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Antiviral drug Umifenovir (Arbidol) in municipal wastewater during the COVID-19 pandemic: Estimated levels and transformation

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HIGHLIGHTS

• During COVID-19 pandemic umifenovir enters municipal wastewater in large quantities.
• Umifenovir is poorly removed and partially transformed during wastewater treatment.
• Twenty nine umifenovir transformation products were identified and quantified.
• Polybrominated species dominate among umifenovir volatile disinfection by-products.
• Umifenovir and its metabolites accumulate in sludge and river bottom sediments.

ABSTRACT

An indole derivative umifenovir (Arbidol) is one of the most widely used antiviral drugs for the prevention and treatment of COVID-19 and some other viral infections. The purpose of the present study was to shed light on the transformation processes of umifenovir in municipal wastewater, including disinfection with active chlorine, as well as to assess the levels of the antiviral drug and its metabolites entering and accumulating in natural reservoirs under conditions of the SARS-CoV-2 pandemic. The combination of high-performance liquid chromatography with electrospray ionization high-resolution mass-spectrometry and inductively coupled plasma mass spectrometry was used for tentative identification and quantification of umifenovir and its transformation products in model reaction mixtures and real samples of wastewater, river water, biological sludge and bottom sediments taken at the wastewater treatment plant in Arkhangelsk, a large cultural and industrial center at the Russian North. Laboratory experiments allowed identifying fifteen bromine-containing transformation products, forming at the initial stages of the chlorination and fourteen classic volatile and semi volatile disinfection by-products with bromoform as the dominant one. Chlorinated derivatives are only the minor disinfection by-products forming by substitution of alkylamine group in the aromatic ring. The schemes of umifenovir transformation in reactions with dissolved oxygen and sodium hypochlorite are proposed. Two established primary transformation products formed by oxidation of the thioether group to sulfoxide and elimination of thiophenol were detected in noticeable concentrations in the wastewater together with their precursor. The level of umifenovir reached 1.3 mg kg⁻¹ in the sludge and municipal wastewater treat contained 1 μgL⁻¹ of that drug, while its removal...
1. Introduction

The problem of emerging contaminants entering natural reservoirs becomes more and more relevant (López-Pacheco et al., 2019; La Farré et al., 2008; Richardson and Kimura, 2020). A particular danger is posed by pharmaceuticals as they are created as potent biologically active compounds. Their list already exceeds several thousand products and constantly expanding. Among them, antibiotics and antiviral drugs consumed in large volumes sharply increasing during periods of seasonal colds and especially epidemics (mainly influenza) should be highlighted (Prasse et al., 2010). A great number of such compounds persist for a long time in natural aquatic environments and in addition to high biological activity demonstrate numerous non-selective effects on living organisms (Jain et al., 2013). An important aspect of assessing the risks associated with the water pollution with drugs involves their transformation due to the natural biological and physicochemical processes or during wastewater treatment with participation of the dissolved oxygen and/or active chlorine. It is known that a significant part of transformation products possesses higher ecotoxicity compared to the parent substances (Boxall et al., 2004), while many of them cannot be removed at municipal wastewater treatment plants (Schlüter-Vorberg et al., 2015; Funke et al., 2016; Prasse et al., 2011).

The problem of antiviral drugs entering wastewater has become especially acute due to the development of the pandemic caused by the SARS-CoV-2 coronavirus (COVID-19) and leading to a manifold increase in the consumption of the corresponding pharmaceuticals. In addition to the currently officially approved by U.S. Food and Drug Administration remdesivir, avipirdal, and dexamethasone, these include several other active substances as favipiravir, chloroquine (or hydroxychloroquine), azithromycin, ribavirin, lopinavir/ritonavir, etc. (Villamagna et al., 2020; ElBagoury et al., 2021).

