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RESEARCH ARTICLE

Human metabolites and transformation products of cyclophosphamide and ifosfamide: analysis, occurrence and formation during abiotic treatments

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Abstract This study describes a gas chromatography-mass spectrometry analytical method for the analysis of cytostatic cyclophosphamide (CP), ifosfamide (IF) and their selected metabolites/transformation products (TPs): carboxy-cyclophosphamide (carboxy-CP), keto-cyclophosphamide (keto-CP) and 3-dechloroethyl-ifosfamide/*N*-dechloroethyl-cyclophosphamide (*N*-decl-CP) in wastewater (WW). Keto-cyclophosphamide, CP and IF were extracted with Oasis HLB and *N*-decl-CP and carboxy-CP with Isolute ENV+ cartridges. Analyte derivatization was performed by silylation (metabolites/TPs) and acetylation (CP and IF). The recoveries and LOQs of the developed method were 58, 87 and 103 % and 77.7, 43.7 and 6.7 ng L^{-1} for carboxy-CP, keto-CP and *N*-decl-CP, respectively. After validation, the analytical method was applied to hospital WW and influent and effluent samples of a receiving WW treatment plant. In hospital WW, levels up to 2690, 47.0, 13,200, 2100 and 178 ng L^{-1} were detected for CP, IF, carboxy-CP, *N*-decl-CP and keto-CP, respectively, while in influent and effluent samples concentrations were below LOQs. The formation of TPs during abiotic treatments was also studied. Liquid chromatography-high-resolution mass spectrometry was used to identify CP and IF TPs in ultrapure water, treated with UV and UV/H_2O_2. UV treatment produced four CP TPs and four IF TPs, while UV/H_2O_2 resulted in five CPs and four IF TPs. Besides already known TPs, three novel TPs (CP-TP138a, imino-ifosfamide and IF-TP138) have been tentatively identified. In hospital WW treated by UV/O_3/H_2O_2, none of the target metabolites/TPs resulted above LOQs.

Keywords Cyclophosphamide · Ifosfamide · Cytostatic · Metabolites · Transformation products · Occurrence · Wastewater · Abiotic treatment

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Introduction

Cancer is one of the most frequent global diseases (Ferlay et al. 2015). Among many different types of pharmaceuticals used in chemotherapy, cyclophosphamide (CP) and ifosfamide (IF) are two of the oldest yet still frequently used cytostatics (Besse et al. 2012; Booker et al. 2014; Zhang et al. 2013). They belong to a group of alkylating agents, whose mechanism of action is primarily through the alkylation of DNA in cells resulting in repressed cell division (Besse et al. 2012). Once administered, these compounds are partially metabolized and excreted in urine and/or faeces. The pharmacokinetcs, i.e. the excretion rates for both parent compounds and their metabolites, are highly patient-state and administration-type and dose-dependent (Zhang et al. 2006). These residues eventually end up in the aquatic environment via the sewerage system, where they could have effects on non-target organisms due to their known cytotoxic, genotoxic,

mutagenic and teratogenic properties (Lin et al. 2015; Zhang et al. 2006). Out-patient clinics (i.e. clinics, where patients receive chemotherapy treatment and are dismissed on the same day) are becoming increasingly popular on a global scale (Franquet-Griell et al. 2015; Kosjek and Heath 2011). Regardless, a major source of cytostatic residues represent hospitals, where chemotherapies are performed daily (Zhang et al. 2013). This has been confirmed by studies reporting levels of CP and IF in hospital wastewaters (WWs) in microgram per liter (Gómez-Canela et al. 2012; Kim et al. 2009; Kümmerer et al. 1997; Steger-Hartmann et al. 1996, 1997; Yin et al. 2010; Česen et al. 2015), although their human metabolites/transformation products (TPs) are rarely reported. Only carboxy-cyclophosphamide (carboxy-CP) has been qualitatively determined in a Spanish hospital WW (Ferrando-Climent et al. 2013).

Studies reporting the removal efficiencies for CP and IF during biological treatment, e.g. suspended biomass and membrane bioreactors (Buerge et al. 2006; Delgado et al. 2011; Kiffmeyer et al. 1998; Kovalova et al. 2012; Steger-Hartmann et al. 1997), and abiotic treatments, e.g. UV, ozonation (O_3) and various advanced oxidation processes (AOPs) including UV/H_2O_2, UV/TiO_2 and UV/O_3/H_2O_2, have been published (Chen et al. 2008; Fernández et al. 2010; Garcia-Ac et al. 2010; Köhler et al. 2012; Lai et al. 2015; Lin et al. 2014; Lutterbeck et al. 2015). Their findings show that treatments fail to completely mineralize these compounds leaving behind mixtures of parent compounds and possible TPs of unknown toxicity (Busetti et al. 2015). To date, only a few published studies report the formation of TPs of CP and IF during abiotic treatment. Fernández et al. (2010) and Venta et al. (2005) both observed the formation of keto-cyclophosphamide (keto-CP) during ozonation, while Lutterbeck et al. (2015), investigating the degradation of CP by UV/H_2O_2 and UV/TiO_2, identified five TPs and Lai et al. (2015) investigating UV/TiO_2 treatment identified seven CP TPs and ten IF TPs. These studies were performed in either ultrapure water or buffer solutions at high concentrations (mg L^{-1}).

The aim of this study was to develop an analytical method for quantifying trace levels of selected CP and IF metabolites/TPs: carboxy-CP, keto-CP and either *N*-dechloroethyl-cyclophosphamide or 3-dechloroethyl-ifosfamide (*N*-decl-CP) in WW in order to quantitatively assess their presence in WWs. Furthermore, the formation of CP and IF TPs during UV and UV/H_2O_2 treatment in ultrapure water was examined, which represents a first step in the identification of potential TPs that might occur in real-scale treatment plants. Finally, the degradation and/or formation of the target metabolites/TPs included in this study was monitored during UV/O_3/H_2O_2 treatment of actual hospital WW in order to evaluate the efficiency of this WW treatment, which is capable of nearly complete CP and IF removal from various types of WW (Česen et al. 2015).

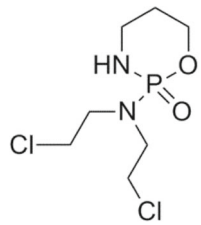
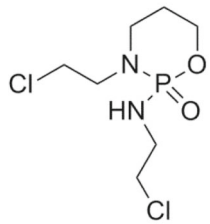
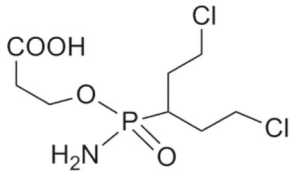
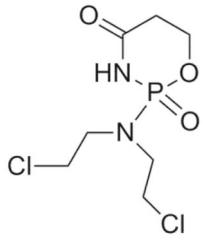
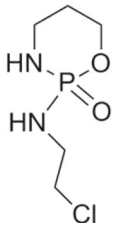
Materials and methods

Caution CP, IF, carboxy-CP, keto-CP and *N*-decl-CP are cytotoxic compounds and must be handled appropriately. Preparation of all stock solutions and samples were performed inside a laminar flow safety hood. A contaminated material was treated as hazardous waste (Eitel et al. 2000).

