. 2001, American Chemical Society Washington, DC. p. 348-364.
25. Ort, C., et al., *Wastewater Analysis for Community-Wide Drugs Use Assessment.* Handb Exp Pharmacol, 2018.
26. Carneiro, R.B., et al., *A critical review of wastewater-based epidemiology as a tool to evaluate the unintentional human exposure to potentially harmful chemicals.* Anal Bioanal Chem, 2025. **417**(3): p. 495-511.
27. Zuccato, E., et al., *Estimating community drug abuse by wastewater analysis.* Environ Health Perspect, 2008. **116**(8): p. 1027-32.
28. Plosz, B.G., et al., *Biotransformation kinetics and sorption of cocaine and its metabolites and the factors influencing their estimation in wastewater.* Water Res, 2013. **47**(7): p. 2129-40.
29. EUDA. *Wastewater Analysis and Drugs — A European Multi-City Study*. 2024; Available from: https://www.emcdda.europa.eu/publications/html/pods/waste-water-analysis_en.
30. Shafi, A., et al., *New psychoactive substances: a review and updates.* Ther Adv Psychopharmacol, 2020. **10**: p. 2045125320967197.
31. Pantano, F., et al., *New Psychoactive Substances: A Matter of Time.* Curr Neuropharmacol, 2019. **17**(9): p. 818-822.
32. Peters, F.T. and J.A. Martinez-Ramirez, *Analytical toxicology of emerging drugs of abuse.* Ther Drug Monit, 2010. **32**(5): p. 532-9.
33. Wagmann, L., et al., *How to Study the Metabolism of New Psychoactive Substances for the Purpose of Toxicological Screenings-A Follow-Up Study Comparing Pooled Human Liver S9, HepaRG Cells, and Zebrafish Larvae.* Front Chem, 2020. **8**: p. 539.
34. Meyer, M.R., *New psychoactive substances: an overview on recent publications on their toxicodynamics and toxicokinetics.* Arch Toxicol, 2016. **90**(10): p. 2421-44.
35. Bade, R., et al., *Improving wastewater-based epidemiology for new psychoactive substance surveillance by combining a high-throughput in vitro metabolism assay and LC-HRMS metabolite identification.* Water Res, 2024. **253**: p. 121297.
36. Soeiro, T., et al., *Inputs of pharmacoepidemiology in addictovigilance: How do they fit together?* Therapie, 2025. **80**(2): p. 205-211.