In some countries, and especially in Russia, an indole derivative umifenovir (Arbidol, ethyl 6-bromo-4-[[dimethylamino]methyl]-5-hydroxy-1-methyl-2-[(phenylsulfanyl)methyl]-1H-indole-3-carboxylate) is the most widely used drug for the prevention and treatment of COVID-19 and some other viral infections (Fig. 1). Umifenovir is characterized by a wide spectrum of action against influenza and has been originally licensed to fight this infection in Russia (1993) and China (2006). In addition, this compound is known to be effective against human herpes, hepatitis B and C, Ebola virus, influenza virus (A/H1N1) and possesses antioxidant activity (Pécheur et al., 2016; Proskurnina et al., 2020). Umifenovir interacts preferentially with aromatic amino acids affecting multiple stages of the virus life cycle, interferes SARS-CoV-2 binding and intracellular vesicle trafficking (Wang et al., 2020; Vankadari, 2020; Huang et al., 2021). Umifenovir has relatively low toxicity for humans (LD₅₀ > 4 g kg⁻¹) and its preventative daily dosage was defined as 200 mg while therapeutic dosage reaches 800 mg/day with a duration of medication of 5 days (Yang et al., 2020; Instructions for the Medical Use of Arbidol). The half-life of the drug in the body is 17–21 h and ~40% is excreted in unchanged form. The formation of metabolites occurs by N-demethylation, S-oxidation, hydroxylation, O-glucuronide and O-sulfate conjugation (Balakin et al., 2018; Wang et al., 2008; Deng et al., 2013). Thus, taking into account the incidence rate of coronavirus infection, the volumes of umifenovir and its metabolites entering municipal wastewater are quite large and for a city with a population of 1 million can reach several tens kg per day, even excluding consumption for the treatment of other viral diseases. In a recent study by Kuroda et al. (2021) with the aid of quantitative structure-activity relationship (QSAR) modelling the ecotoxicological risk associated with the ingress of umifenovir into river waters was assessed as high and the wild animals’ resistance to antiviral drug was found to be low.

Analysis of a sludge sample collected at the final clarifier after the stage of biological treatment (Fig. S1) in Arkhangelsk (European north of Russia) demonstrated that umifenovir content was extremely high (1.3 mg kg⁻¹). Definitely some transformation products of the drug with unknown structure and properties could be also present in the sludge as well as water and sediments.

Thus, the present study is the first attempt to shed light on the transformation processes of umifenovir in municipal wastewater, including disinfection with active chlorine, as well as to assess the levels of the antiviral drug and its metabolites entering and accumulating in natural reservoirs under conditions of the SARS-CoV-2 pandemic. The study was carried out on an example of the city of Arkhangelsk, which is the world’s largest urban agglomeration (population ~ 350,000), located in the Arctic zone and having a significant impact on the state of fragile aquatic ecosystems of the Arctic basin.

2. Materials and methods

2.1. Chemicals and materials

Umifenovir hydrochloride (>98%, HPLC) was purchased from Sigma-Aldrich (Steinheim, Germany) and was used as a model substrate for oxidation and chlorination, as well as a standard for quantification purposes. Sodium phosphate dibasic (ACS reagent, >99%) and orthophosphoric acid (ACS reagent, ≥95% in H₂O) for the preparation of buffer solutions were purchased from Sigma-Aldrich (Steinheim, Germany). An HPLC gradient grade methanol (Khimmed, Moscow, Russia), formic acid (ACS reagent, puriss. p.a., Sigma-Aldrich, Steinheim, Germany) and Milli-Q Type I ultra-pure water were used for the preparation of mobile phase in chromatographic analyses. Extraction of sludge and sediments was performed using an HPLC gradient grade acetonitrile, ammonium hydroxide (ACS reagent, 28–30% NH₃), and anhydrous sodium sulfate (ACS reagent) purchased from Sigma-Aldrich (Steinheim, Germany).

Hydrochloric acid, potassium permanganate, sodium hydroxide, sodium thiosulfate, potassium iodide, and sulfuric acid of “chem. pure” grade were purchased from Komponent-Reaktiv (Moscow, Russia) and used for the obtaining of chlorine (Brauer, 1963) and preparing sodium hypochlorite solution according to procedure described earlier (Ul’yanovskii et al., 2020). Based on iodometric titration (White, 1999) results the obtained NaOCl stock solution contained 120 g L⁻¹ of active chlorine.