Standards, solvents and chemicals

Analytical standards of CP (purity 99 %) and IF (purity 99 %) were purchased from Sigma-Aldrich (Hong Kong, China). Carboxy-cyclophosphamide, 4-keto-CP, *N*-decl-CP and the deuterated standard [$^2H_6$] cyclophosphamide (CP-d_6) used as a surrogate standard for CP and IF and analysis by GC-MS were obtained from Niomech—IIT GmbH (Bielefeld, Germany). Cyclophosphamide (CP-d_4) used for LC-MS analysis was obtained from Toronto Research Chemicals (Toronto, Canada). Ibuprofen (IB-d_3), obtained from CDN Isotopes (Quebec, Canada), was used as a surrogate standard for the analysis of metabolites/TPs in WW since their isotopically labelled compounds are not commercially available. Table 1 gives chemical structures and molecular weights (MWs) of the investigated compounds. The derivatizing agents heptafluorobutyric anhydride (HFBA, 99 % purity) and trifluoroacetic anhydride (TFAA, 99 % purity) were purchased from Fluka (Buchs, Switzerland), *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA, 97 % purity) was purchased from Acros Organics (NJ, USA), *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide) (MSTFA, 98.5 %), acetic anhydride (AC_2O_2, 98 % purity), *N*-methyl-bis(trifluoroacetamide) (MBTFA, 99 % purity), 2,3,4,5,6-pentafluorobenzyl bromide (PFBBr, 99 % purity), 2,3,4,5,6-pentafluorobenzoyl chloride (PFBCl, 99 % purity), heptafluorobutyrylimidazole (HFBI) and *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide with 1 % *tert*-butyldimethylchlorosilane (MTBSTFA with 1 % TBDMCS, 95 %) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetone (ACT), acetonitrile (ACN), cyclohexane (CHX), dichloromethane (DCM), ethyl acetate (EtAc), methanol (MeOH) and iso-propanol (IPA) were purchased from J. T. Baker (Deventer, Netherlands). Toluene (TOL) and acetic acid (AA) were obtained from Merck (Darmstadt, Germany). In case of liquid chromatography-high-resolution mass spectrometry (LC-HRMS), the mobile phases were ultrapure water (Purelab Ultra, Australia) and MeOH (Honeywell Burdick & Johnson, Muskegon, USA). All solvents were of analytical grade purity. Concentrated hydrochloric acid (37 %, HCl) and formic acid (FA) used for optimization of solid-phase extraction (SPE) were purchased from Sigma-Aldrich (St. Luis, USA); ammonia solution (25 % NH_3 sol.) was purchased from Merck (Darmstadt, Germany).

Table 1 Investigated cytostatic compounds and their metabolites/TPs. Elemental composition and exact mass are also reported

Name (acronym)	Structure	Elemental composition	Exact mass (monoisotopic)
Cyclophosphamide (CP)		$C_7H_{16}O_2N_2Cl_2P$	261.0321
Ifosfamide (IF)		$C_7H_{16}O_2N_2Cl_2P$	261.0321
Carboxy-cyclophosphamide (carboxy-CP)		$C_7H_{15}Cl_2N_2O_4P$	292.0141
4-Ketocyclophosphamide (keto-CP)		$C_7H_{14}O_3N_2Cl_2P$	275.0114
***N*-dechloroethyl-cyclophosphamide/ 3-dechloroethylifosfamide (*N*-decl-CP)**		$C_5H_{12}O_2N_2ClP$	198.0319

Formic acid (99 %), also used in the preparation of the liquid mobile phase for LC-HRMS analysis, was purchased from Ajax Finechem Pty Ltd (NSW, Australia). Hydrogen peroxide (30 %) was purchased from AppliChem (Darmstadt, Germany). The stock solutions of CP (108 μg mL^{-1}), IF (99.0 μg mL^{-1}), carboxy-CP (115 μg mL^{-1}), keto-CP (145 μg mL^{-1}), *N*-decl-CP (73.2 μg mL^{-1}) and CP-d_6 (57.1 μg mL^{-1}) were prepared in ACN. Calibration standards were prepared by serial dilution of the stock solutions in ACN, while for the analysis of TPs in ultrapure water, stock solutions of CP, IF and CP-d_4 were prepared in ultrapure water at 27.9, 13.1 and 1.00 mg mL^{-1}, respectively. The composition of artificial WW used for GC-MS method validation is described elsewhere (Kosjek et al. 2007).

Sample collection

The first part of the sampling campaign was performed at a Slovenian hospital specialized in the treatment of cancer patients, where annually, approximately 13,000 admissions (i.e. cancer treatment therapies) are performed. Based on our previous study (Česen et al. 2015), where we evaluated the occurrence of CP and IF residues in this hospital WW at different times, we sampled on seven successive days to address inter-day and intra-day variations (e.g. working days and the weekend) of both parent compounds and selected metabolites/TPs. Accordingly, seven grab samples were taken daily from a WW collection basin at the hospital between 10 a.m. and 12 a.m. over 1 week (15 Dec 2014 to 21 Dec 2014).

The sampled hospital WW is released from the collection basin into the sewerage system (WW load averages approximately 50–60 m^3 day^{-1}), which is connected to the corresponding WWTP (360,000 population equivalents; PE). The time for the hospital WW to reach the WWTP is approximately 2–3 h. Taking into account the average daily WW load of the WWTP (80,000 m^3 day^{-1}), the WW from the hospital represents approximately 0.07 % of the overall WW load. Hence, the concentrations are expected to be 700 times lower in WWTP influent and/or effluent not taking into account WWTP removal of studied compounds. To check whether or not the concentrations of investigated compounds are measurable at the WWTP influent and effluent, additional sampling was performed during mid-week. Two 24-h time-proportional composite samples were collected: WWTP influent was sampled on 18 Dec 2014, while WWTP effluent was collected on 19 Dec 2014 to take into account the hydraulic retention time of the WWTP (15–22 h). Water treatment at the WWTP includes mechanical and conventional biological treatment with suspended biomass. Additional information regarding WW treatment technology is published elsewhere (Česen et al. 2015).

In addition, the hospital WW used in $UV/O_3/H_2O_2$ treatment experiments was sampled by grab sampling at the same hospital as the 1-week sampling campaign for the analysis of metabolites/TPs described above, but on 9 May 2014 at 11 a.m.

All samples were transferred to the laboratory on ice, immediately filtered through glass-microfibre filters (Machery Nagel, Düeren, Germany) and 0.45-μm cellulose nitrate filters from Sartorius Stedim Biotech (Göttingen, Germany) and stored at −20 °C prior to analysis. All analyses were performed within 1 week.

Sample pre-treatment

The first part of this study involved developing and optimizing a gas chromatography-mass spectrometry (GC-MS) analytical method for determining CP, IF and selected metabolites/TPs in various WW samples. To achieve high sensitivity and selectivity, different SPE cartridges and derivatization agents and conditions were tested, whereas the method for parent compounds was adopted from Česen et al. (2015). The optimal conditions of the whole analytical procedure were as follows. For CP, IF and keto-CP, 100 mL of sample was preconcentrated on Oasis HLB (60 mg, 3 mL; Waters, MA, USA). Each cartridge was conditioned with 3 mL of EtAc, 3 mL of MeOH and equilibrated with 3 mL of ultrapure water. The sample was extracted at a flow rate of 3 mL min^{-1} using a Supelco Vacuum Manifold (Bellefonte, USA). After sample loading, the sorbent was dried under vacuum (10 mmHg, 20 min) and the analytes were eluted with 3 mL of EtAc (3 × 1 mL). The solvent was removed under a gentle stream of nitrogen. The SPE procedure for carboxy-CP and *N*-decl-CP was performed using Isolute ENV+ (100 mg, 3 mL) cartridges from Biotage™ (Uppsala, Sweden). Conditioning, equilibration, sample loading and sorbent drying steps were performed as described for CP, IF and keto-CP. Analytes were eluted with 6 mL of 5 % AA in EtAc (3 × 2 mL). The excess solvent was removed under a gentle stream of nitrogen. Derivatization of CP and IF was performed using 100 μL TFAA in 100 μL EtAc at 60 °C for 1 h. The metabolites/TPs were derivatized with 30 μL MTBSTFA with 1 % TBDMCS in 220 μL of ACN. Sample extracts containing metabolites/TPs were derivatized for 20 h at 90 °C.