37. WHO *22nd Model list of essential medicines*. 2021.
38. Savic Vujovic, K., et al., *Ketamine, an Old-New Drug: Uses and Abuses.* Pharmaceuticals (Basel), 2023. **17**(1).
39. EMCDDA, *European Drug Report 2023.* Publications of of the European Union, 2023.
40. EMCDDA, *Europol 2015 Annual Report on the implementation of Council Decision 2005/387/JHA*. 2017, Publications Office of the European Union, Luxembourg.
41. EMCDDA, *Europol 2016 Annual Report on the implementation of Council Decision 2005/387/JHA*. 2016: https://www.emcdda.europa.eu/system/files/publications/4724/TDAN17001ENN_PDFWEB.pdf.
42. Sharif, S., et al., *The Use and Impact of Cognitive Enhancers among University Students: A Systematic Review.* Brain Sci, 2021. **11**(3).
43. Chiappini, S., et al., *Methylphenidate abuse and misuse in patients affected with a psychiatric disorder and a substance use disorder: a systematic review.* Front Psychiatry, 2024. **15**: p. 1508732.
44. Ludwig, W.D., B. Mühlbauer, and R. Seifert, *Arzneiverordnungs-Report 2023*. 2023.
45. Wilms, W., et al., *Nootropic drugs: Methylphenidate, modafinil and piracetam - Population use trends, occurrence in the environment, ecotoxicity and removal methods - A review.* Chemosphere, 2019. **233**: p. 771-785.
46. Gonzalez-Marino, I., et al., *Multi-residue determination of psychoactive pharmaceuticals, illicit drugs and related metabolites in wastewater by ultra-high performance liquid chromatography-tandem mass spectrometry.* J Chromatogr A, 2018. **1569**: p. 91-100.
47. Oliveira, T.S., et al., *Characterization of Pharmaceuticals and Personal Care products in hospital effluent and waste water influent/effluent by direct-injection LC-MS-MS.* Sci Total Environ, 2015. **518-519**: p. 459-78.
48. Herndon, L.K., et al., *Bacterial Wastewater-Based Epidemiology Using Surface-Enhanced Raman Spectroscopy and Machine Learning.* Nano Lett, 2025. **25**(4): p. 1250-1259.
49. Cai, L., et al., *Wastewater-based epidemiology for comprehensive communitywide exposome surveillance: A gradient of metals exposure.* medRxiv, 2023.
50. Ma, Y., et al., *Concentrations of perfluoroalkyl substances in sediments and wastewater treatment plant-derived biosolids from Ireland.* Sci Total Environ, 2025. **979**: p. 179380.
51. Letzel, M., et al., *Occurrence and fate of the human pharmaceutical metabolite ritalinic acid in the aquatic system.* Chemosphere, 2010. **81**(11): p. 1416-22.
52. Jobling, S., et al., *Predicted exposures to steroid estrogens in U.K. rivers correlate with widespread sexual disruption in wild fish populations.* Environ Health Perspect, 2006. **114 Suppl 1**(Suppl 1): p. 32-9.
53. Gushgari, A.J., et al., *Long-term tracking of opioid consumption in two United States cities using wastewater-based epidemiology approach.* Water Res, 2019. **161**: p. 171-180.
54. Duvallet, C., et al., *Mapping Community Opioid Exposure Through Wastewater-Based Epidemiology as a Means to Engage Pharmacies in Harm Reduction Efforts.* Prev Chronic Dis, 2020. **17**.

55. Rice, J., et al., *Wastewater-based epidemiology combined with local prescription analysis as a tool for temporalmonitoring of drugs trends - A UK perspective.* Sci Total Environ, 2020. **735**: p. 139433.
56. Gray, N., et al., *Comparison of reversed-phase and hydrophilic interaction liquid chromatography for the quantification of ephedrines using medium-resolution accurate mass spectrometry.* J Chromatogr A, 2013. **1289**: p. 37-46.
57. Nguyen, H.P. and K.A. Schug, *The advantages of ESI-MS detection in conjunction with HILIC mode separations: Fundamentals and applications.* J Sep Sci, 2008. **31**(9): p. 1465-80.
58. Subirats, X., M.H. Abraham, and M. Roses, *Characterization of hydrophilic interaction liquid chromatography retention by a linear free energy relationship. Comparison to reversed- and normal-phase retentions.* Anal Chim Acta, 2019. **1092**: p. 132-143.
59. Senta, I., et al., *Wastewater-based epidemiology to assess human exposure to personal care and household products - A review of biomarkers, analytical methods, and applications.* Trends Environ Anal Chem, 2020. **28**: p. e00103.
60. Yi, R., et al. *Wastewater-Based Epidemiology: Assessing Illicit Drug Usage and Impact through an Innovative Approach*. Water, 2023. **15**, DOI: 10.3390/w15234192.
61. Hernandez, F., et al., *Mass spectrometric strategies for the investigation of biomarkers of illicit drug use in wastewater.* Mass Spectrom Rev, 2018. **37**(3): p. 258-280.
62. Hernandez, F., et al., *Rapid wide-scope screening of drugs of abuse, prescription drugs with potential for abuse and their metabolites in influent and effluent urban wastewater by ultrahigh pressure liquid chromatography-quadrupole-time-of-flight-mass spectrometry.* Anal Chim Acta, 2011. **684**(1-2): p. 87-97.
63. Hernández, F., et al., *Investigation of pharmaceuticals and illicit drugs in waters by liquid chromatography-high-resolution mass spectrometry.* TrAC Trends in Analytical Chemistry, 2014. **63**: p. 140-157.
64. Frankenfeld, F., et al., *Quantitative analysis of drugs of abuse and cognitive enhancers in influent wastewater by means of two chromatographic methods.* Drug Testing and Analysis, 2023.
65. Frankenfeld, F., et al., *Prevalence of Drugs of Abuse and Cognitive Enhancer Consumption Monitored in Grab Samples and Composite Wastewater via Orbitrap Mass Spectrometry Analysis.* Molecules, 2024. **29**(16).
66. Frankenfeld, F., et al., *In Vivo and In Vitro Metabolic Fate and Urinary Detectability of Five Deschloroketamine Derivatives Studied by Means of Hyphenated Mass Spectrometry.* Metabolites, 2024. **14**(5).
67. Wagmann, L., M.R. Meyer, and H.H. Maurer, *What is the contribution of human FMO3 in the N-oxygenation of selected therapeutic drugs and drugs of abuse?* Toxicol Lett, 2016. **258**: p. 55-70.
68. Frankenfeld, F., L. Wagmann, and M.R. Meyer, *Studies on the Stability and Microbial Biotransformation of Five Deschloroketamine Derivatives as Prerequisite for Wastewater-Based Epidemiology Screening.* Drug Test Anal, 2024.
69. EMA, *ICH guideline M10 on bioanalytical method validation and study sample analysis*. 2022, European Medicines Agency: Amsterdam, The Netherlands.