2.2. Samples and sample preparation

Samples were collected in March 2021 at a municipal wastewater treatment plant in Arkhangelsk, as well as from the Khatoritsa River (a tributary of the Northern Dvina River) into which the treated wastewater is discharged. Treatment facilities receive the entire volume (~27 · 10⁶ m³ annually, 3000 m³ h⁻¹ average flowrate) of the city’s wastewater (including urban, hospital and industrial) and implement the following main stages: removal of mechanical impurities (preliminary treatment and clarifying), disinfection with sodium hypochlorite, biological treatment with activated sludge, removal of biological sludge and sediments and discharge of treated wastewater (Supplementary information, Fig. S1).
Sludge sample (SL)

The mixture of sewage sludge with biological sludge weighing ~2 kg with moisture content 85% has been collected from the final clarifier after the stage of biological treatment (Fig. S1). The sample was placed in a plastic container, hermetically sealed, stored at a temperature of −20 °C, and thawed immediately before the analysis.

Sediments sample (SED)

Bottom sediments were sampled from the Khatorita River 2 km downstream of the treated wastewater discharge site (Fig. S1). A sample weighing ~1 kg with the moisture content 55% was packed and stored in the same way as SL sample.

Wastewater samples

Two wastewater samples (3 L each) were taken simultaneously with the wastewater sludge sampling into the amber glass bottles. The first sample (WW-1) was taken after the preliminary treatment and clarifying stage and represents the initial municipal wastewater entering the treatment plant. The second sample (WW-2) was taken after the active chlorine disinfection stage (Fig. S1). According to the laboratory of the wastewater treatment plant the samples WW-1 and WW-2 had the following parameters: COD 100–200, BOD<sub>5</sub> 10–50, and chlorate 10–20 mg L<sup>−1</sup>, pH 7.0 and 7.2, suspended matter ~200 and 8.9 mg L<sup>−1</sup>, respectively.

River water sample (RW)

River water (3 L) was collected into the amber glass bottle from Khatorita River at the same place as SL sample.

All water samples were subjected to solid phase extraction (SPE) and chromatographic analysis on the day of collection immediately after delivery to the laboratory (transportation time was <1 h). Pre-filtered 1-L samples were brought to pH ~7, and passed at a rate of 10–15 mL min<sup>−1</sup> through SPE cartridges (Agilent Technologies, Santa Clara, USA) with 200 mg of functionalized styrene-divinylbenzene polymeric sorbent BondElut PPL with good recovery rates for the wide range of moderately polar and nonpolar compounds (Gonsior et al., 2014; Kosyakov et al., 2017). The cartridges were pre-conditioned with methanol and deionized water. After sorption, each cartridge was washed with deionized water, dried in a stream of high-purity (>99.99%) nitrogen, and eluted with 10 mL of methanol. The resulting extract was evaporated to 500 μL and centrifuged. The attained degree of pre-concentration was 2000.

The thawed at room temperature samples of sludge and bottom sediments were thoroughly homogenized, after that 50-g portions were placed into 250–ml glass conical flasks, poured with 15 mL of acetonitrile containing 0.5% of ammonium hydroxide and kept in an ultrasonic bath for 15 min. After settling and separation from the solution the precipitate was poured with a fresh portion of the extractant. The procedure was repeated three times, the extracts were combined, dried over anhydrous sodium sulfate, and evaporated to a volume of 3 mL in a stream of high-purity nitrogen. For further analysis, 200 μL of the extract was taken, diluted two-fold with water, centrifuged, and injected into HPLC system.