In the second stage of this study, when LC-HRMS instrumentation became available, identification of unknown TPs formed during UV and UV/H_2O_2 treatment of IF and CP in ultrapure water was performed. For these oxidation experiments, samples were directly injected into the liquid chromatograph-high-resolution mass spectrometer. No additional sample pre-treatment was necessary since the initial spiked concentration of CP and IF was 26.1 mg L^{-1}.

Instrumental analysis and validation

Gas chromatography-mass spectrometry

A HP 6890 series gas chromatograph with a single quadrupole mass spectrometer (Hewlett-Packard, Waldbron, Germany) was used for the analysis of environmental samples and WW samples from abiotic treatment. Chromatographic separation was achieved using a DB-5 MS capillary column (30 m × 0.25 mm × 0.25 μm; Agilent, USA) with He as the carrier gas. For optimal chromatographic separation, two different temperature programs were applied, one for CP and IF and one for metabolites/TPs, which was possible since two different derivatization procedures were used. For CP and IF, the conditions and temperature program are presented elsewhere (Česen et al. 2015). For the analysis of metabolites/TPs, 1 μL of the sample extract was injected in splitless mode at 250 °C. The temperature program was as follows: an initial

temperature 100 °C was ramped at 30 °C min^{-1} to 300 °C and held for 1 min. Total GC-MS runtime was 7.17 min. The mass spectrometer was operated in EI mode at 70 eV.

Identification of pharmaceutical derivatives was made in selective ion monitoring (SIM) mode using retention time matching (CP 7.28 min, IF 6.98 min, CP-d_6 7.29 min, IB-d3 5.82 min, *N*-decl-CP 6.13 min, keto-CP 6.67 min, carboxy-CP 6.72 min) and by monitoring the following ions: m/z 309, 307 and 150 for CP; m/z 307, 181 and 150 for IF; m/z 313 and 315 for CP-d_6; m/z 357, 355 and 339 for carboxy-CP; m/z 308, 280 and 195 for *N*-decl-CP; m/z 297, 295 and 242 for keto-CP; and m/z 266 and 205 for IB-d_3. The TIC and mass spectra of the investigated metabolites/TPs are presented in the Supplementary Material (SM) 1. For instrumental control and data processing, Chemstation software (version A.03.00) was used.

For method validation, the linearity, accuracy, instrumental detection limits (IDLs), limits of detection (LOD), limit of quantification (LOQ) and repeatability (method and instrumental) were determined. The calibration range was 100–10, 100 ng $L^{-1}$and the coefficient of determination (R^2) was used to express calibration curve linearity. The method accuracy was expressed as [(experimental value − spiked value)/spiked value] ($n = 3$). Instrumental detection limits were calculated as three times the standard deviation of the baseline of the blank divided by the slope of their calibration curve using standards. The LODs and LOQs were calculated as three times and ten times the standard deviation of the baseline of the blank, respectively, divided by the slope of their calibration curve ($n = 3$) using WW as matrix. Repeatability was taken as the relative standard deviation (RSD) of three replicates ($n = 3$, 5000 ng L^{-1}), while instrumental repeatability was taken as the RSD of three consecutive injections of the same sample (5000 ng L^{-1}). Potential losses of analytes from filtration, i.e. the adsorption of analytes onto the glass-microfibre filters (approx. 0.5 μm) and cellulose nitrate filters (0.45 μm), were assessed by comparing filtered and non-filtered samples spiked at 5000 ng L^{-1} in tap water ($n = 3$).

Liquid chromatography-high-resolution mass spectrometry

The degradation pattern of parent compounds CP and IF as well as the identification of TPs formed during UV and UV/H_2O_2 treatment was performed using LC-HRMS using a Thermo Accela 600 LC system coupled to a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific Corporation, Waltham, MA, USA). The system was equipped with an electrospray ion source (ESI) operated in positive ionization mode. Separation was achieved using a Kinetex C18 column (100 × 2.1 mm, 3 μm, 100 Å) from Phenomenex (Sydney, Australia) at a flow rate of 100 μL min^{-1}. The injection volume was 10 μL. The mobile phase comprised ultrapure water (A) and MeOH (B) both containing 0.1 % of formic acid. Initial conditions were 70 % A for 3 min, followed by a linear gradient to 5 % A in 15 min and held for 10 min. Afterwards, the initial conditions were re-established within 0.1 min and the column was re-equilibrated for 10 min before injecting the next sample. Total run time was 35 min. To minimize carryover, the needle was rinsed in the injection port with 1 mL of pure MeOH before and after each injection. The following ESI settings were applied: spray and capillary voltages were set at +3.5 kV and +35 V, respectively; capillary temperature was 275 °C; sheath gas flow rate was 18 arbitrary units, whereas auxiliary and sweep gas flow were off. Full calibration of the mass spectrometer was performed weekly with the positive ion calibration solution provided by Thermo Scientific. Optical lenses were optimized with a standard solution of caffeine and/or CP and IF prior to each measurement. For increased mass accuracy on the LTQ Orbitrap XL, a plasticizer interfering peak present in LC mobile phase at m/z 214.0887 (*n*-butyl benzenesulfonamide, $C_6H_5SO_2NH(CH_2)_3CH_3$, $[M+H]^+ = 214.0896$ m/z) was used for the lock mass function.

To evaluate CP and IF degradation by UV irradiation and UV/H_2O_2 treatment, reaction mixtures were analysed using selected reaction monitoring (SRM). The following SRM transitions and collision-induced dissociation (CID) energies were applied: CP (261 → 233 m/z; CID = 25); IF (261 → 154 m/z; CID = 25); CP-d_4 (265 → 235 m/z; CID = 23). The calibration curves for CP ($R^2 = 0.9930$) and IF ($R^2 = 0.9940$) were performed at 0.250–26.1 mg L^{-1} to check linearity. Samples showing approximately >40 % degradation of either CP or IF were further analysed using LC-HRMS to identify possible TPs. The analysis was conducted using the LTQ Orbitrap-XL mass spectrometer in data-dependent mode (full-scan mode from 70 to 450 m/z with a mass resolution of 60,000 (at 400 m/z) followed by HRMS-MS on the top five most intense peaks from the MS scan at a mass resolution of 30,000 (at 400 m/z). To proceed with the structural elucidation of TPs and fragments, samples were also infused into the mass spectrometer for analysis of ions in high-resolution product ions scan mode (MS^2 to MS^4) with a mass resolution of 30,000 (at 400 m/z). To identify unknown compounds, both chromatographic retention time and the deviation of the measured mass (i.e. parent compound and fragments) relative to the theoretical mass (<3 ppm, relative error) were used. Data was processed using Xcalibur QualBrowser 2.0.7 SP1, TraceFinder 3.1 and MassFrontier 7.0, where TraceFinder 3.1 and XCalibur QualBrowser 2.0.7 SP1 were used for qualitative/quantitative analysis of CP, IF and TPs, while MassFrontier 7.0 was used for spectral interpretation and structural elucidation of TPs.