70. Mastroianni, N., M. Lopez de Alda, and D. Barcelo, *Analysis of ethyl sulfate in raw wastewater for estimation of alcohol consumption and its correlation with drugs of abuse in the city of Barcelona.* J Chromatogr A, 2014. **1360**: p. 93-9.
71. Rodriguez-Alvarez, T., et al., *Alcohol and cocaine co-consumption in two European cities assessed by wastewater analysis.* Sci Total Environ, 2015. **536**: p. 91-98.
72. Finger, G., E.R. Silva, and A. Falavigna, *Use of methylphenidate among medical students: a systematic review.* Rev Assoc Med Bras (1992), 2013. **59**(3): p. 285-9.
73. Meyer, M.R., et al., *Studies on the metabolism of the alpha-pyrrolidinophenone designer drug methylenedioxy-pyrovalerone (MDPV) in rat and human urine and human liver microsomes using GC-MS and LC-high-resolution MS and its detectability in urine by GC-MS.* J Mass Spectrom, 2010. **45**(12): p. 1426-42.
74. Wissenbach, D.K., et al., *Development of the first metabolite-based LC-MS(n) urine drug screening procedure-exemplified for antidepressants.* Anal Bioanal Chem, 2011. **400**(1): p. 79-88.
75. Banks, A.P.W., et al., *Potential impact of the sewer system on the applicability of alcohol and tobacco biomarkers in wastewater-based epidemiology.* Drug Test Anal, 2017.
76. Kumar, V., et al., *De-conjugation behavior of conjugated estrogens in the raw sewage, activated sludge and river water.* J Hazard Mater, 2012. **227-228**: p. 49-54.

8. ABBREVIATIONS

ADHD	Attention deficit hyperactivity disorder
AMPH	Amphetamine
BZE	Benzoylecgonine
COC	Cocaine
DCKD	Deschloroketamine derivative
DOA	Drug of abuse
GC-MS	Gas chromatography - mass spectrometry
HESI-II	Heated electrospray ionization II
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
HMMA	4-Hydroxy-3-methoxymethamphetamine
HRMS	High-resolution mass spectrometry
HRMS/MS	High-resolution tandem mass spectrometry
LC	Liquid chromatography
MDMA	3,4-Methylendioxymethamphetamine
METH	Methamphetamine
MPH	Methylphenidate
MS/MS	Tandem mass spectrometry
NPS	New psychoactive substance
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-PCP	Deschloro-*N*-propyl-ketamine
pHLM	Pooled human liver microsomes
QqQ	Triple quadrupole
RP	Reversed-phase

SCORE	Sewage analysis CORe group-Europe
SPE	Solid-phase extraction
SUSA	Standard urine screening approach
TOF	Time of flight
UPLC	Ultra performance liquid chromatography
UHyAc	Partial urine hydrolysis followed by acetylation
UPP	Urine precipitation
WBE	Wastewater-based epidemiology
WW	Wastewater
WWTP	Wastewater treatment plant