2.3. Umifenovir model oxidation and chlorination procedures

A stock solution of umifenovir in methanol with a concentration of 4 g L<sup>−1</sup> was prepared by a gravimetric method. Working solutions with concentration of 20 mg L<sup>−1</sup> were prepared at different pH levels (7.0, 6.0, 5.0, and 4.3) by diluting the stock solution (50 μL) with 10 mL of appropriate 50 mM phosphate buffer solution in 60-ml glass vials.

The study of the umifenovir oxidation with dissolved oxygen was carried out by keeping vials at ambient temperature (20 ± 2 °C) in the dark with continuous shaking for 10 days. Periodically, 200-μL aliquots were taken from each vial and subjected to the analysis. In addition to these samples, an aqueous solution of umifenovir with a concentration of 1 g L<sup>−1</sup> contacted with air for 3 months was also investigated.

To study the umifenovir transformations under conditions of aqueous chlorination, sodium hypochlorite solution was added to the five working solutions at each pH value to obtain the active chlorine concentrations of 0 (reference sample), 2, 12, 20, and 100 mg L<sup>−1</sup> (from 1:1.3 to 1:67 umifenovir/active chlorine molar ratios). One mL aliquots were taken at specified time intervals (0.5, 1, 5, 10 min) after the introduction of active chlorine and immediately mixed with 10 μL of 0.05 M aqueous solution of sodium thiosulfate to stop the reaction. After centrifugation the samples were subjected to the chromatographic analysis.

2.4. Analytical methods

Taking into account the low volatility and thermal lability of umifenovir, a high-performance liquid chromatography – electrospray ionization high-resolution mass spectrometry (HPLC-ESI-HRMS) was used for its quantification and tentative identification of transformation products at a confidence level 2b according to Schymanski et al. (2014). This technique provided mild ionization conditions and had already been successfully used to study the transformation pathways of various nitrogen-containing xenobiotics (Zahar et al., 2016; Kosyakov et al., 2017; Ul’yanovskii et al., 2020; Bujak et al., 2020). To solve the problem of quantification of bromine-containing umifenovir transformation products without available analytical standards, an original approach based on the combination of HPLC-ESI-HRMS and HPLC-ICP-MS techniques was used. The inductively coupled plasma mass spectrometry (ICP-MS) detection allowed for reliable and sensitive quantification of bromine and based on HRMS data on elemental compositions of the detected compounds, calculating their concentrations.

HPLC-ESI-HRMS analyses were conducted on an LC-30 Nexera (Shimadzu, Kyoto, Japan) HPLC system consisted of DPU-5A vacuum degasser, two LC-30AD chromatographic pumps, SIL-30AC autosampler, CTO-20A column thermostat, and CBM-20A system controller, and combined with TripleTOF 5600+ quadrupole time-of-flight mass spectrometer (AB Sciex, Concord, Canada) equipped with Duospray ion source. Chromatographic separation was achieved on a Nucleodur PPP column, 150 × 2 mm, particle size 1.8 μm (Macherey-Nagel, Duren, Germany) with pentafluorophenyl stationary phase at 40 °C. A gradient elution with 0.1% solutions of formic acid in water (A) and methanol (B) was used according to the following program: 0–1 min: 25% B, 1–20 min: linear ramp of B from 100% to 30–20 min: 100% B. The mobile phase flowrate was 0.25 mL min<sup>−1</sup>, injection volume ~5 μL. Mass spectrometry detection was performed in positive ion mode using information dependent acquisition (IDA) technique with recording tandem (MS/MS) mass spectra of precursor ions with signal intensities exceeding 100 cps. The following ion source parameters were used: nebulizing and drying gas pressure 40 psi, curtain gas pressure 30 psi, capillary voltage 5500 V, and source temperature 300 °C. Mass spectra were recorded in the mass ranges of 200–1000 Da (MS) and 20–1000 Da (MS/MS). Collision-induced dissociation (CID) with nitrogen as a collision gas and 40 eV collision energy with 20 eV spread was used in MS/MS mode. The maximum number of precursor ions simultaneously detected reached 7000.

Collision energies of 40 eV were used for quantitative measurements of umifenovir and its stable isotope labeled analogues (2b confidence level according to Schymanski et al. (2014)).