Water treatment set-up

UV/O_3/H_2O_2 experiments in hospital WW

Initial chemical analysis of the hospital WW used for this set of experiments revealed measurable concentrations of the investigated compounds, and additional spiking of hospital WW

with the analytes was deemed not necessary. The degradation experiment was conducted on a laboratory-scale water treatment plant, where the removal and transformations of oxazaphosphorine-based compounds (CP, IF, *N*-decl-CP, keto-CP and carboxy-CP) present in hospital WW were studied during UV/O_3/H_2O_2 (H_2O_2 at 5000 mg L^{-1}) treatment (20–120 min). A detailed description of the equipment and experimental conditions are reported elsewhere (Česen et al. 2015). Briefly, an LP Copper UV lamp (12 W) was used for UV irradiation (UV dose = 44 mJ cm^{-2}). The equipment is designed for the simultaneous ozonation of a sample using an O_3 generator (500 mg h^{-1}) producing O_3 at 10 mg L^{-1}. The sample (4.8 L) was circulated through the system at 60 L h^{-1}.

UV and UV/H_2O_2 experiments in ultrapure water

Transformation product formation during UV and UV/H_2O_2 treatment was studied using a cylindrical glass reactor containing 500 mL of ultrapure water spiked with either CP or IF at 26.1 mg L^{-1} (10 mM). The reactor contents were stirred continuously with a magnetic stirrer (500 rpm), and a low-pressure Mercury (LP Hg) lamp (Heraeus Noblelight model TNN 15/32, 15 W) was introduced into the reactor and kept isolated from the testing solution by a quartz cooling well. The reaction solution was kept at a constant $T = 20$ °C throughout the experiment. The fluence rate values were determined by chemical actinometry using a 250 μM solution of H_2O_2 (Nicole et al. 1990). The measured UV intensity was 4.5×10^{-6} Ein sec^{-1}. For the UV/H_2O_2 experiments, the H_2O_2 concentration was 8.5 mg L^{-1}. For UV experiments, samples were taken at 0, 5, 10, 15, 20, 40 and 60 min, while for UV/H_2O_2 experiments, sampling times were 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 20, 24, 28, 32 and 36 min.

Results and discussion

GC-MS method for determining CP and IF metabolites/TPs in WW

Selection of derivatization agent and conditions

To increase the sensitivity of the analytical method and achieve simultaneous determination of all investigated compounds, different derivatization agents, solvents, their mixtures and reaction times and temperatures were tested. Keto-CP and *N*-decl-CP contain oxazaphosphinane moieties in their structures, which include a secondary amine group, while carboxy-CP has a free carboxylic group and a primary amine in the phosphordiamide group (Table 1). Based on the compounds' structural characteristics, various derivatizing agents were investigated. Preliminary experiments were performed using silylating (MSTFA, MTBSTFA, MTBSTFA with 1 % TBDMCS), acylating (HFBA, TFAA, HFBI, MBTFA, PFBCl, Ac_2O_2) and alkylating (PFBBr) agents with 1 mL of EtAc at 60 °C. Derivatization time varied from 1 h to 16 h. Most of the acylating agents have a fluorine atom in their structure and are usually determined using an electron capture detector (ECD), since an ECD is extremely sensitive for halogenated compounds. Regardless, selected acylating agents for derivatization of investigated metabolites/TPs were examined since parent compounds were successfully derivatized with TFAA and analysed using a mass selective detector (MSD) that gave LODs at nanogram per liter levels (Česen et al. 2015). In addition, there are two studies, in which *N*-decl-CP, keto-CP and carboxy-CP were derivatized with TFAA and keto-CP with diazomethane (no longer commercially available) and analysed by GC-MS (Kalhorn et al. 1999; Momerency et al. 1994). Unfortunately, the derivatization of these compounds with TFAA (2 h at 70 °C) resulted in a low chromatographic response (Momerency et al. 1994). Increasing time and temperature of derivatization did not improve sensitivity. Derivatization with HFBA produced similar results to TFAA, while other acylating and alkylating agents failed to sufficiently derivatize the investigated compounds. Derivatization with silylating agents revealed that the most effective derivatization agent was MTBSTFA with 1 % TBDMCS, where the addition of TBDMCS catalyzes the reaction of the hindered amines resulting in an increased MS response compared to MTBSTFA alone.

Different solvents were also tested: ACT, ACN, CHX, DCM, EtAc and TOL, with MTBSTFA containing 1 % TBDMCS. Reactions were conducted at 75 °C for 16 h, where ACN was shown to be the most appropriate solvent (SM 2). Further step involved the optimization of reaction time (16–32 h) and temperature (60–90 °C), where 90 °C for 20 h was chosen as the optimal conditions (SM 3). Finally, to further increase the sensitivity of the analytical method, various ratios between derivatizing agent (5–30 μL) and solvent (220–1000 μL) were examined. A lower solvent volume (220 μL), i.e. increased concentration, resulted in increased response for keto-CP and carboxy-CP. For carboxy-CP, there was a tenfold increase in response. The response of *N*-decl-CP remained unchanged when the amount of solvent was reduced (500 and 220 μL). Derivatization of compounds with lower volumes of derivatizing agent was also examined (5, 10 and 15 μL). The responses were lower, so 30 and 220 μL were taken as the optimal volumes of MTBSTFA with 1 % TBDMCS and ACN, respectively.

The deuterated standard, IB-d_3, was added prior to the SPE procedure and was successfully derivatized with MTBSTFA with 1 % TBDMCS and produced similar responses under tested experimental conditions. Finally, the stability of the derivatized compounds was also assessed. The analytes of interest were stable for up to 24 h at room temperature and

for up to 5 days at −20 °C resulting in less than a 10 % degradation under both storage conditions ($n = 3$).

Optimization of the SPE procedure

Solid-phase extraction was studied separately for tap water and WW. The SPE recovery was determined as a quotient of peak area of analyte spiked prior to SPE and that obtained by spiking the same amount of analyte added post SPE. Initially, 200 mL of tap water spiked with 5000 ng L^{-1} of metabolites/TPs were extracted using various types of reversed phase and mixed mode (i.e. reversed phase and strong anion exchange) SPE stationary phases along with the use of different solvents or solutions adopted in the SPE elution step (SM 4). In the case of Supelco DPA6S and Supelco EnviCarb SPE stationary phases, a comparison between non-acidified (pH = 7) and acidified samples (pH = 2) was made (SM 4). The final extracts were blown down under a gentle stream of nitrogen and further derivatized as described in "Sample pre-treatment".

Results revealed variations in SPE efficiencies when using different types of sorbents and eluents for compounds of interest (SM 4). Except for Oasis MAX, which has both reversed-phase and ion-exchange retention modes, all others contained only a reversed-phase sorbent. Recoveries >50 % were only observed for ABN Evolute, Strata C18, Supelco EnviCarb and Strata X for keto-CP. Using Oasis HLB and Isolute ENV+, a recovery >50 % was obtained for *N*-decl-CP and keto-CP. Supelco DPA6S generally resulted in the poor recovery (0–4 %) of all compounds regardless of the eluting solvents and sample pH. Based on these preliminary tests, Oasis HLB and Isolute ENV+ cartridges were favored for further optimization of the SPE procedure for keto-CP and *N*-decl-CP in WW. Carboxy-CP was successfully extracted when an Oasis MAX phase was used and eluted with 2 % FA in MeOH (recovery >90 %; SM 4). This can be explained by the ionic exchange retention mechanism between the anionic form of carboxy-CP (i.e. containing a free carboxylic group) and the positively charged quaternary amine on the Oasis MAX sorbent. After this initial screening step, selected SPE procedures were examined in terms of repeatability (SM 4). The described SPE procedure for tap water was used to evaluate analyte losses during filtration step ("Gas chromatography-mass spectrometry").

Recovery was also determined for WW, a more complex matrix. Carboxy-CP was not extracted successfully using the same method as for tap water even when the amount of sorbent was increased to 150 mg and the elution volume to 6 mL of 2 % FA in MeOH (recovery was 28.8 ± 0.4 %, $n = 3$). It is assumed that matrix complexity influences the retention of carboxy-CP resulting in lower recoveries. Therefore, carboxy-CP was included in the SPE optimization procedure in WW for keto-CP and *N*-decl-CP with Oasis HLB (elution: EtAc) and Isolute ENV+ (elution, 5 % AA in EtAc). Furthermore, SPE recoveries from WW for all three compounds were determined for ABN Evolute, Strata C18, Strata X and Supelco EnviCarb eluted with MeOH, for which determined recoveries were all below 30 % (SM 4).

As expected, SPE recoveries of metabolites/TPs in WW were found to be consistently lower than for tap water also when Oasis HLB and Isolute ENV+ were used as the stationary phases (SM 4, SM 5). This can be explained by the high affinity of matrix interferences present in WW for the SPE sorbents causing 'saturation' of the sorbent beds and the consequent breakthrough of analytes during the loading step (Busetti et al. 2006). Breakthrough is more pronounced for polar analytes since reverse-phase interactions with the stationary phase are weaker. Overall, this problem can be overcome by using higher amounts of sorbent and/or by loading smaller volumes of sample. Therefore, in order to improve the recovery of metabolites/TPs in WW, the sample volume was reduced to 100 mL and the mass of stationary phase was increased. For this purpose, the following cartridges were tested: 60 and 500 mg Oasis HLB and 100 and 500 mg Isolute ENV+.

The results revealed an improved SPE recovery for carboxy-CP (recovery 58.1 ± 6.0 %) when a lower volume of sample (100 mL) was used for extraction with Isolute ENV+ cartridges regardless of the mass of sorbent (Table 2). *N*-Decl-CP was also efficiently extracted with ENV+ (recovery 102.9 ± 2.9 %) using either 100 or 200 mL; hence, to unify the procedure for *N*-decl-CP and carboxy-CP, 100 mL of sample was chosen as optimum. Finally, keto-CP was successfully extracted with HLB Oasis cartridges using either 60 or 500 mg of sorbent. Therefore, in order to unify the SPE procedure for keto-CP with CP and IF, 60-mg cartridges were used (Česen et al. 2015). Table 2 presents the optimal conditions for SPE and average ($n = 3$) SPE recoveries of the investigated metabolites/TPs.

Validation

Table 3 reports the linearity, slope, accuracy, repeatability (instrumental and method), IDLs, LODs and LOQs of the analytical method as well as analyte recovery after a filtration step.

The R^2 values were above 0.97 for all investigated compounds showing good linearity over the working concentration range. Method accuracy was below 25 % in all cases, whereas method and instrumental repeatability were below 13 and 8.5 % for all investigated compounds, respectively. The LODs varied between 2 and 23 ng L^{-1}, while the LOQs were between 6.7 and 77.7 ng L^{-1}. The results confirmed that filtration did not affect the recoveries (Table 3).

Table 2 Optimized SPE procedure and average SPE recoveries ± RSD for metabolites/TPs in WW

Compound	SPE cartridge	SPE conditioning	Sample volume	SPE elution	Recovery % ± RSD; ($n = 3$)
N-decl-CP	Isolute ENV+ (100 mg/3 mL)	3 mL EtAc 3 mL MeOH 3 mL water	100 mL	(3 × 2 mL) 5 % AA in EtAc	102.9 ± 2.8
Carboxy-CP					58.1 ± 10.3
Keto-CP	Oasis HLB (60 mg/3 mL)	3 mL EtAc 3 mL MeOH 3 mL water		(3 × 1 mL) EtAc	86.7 ± 16.9

Cytostatic residues: CP, IF, *N*-decl-CP, keto-CP and carboxy-CP in hospital WW

Occurrence of CP, IF and metabolites/TPs in hospital and municipal WWs

Chemical analysis of grab samples from hospital WW revealed concentrations of investigated compounds in the nanogram-per-liter to microgram-per-liter range, except for the sample taken on Sunday (21 Dec 2014), where keto-CP was below the LOD (Fig. 1, SM 6).

Apart from IF and to a lesser extent also keto-CP, the other compounds all show a similar trend, i.e. the highest concentrations were detected in samples taken from Tuesday to Friday, whereas samples collected at the weekend and on Monday contained the lowest concentrations of the investigated compounds. This is expected since a lower number of patients receive chemotherapy over the weekend. A high inter-patient variability in pharmacokinetics among patients receiving chemotherapy with CP or IF is known; therefore, it is hard to correlate the excretion of parent compounds and metabolites to their presence in hospital WW (Gilard et al. 1993; Kaijser et al. 1994; Tasso et al. 1992; Zhang et al. 2006). However, the ratio of the excreted compounds (of the doses applied) reported in the literature (Table 4) was partially in an agreement with the results obtained within this study (Fig. 1, SM 6). For example, high excretion of CP metabolite, carboxy-CP, correlates with the highest concentrations (>10, 000 ng L^{-1}; SM 6) observed for this metabolite in WW. Its highest concentration was detected in a sample collected on Tuesday (Fig. 1, SM 6). This compound has been qualitatively determined in a hospital WW by Ferrando-Climent et al. (2013), whereas all other metabolites/TPs are detected in WW for the first time, and therefore, a comparison with other data in the literature is not possible. Moreover, CP and *N*-decl-CP (up to 2680 and 2100 ng L^{-1}, respectively; SM 6) were present in higher amounts than keto-CP (up to 178 ng L^{-1}; SM 6), agreeing with the higher excretion for these two compounds compared to keto-CP (Table 4). Cyclophosphamide is prescribed more often than IF due to fewer side effects, which explains why IF was present in lower amounts (up to 47 ng L^{-1}; Fig. 1, SM 6; (Weiss 1991)). In addition, since IF was the least abundant, we also assume that *N*-decl-CP that was detected in hospital WW samples originated mainly from CP therapy.

From Table 5, it is apparent that concentrations of CP and IF in hospital WWs span three orders of magnitude, from below LOQ/few nanogram per liter up to thousands of nanogram per liter. The results of our study are in agreement with the literature as the detected concentrations of CP and IF in hospital WW samples are also within this range. Although an in-depth study with time-proportional or flow-proportional sampling and intra-day variations would be necessary to confirm the findings of this study, these preliminary results

Table 3 Validation parameters for the GC-MS method used to analyse parent compounds and selected metabolites/TPs in WW samples

R^2 value	CP 0.9959	IF 0.9991	Carboxy-CP 0.9800	Keto-CP 0.9796	*N*-decl-CP 0.9903
Accuracy (%)	3.7	2.4	25.0	2.1	9.1
Slope of the calibration curve	0.0025	0.0016	0.00091	0.0013	0.0021
IDL (ng L^{-1})	2.7	1.9	7.7	4.5	1.2
LOD (ng L^{-1})	2.3	4.8	23.0	13.1	2.0
LOQ (ng L^{-1})	7.7	16.0	77.7	43.7	6.7
Method repeatability (RSD; %)	2.9	7.1	7.5	10.1	13.0
Instrumental repeatability (RSD; %)	4.3	8.5	4.5	5.2	7.0
Filtration step recovery (%)	99.3	100.3	95.1	100.8	97.7

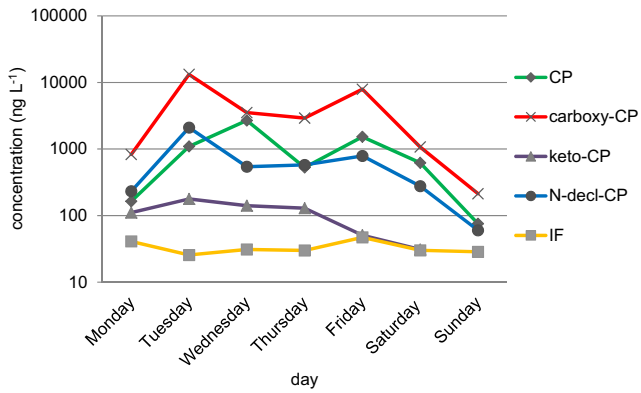


Fig. 1 Daily concentrations (ng L^{-1}) of CP, IF and selected human metabolites/TPs in hospital WW ($n = 2$; RSD was below 15.9 % for all compounds); sampling campaign: 15 Dec 2014 (Monday) to 21 Dec 2014 (Sunday)

suggest that structural analogues (e.g. human metabolites) should also be included in studies since these compounds can occur at even higher concentrations than the parent compounds, and once in the environment, they also have the potential to harm aquatic biota to a similar or even higher degree (Farré et al. 2008).