Fig. 1. Structural formula of Umifenovir.

Chemical formula of Umifenovir.
subjected to CID was 15. Extracted ion current chromatograms (XIC) of the detected compounds were constructed using the mass window of 5 mDa. Elemental compositions were based on the accurate mass measurements and isotopic distributions of the ions using the following constraints: the maximum number of atoms C = 100, H = 300, O = 20, N = 5, Br = 1, S = 1, Cl = 5, P = 2, mass error ≤5 ppm (MS) and <10 ppm (MS/MS), signal-to-noise ratio (S/N) ≥10. Mass scale calibrations in the MS and MS/MS modes were performed prior to every run in an automatic regime using a sodium formate solution as a standard. System control and data analysis were performed using Analyst, PeakView, MasterView, and FormulaFinder software packages (AB Sciex, Concord, Canada).

HPLC-ICP-MS analyses were performed using the same HPLC system and chromatographic conditions as in HPLC-ESI-HRMS experiments. Bromine detection and quantification was carried out using Aurora Elite (Bruker, Bremen, Germany). ICP-MS system equipped with quadrupole mass analyzer and collision reaction interface (CRI). The following parameters were applied: RF power 1.60 kW, sampling depth 5.0 mm; plasma, auxiliary, sheath and nebulizer gas (Ar) flowrates 18.0, 1.65, 0.23, and 0.80 L min⁻¹, respectively; dwell time 500 ms. Detection was conducted in selected ion monitoring mode (m/z 79). High-purity hydrogen (40 mL min⁻¹) was used as a CRI reaction gas suppressing interferences caused by argon-containing ion clusters (38Ar⁴⁰ArH⁺ and 39K⁴⁰Ar⁺). Limits of detection (LOD) and quantification (LOQ) of bromide anion in reaction mixtures was performed as concentrations providing signal-to-noise ratios of 3:1 and 10:1 were 1.0 and 3.3 μg L⁻¹, respectively. The quantitative determination of bromine-containing compounds was carried out by external standard method. Linear calibration plots were constructed using bromide standard solutions in concentration range of 5–10,000 μg L⁻¹ (recalculated to Br). System control, data collection, and analysis were performed using Quantum and Compass CDS software packages (Bruker, Bremen, Germany).

Identification of umifenovir deep degradation (destruction of molecule backbone with the formation of low molecular weight volatile compounds) products was performed by gas chromatography—high-resolution mass spectrometry (GC-HRMS) on an Exactive GC system (Thermo Scientific, Waltham, USA), consisting of a Trace 1310 gas chromatograph equipped with a split/splitless inlet and RSH TriPlus robotic autosampler, and Orbitrap high-resolution mass spectrometer. Chromatographic separation was achieved on a TG-55LSMS capillary column, 30 m × 0.25 mm × 0.25 μm using the following oven program: 30 °C (held 3 min), 15 °C min⁻¹ ramp to 280 °C (held 4 min). Headspace solid phase microextraction technique (HSSPME) with carboxen/divinylbenzene/polydimethylsiloxane (Car/DVB/PDMS) fiber (Supelco, Bellefonte, USA) ensuring sorption of a wide range of polar and non-polar analytes was used for sampling. A 5-mL aqueous sample was placed in crimped 20-mL headspace vial and after equilibration extracted for 30 min at 50 °C with the automatic agitation. Desorption/injection was carried out at 250 °C during 2 min in splitless mode. MS detection was performed in scan mode (m/z 40–500) with resolving power of 30,000 (M/ΔM, at m/z 200) and 12 Hz data acquisition frequency using electron ionization (70 eV). The transfer line and ion source temperatures were 280 and 200 °C, respectively. Mass scale was calibrated daily using perfluorotributylamine as a standard to achieve mass accuracy below 3 ppm (typically <1 ppm). Library search (NIST 2020 database) and accurate mass based elemental compositions were used for reliable identification of analytes. The control of the GC-HRMS system and data processing were performed using TraceFinder software (Thermo Scientific, Waltham, USA).