None of the investigated compounds were detected in the 24-h time-proportional samples taken at the WWTP (influent and effluent). This is probably due to the high dilution factor of the hospital WW into the municipal WW. Because hospital WW represents only approximately 0.07 % of the overall WW load at this WWTP, concentrations below the LOD were expected. For example, 13,202 ng L^{-1} was the highest concentration in hospital WW (for carboxy-CP, Fig. 1, SM 6); the theoretical value detected in the influent should be 9.2 ng L^{-1}, which is approximately eight times lower than LOD determined for carboxy-CP (Table 3). This agrees with the majority of the published studies that report concentrations of the parent compounds in influents and effluents below LOQs (Table 5).

A study addressing the OECD-based bioassays for ecotoxicity and genotoxicity assessment of the compounds investigated within this paper has been recently published (Česen et al. 2016). Interestingly, the most ecotoxic ($EC_{50} = 17.1$ mg L^{-1}) and genotoxic (significant genotoxic activity at 320 mg L^{-1}) compound was carboxy-CP, which was also the most abundant compound in the WW samples. In addition, the authors investigated the ecotoxicity and genotoxicity of a mixture of CP + IF + carboxy-CP + keto-CP + *N*-decl-CP. The effects of the mixture on the tested organisms in both bioassays were higher than expected compared to that of the compounds tested individually; however, the concentrations producing a significant effect were still in a milligram-per-liter range (Česen et al. 2016). Based on the concentrations of CP, IF and metabolites/TPs recorded in this study (Fig. 1, SM 6) and reported in the literature (Table 4), the risk associated with the presence of these chemicals in the environmental is minimal. Regardless, further studies addressing the actual environmental concentrations of the investigated metabolites/TPs are needed to make any firm conclusions regarding their possible threat to the environment.

Table 4 The excretion of CP, IF and their metabolites reported in literature

Compound	Excretion as % of the applied dose	Reference
CP	16–17 %	(Joqueviel et al. 1998; Zhang et al. 2006)
IF	18 %	(Gilard et al. 1993)
Carboxy-CP	11–23 %	(Joqueviel et al. 1998)
keto-CP	0.3–0.6 %	(Joqueviel et al. 1998)
N-Decl-CP	2–3 % (CP) 11 % (IF)	(Gilard et al. 1993; Joqueviel et al. 1998)

Fate of investigated compounds during UV/O_3/H_2O_2 treatment

Native concentrations of CP and IF in hospital WW were 5340 and 6830 ng L^{-1}, respectively (Česen et al. 2015). Both CP and IF were effectively removed by UV/O_3/H_2O_2 treatment (CP removal >97.9 % and IF removal >98.4 % at $t = 120$ min). In addition, carboxy-CP (26.2 ng L^{-1}) and keto-CP (152.0 ng L^{-1}) were also detected in the same hospital WW sample (Fig. 2). The detected concentrations of metabolites/TPs were significantly lower in these samples than in a series of samples taken during the sampling campaign in December 2014 (Fig. 1, SM 6). This is due to the nature of sampling (i.e. grab sampling), expectable. Therefore, unfortunately, the uncertainty of the obtained results for carboxy-CP is too high to draw any firm conclusions regarding the formation/degradation of this metabolite/TP. As regards keto-CP, the concentration was initially below the LOQ, but then increased after 60 min of treatment, which can be attributed to the degradation of CP. This is the first study reporting the degradation/formation of targeted metabolites/TPs during UV/O_3/H_2O_2 treatment of a hospital WW. The data suggests that the decrease in CP and IF results in the formation of unknown TPs during investigated treatment; hence, further studies are needed to identify them. In addition, due to the described behaviour of the targeted metabolites/TPs and very low concentrations (i.e. ng L^{-1}), the kinetic studies were omitted from this part of our work.

Transformation product formation during UV and UV/H_2O_2 treatment

Degradation rate constants for CP and IF during UV and UV/H_2O_2 treatment in ultrapure water were 0.012 and

Table 5 Concentration ranges (ng L^{-1}) of CP and IF in hospital WW, WWTP influents and effluents as well as surface water from literature

	Type of water	Sampling (flow-proportional, time-proportional or grab and number of samplings)	Conc. range (min-max; ng L^{-1})	Reference
CP	Hospital WW	24-h time proportional $n = 7$	19–4500	(Steger-Hartmann et al. 1997)
		24-h time proportional $n = 1$	146	(Steger-Hartmann et al. 1996)
		Grab $n = 12$	<LOD (2)–21	(Thomas et al. 2007)
		Grab $n = 65$ (21 hospitals)	6–2000	(Yin et al. 2010)
		24-h time proportional $n = 1$	5730	(Gómez-Canela et al. 2012)
		Grab $n = 1$ (4 hospitals)	<LOQ (3.6)–200.7	(Ferrando-Climent et al. 2013)
		24-h time proportional $n = 7$	<LOQ (3.0)–100.0	(Negreira et al. 2014)
		Grab $n = 30$ (2 hospitals)	<LOQ (4.0)–4720	(Gomez-Canela et al. 2014)
		Grab $n = 10$ (5 hospitals)	14–22000	(Česen et al. 2015)
	WWTP influent	8-h time proportional n = 2	<LOD (6)–143 <LOD (6)–8	(Steger-Hartmann et al. 1997)
		Flow proportional (24 h) $n = 5$ (3 WWTPs)	2–11	(Buerge et al. 2006)
		24-h time proportional $n = 2$	<LOD (2)	(Thomas et al. 2007)
		24-h time proportional $n = 3$	<LOQ (7.1)	(Martín et al. 2011)
		24-h time proportional $n = 2$ (3 WWTPs)	<LOD (0.35)–13100	(Gómez-Canela et al. 2012)
		Grab $n = 2$ (3 WWTPs)	<LOQ (3.6)–25.5	(Ferrando-Climent et al. 2013)
		24-h time proportional $n = 1$ (12 WWTPs)	<LOQ (3.0)–43.8	(Negreira et al. 2014)
		24-h time proportional $n = 15$ (2 WWTPs)	<LOQ (4.0)–10	(Gomez-Canela et al. 2014)
		8-h time proportional $n = 2$	6–17 8–15	(Steger-Hartmann et al. 1997)
		24-h time proportional or grab $n = 4$ (4 WWTPs)	<LOD (0.55)–27	(Česen et al. 2015)
	WWTP effluent	Grab $n = 1$	<LOD (10)–20	(Ternes 1998)
		24-h time proportional $n = 9$ (different WWTPs)	<LOQ (1.9)–9	(Castiglioni et al. 2005)
		Flow proportional (24 h) $n = 5$ (3 WWTPs)	2–10	(Buerge et al. 2006)
		24-h time proportional $n = 1$ (8 WWTPs)	Median 0.6	(Zuccato et al. 2005)
		24-h time proportional $n = 2$	<LOD (2)	(Thomas et al. 2007)
		24-h time proportional and grab $n = 3$ (2 WWTPs)	<LOQ (100)	(Busetti et al. 2009)
		24-h time proportional $n = 3$	<LOQ (7.7)	(Martín et al. 2011)
		Grab $n = 3$ (2 WWTPs)	0.19–3.7	(Llewellyn et al. 2011)
		24-h time proportional $n = 2$ (3 WWTPs)	<LOD (0.35)	(Gómez-Canela et al. 2012)
		24-h time proportional $n = 1$ (12 WWTPs)	<LOQ (SM)-25.0	(Negreira et al. 2014)
		24-h time proportional $n = 15$ (2 WWTPs)	<LOQ (4.0)–5	(Gomez-Canela et al. 2014)

Table 5 (continued)

	Type of water	Sampling (flow-proportional, time-proportional or grab and number of samplings)	Conc. range (min-max; ng L^{-1})	Reference
		Grab $n = 1$	<LOD (10)	(Ternes 1998)
		24-h time proportional or grab $n = 4$ (4 WWTPs)	<LOD (0.55)–17	(Česen et al. 2015)
	Surface water	Grab $n = 5$ (3 SWs)	0.05–0.17	(Buerge et al. 2006)
		2.5-h time proportional $n = 1$ (2 SWs)	<LOD (data not available)	(Zuccato et al. 2005)
		Grab $n = 2$ (4 SWs)	<LOQ (30)–65	(Moldovan 2006)
		Grab $n = 3$	<LOQ (5.5)	(Martín et al. 2011)
		Grab $n = 5$ (5 rivers)	<LOD (3)	(Valcárcel et al. 2011)
		Grab	<LOD (6)–1914	(Kümmerer et al. 1997)
IF	Hospital WW	24-h time proportional $n = 1$	24	(Steger-Hartmann et al. 1996)
		Grab $n = 12$	<LOD (2)–338	(Thomas et al. 2007)
		Grab $n = 65$ (21 hospitals)	4–10647	(Yin et al. 2010)
		Grab $n = 1$ (4 hospitals)	<LOQ (5.8)–227.9	(Ferrando-Climent et al. 2013)
		24-h time proportional $n = 7$	<LOQ (2.0)–19.4	(Negreira et al. 2014)
		Grab $n = 30$ (2 hospitals)	<LOQ (4.0)–86200	(Gomez-Canela et al. 2014)
		6-h time proportional $n = 2$	7–29 <LOD (6)–29	(Kümmerer et al. 1997)
		Grab $n = 10$ (5 hospitals)		(Česen et al. 2015)
	WWTP influent	Flow proportional (24 h) $n = 5$ (3 WWTPs)	<LOD (0.3)–15	(Buerge et al. 2006)
		24-h time proportional $n = 2$	<LOD (2)	(Thomas et al. 2007)
		24-h time proportional $n = 3$	3.5 ± 0.1 (mean ± SD)	(Martín et al. 2011)
		Grab $n = 2$ (3 WWTPs)	<LOQ (5.8)–130.1	(Ferrando-Climent et al. 2013)
		24-h time proportional $n = 1$ (12 WWTPs)	<LOQ (2.0)–27.9	(Negreira et al. 2014)
		24-h time proportional $n = 15$ (2 WWTPs)	<LOQ (6.0)	(Gomez-Canela et al. 2014)
		6-h time proportional $n = 2$	10–40 <LOD (6)–43	(Kümmerer et al. 1997)
		Grab $n = 1$	<LOD (10)–2900	(Ternes 1998)
		24-h time proportional or grab $n = 4$ (4 WWTPs)	<LOD (0.36)	(Česen et al. 2015)
	WWTP effluent	Flow proportional (24 h) $n = 5$ (3 WWTPs)	1.7–6	(Buerge et al. 2006)
		24-h time proportional $n = 2$	<LOD (2)–71	(Thomas et al. 2007)
		24-h time proportional and grab $n = 3$ (2 WWTPs)	<LOQ (100)	(Busetti et al. 2009)
		24-h time proportional $n = 3$	1.2 ± 0.1 (mean ± SD)	(Martín et al. 2011)
		Grab $n = 3$ (2 WWTPs)	<LOQ (0.24)	(Llewellyn et al. 2011)

Table 5 (continued)

Type of water	Sampling (flow-proportional, time-proportional or grab and number of samplings)	Conc. range (min-max; ng L^{-1})	Reference
	24-h time proportional $n=1$ (12 WWTPs)	<LOQ (2.0)–15.9	(Negreira et al. 2014)
	Grab $n=1$	<LOD (10)	(Ternes 1998)
	Grab $n=5$ (3 SWs)	0.05–0.14	(Buerge et al. 2006)
	24-h time proportional or grab $n=4$ (4 WWTPs)	<LOD (0.36)	(Česen et al. 2015)
Surface water	Grab $n=3$	<LOQ (4.4)	(Martín et al. 2011)
	Grab $n=5$ (5 rivers)	<LOD (1)–41	(Valcárcel et al. 2011)

0.010 min^{-1}, respectively. These values agree with the literature (Kim and Tanaka 2009; Lai et al. 2015; Lutterbeck et al. 2015) and detailed results are presented in SM 7. To identify TPs formed during selected oxidative treatments, samples showing parent compound removal >40 % (SM 7) were analysed by LC-HRMS.

When compared to UV/H_2O_2, UV treatment generally produced fewer and less abundant TPs (SM 8a and b). For example, for CP, four TPs were formed during UV irradiation (N-decl-CP, keto-CP, CP-TP138a and CP-TP138b), while five TPs were formed by UV/H_2O_2 (N-decl-CP, keto-CP, CP-TP138a, CP-TP138b and CP-TP259). With the exception of CP-TP138a, all CP TPs identified have been reported elsewhere during various advanced abiotic treatments, including UV/H_2O_2, UV/TiO_2, O_3 and O_3/H_2O_2 (Fernández et al. 2010; Lai et al. 2015; Lutterbeck et al. 2015; Venta et al. 2005), but not for UV irradiation alone. Lutterbeck et al. (2015) also detected the same TPs during UV/H_2O_2 treatment using a Hg medium-pressure UV lamp. Interestingly, Lutterbeck et al. (2015) and Lai et al. (2015), using UV/TiO_2 treatment, do not report the presence of the same TPs despite the similar experimental conditions (SM 8a and b). In addition, other TPs reported by Lai et al. (2015) for CP, corresponding to m/z 249, 227 and 142, were not detected within the present study,

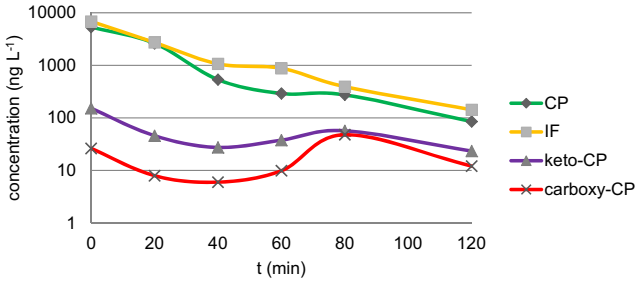


Fig. 2 Average concentrations (ng L^{-1}) of CP, IF, keto-CP and carboxy-CP during UV/O_3/H_2O_2 (5 g L^{-1}) treatment of hospital WW ($n=2$; RSD was below 10.5 % for all compounds). *N*-decl-CP was below LOD within this experiment

whereas keto-CP, identified within this study, is the only reported TP, which was formed during ozonation treatment (Fernández et al. 2010; Venta et al. 2005). Interestingly, CP-TP138a has the same elemental formula and fragmentation pathway as CP-TP138b, a CP TP previously reported by Lutterbeck et al. (2015), but with a significantly different chromatographic retention time (i.e. CP-TP138a, Rt = 2.35 min; CP-TP138b, Rt = 4.71 min). This suggests that CP-TP138a is more polar than CP-TP138b. However, it retains a very similar structure to CP-TP138b as the fragmentation spectra of the two TPs are very similar, apart from the relative abundance of the parent ion and fragments under the same collision energy conditions. A possible chemical structure taking into account elemental composition, increased polarity and fragmentation spectra has been tentatively assigned to CP-TP138a (Table SM 4).

For IF, the TPs are structurally similar to those of CP, the only difference being the position of the CH_2CH_2-Cl group in the molecules. UV irradiation as well as UV/H_2O_2 treatments produced four main TPs: IF-TP199, IF-TP275, IF-TP138 and IF-TP259. So far, only Lai et al. (2015) have identified TPs of IF formed during abiotic treatment (i.e. UV/TiO_2). Two TPs, IF-TP199 (*N*-deschloroethyl-ifosfamide) and IF-TP275 (keto-ifosfamide), which were reported by Lai et al. (2015), who also investigated the formation of TPs from the degradation of IF by UV/H_2O_2 and UV/TiO_2, were identified within this study. A novel TP, IF-TP259 (imino-ifosfamide), during UV and UV/H_2O_2 treatment was also observed. This TP has been previously reported as a metabolite of IF by Li et al. (2010), who investigated the metabolism of IF in mice. Finally, IF-TP138 was also detected as a novel TP formed during UV and UV/H_2O_2 treatment. IF-TP138 is likely to be identical to CP-TP138a, having the same elemental formula, fragmentation spectra and very similar chromatographic retention time.

The identification of TPs of CP and IF under abiotic conditions partially confirmed published findings and with the exception of CP-TP138a, CP-TP138b and IF-TP138

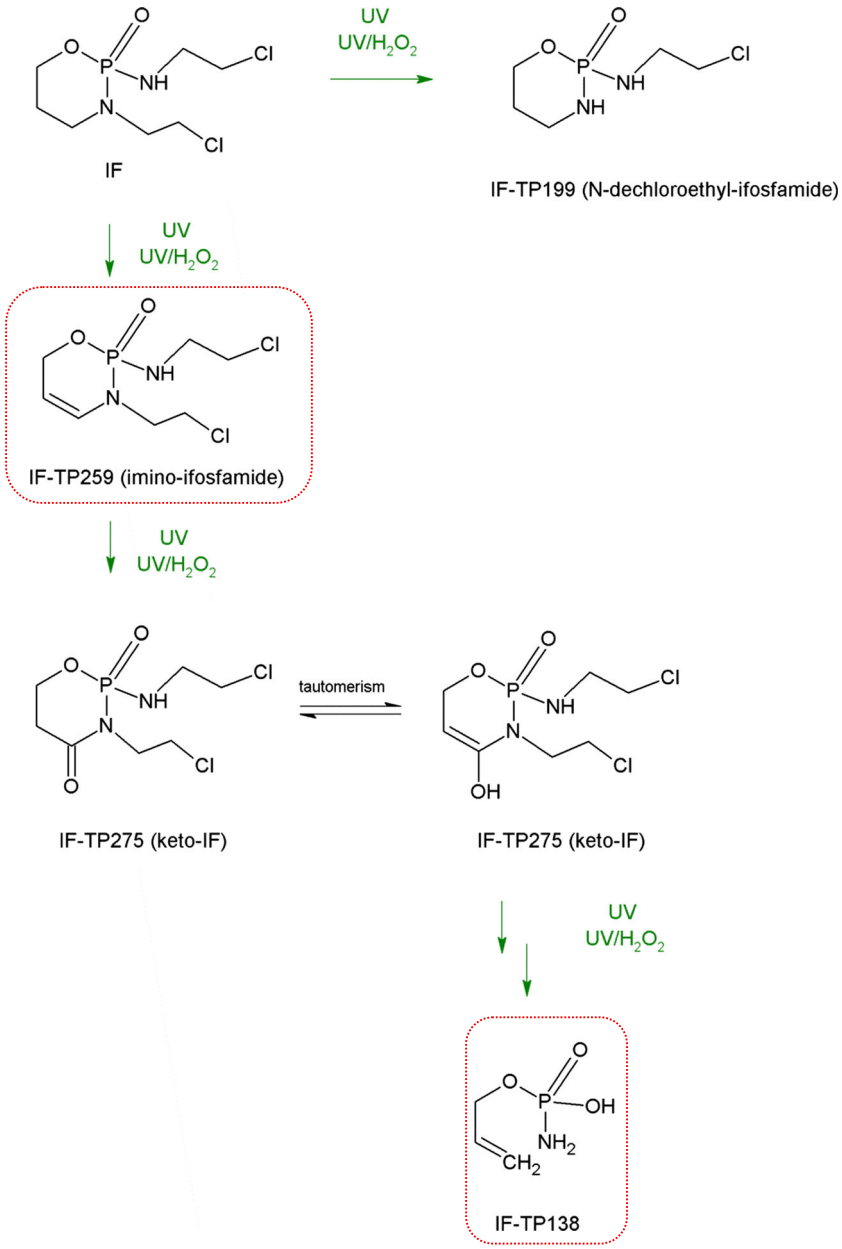


Fig. 3 Pathway for IF TPs during UV and UV/H_2O_2 treatments

(identified in this study), all TPs are also known metabolites (Li et al. 2010). The chromatographic retention times of the TPs were consistent with proposed structures, where the increased polarity of the TPs leads to lower retention times relative to CP and IF. Furthermore, the mass accuracy relative error of the parent compounds and fragments was below 2 ppm indicating a good agreement between the experimental and theoretical data (SM 8a and b).

The proposed formations of CP and IF TPs during UV and UV/H_2O_2 treatment in ultrapure water are presented in SM 8a and Fig. 3, respectively. The pathway for CP TP formation during UV/H_2O_2 is adopted from Lutterbeck et al. (2015), whereas the pathway for IF is proposed for the first time (novel TPs are circled). Figure 3 shows the proposed three-step formation of IF TPs during UV/H_2O_2 treatment. In the first step, *N*-dechloroethyl-ifosfamide and IF-TP259 are formed by dechloroethylation and dehydrogenation, respectively. In the following step, the oxidation of IF-TP259 leads to the formation of IF-TP275. In the last step, the tautomeric form of IF-TP275 is further oxidized to form IF-TP138.

Conclusions

This study describes the development of a GC-MS method for determining human metabolites/TPs of CP and IF (carboxy-CP, keto-CP and *N*-decl-CP) in hospital and municipal WWs at trace levels (low ng L^{-1}). Application of the method to

hospital WW revealed levels of the investigated metabolites/TPs and parent compounds from <LOD to 13,000 ng L^{-1}, whereas in WWTP influent and effluent were all below the LOD. When hospital WW samples were treated using UV/O_3/H_2O_2, the levels of CP and IF decreased with time, while after an initial drop in concentration, keto-CP increased slightly as a product of the degradation of CP. The other targeted metabolites/TPs were all below the LOQ. LC-HRMS not only confirmed the presence of already known TPs but also revealed three novel TPs: imino-ifosfamide, IF-TP138 and CP-TP138a. Future work will include the screening for additional TPs and their occurrence in surface waters.

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