Quantification of bromide in reaction mixtures was performed by ion chromatography using an LC-20 HPLC system (Shimadzu, Kyoto, Japan) consisting of vacuum degasser, LC-20ADsp chromatographic pump, SIL-20A autosampler, STO-20A column thermostat and CCD-10Avp conductivity detector with SeQuant CARS/SAMS background conductivity suppressor system (Merk, Darmstadt, Germany). Separation was achieved on a Star-Ion A300 anion exchange column (Phenomenex, Torrance, USA) at 30 °C using 1.7 mM NaHCO₃ + 1.8 mM Na₂CO₃ aqueous buffer solution as a mobile phase (1 mL min⁻¹ flowrate).

3. Results and discussion

3.1. Umifenovir transformation under disinfection conditions

The harshest conditions promoting transformations of wastewater components involve water disinfection with active chlorine. To establish umifenovir transformation pathways under the aqueous chlorination conditions and reliably identify the resulting products without introducing additional pre-concentration steps, the model experiments with a relatively high initial concentration of the parent compound (20 mg L⁻¹) were conducted. The ratio of active chlorine and umifenovir mass concentrations was varied in the range of 0–500% at

Fig. 2. Number of bromine-containing compounds detected in the reaction mixtures at different active chlorine/umifenovir ratios.
four pH levels (4.3, 5.0, 6.0, 7.0) close to the real values for municipal wastewater (5–7.5).

Surprisingly, the reaction of umifenovir with hypochlorite proceeded extremely rapidly, making impossible the study the process kinetics. Even at a lack of active chlorine, the formation of detectable by applied analytical methods reaction products is completed by the time of the first sampling (30 s after the initiation of the reaction), after which the list of detected metabolites and the corresponding chromatographic peak areas remain constant throughout the experiment (10 min) regardless of pH value. This observation correlates well with the high antioxidant activity of umifenovir described in the literature and comparable to that for such a widely known reference compound as Trolox (Proskurnina et al., 2020). Thus, the main parameter that determines the pathways of umifenovir transformation is the dosage of hypochlorite. That issue can be clearly seen in the diagram of the dependence the active chlorine/umifenovir ratio on the number of bromine-containing products (Fig. 2), passing through a maximum (16 compounds including bromide anion) near 50%. A further increase in the dosage of active chlorine leads to a rapid decrease in the number of the detected products, and in the case of a five-fold excess, only inorganic bromine was found in the reaction mixture with LC-MS tool. It is worth noting that bromine-free transformation products were not found in the reaction mixtures in noticeable amounts—the chromatograms obtained by HPLC-ESI-HRMS and HPLC-ICP-MS were almost identical (Fig. 3). This means that the loss of bromine occurs only at the advanced stages of umifenovir degradation, accompanied, apparently, by the formation of the simplest and highly polar products eluted with the front of the mobile phase (close to void volume). The presence of bromine in all products formed at the initial stages of the umifenovir transformation made it possible to reliably detect and quantify them in complex matrices in the absence of analytical standards through the combined use of two detection techniques (see Section 2.4). Accurate masses in MS and MS/MS spectra, isotopic distributions and tandem mass spectrometry data of the fifteen compounds detected in reaction mixtures by HPLC-ESI-HRMS (Supplementary material, Figs. S2–S16) allowed obtaining their elemental compositions (Table 1), followed by tentative identification and proposing the scheme of umifenovir transformation under the action of active chlorine (Fig. 4).

Two main reaction types, electrophilic substitution (ES) and single electron transfer (ET) should be considered taking into account the molecular formula of umifenovir. These particular reactions are the most obvious when dealing with interaction of aromatic compounds with hypohalous acids (Lebedev et al., 2004; Criquet et al., 2015). However, taking into account that only one site in the indole fragment remains unsubstituted, ET reaction resulting in oxidation of the substrate seems the most preferable. Moreover, besides possible spatial restrictions the joint effect of the other substitutes does not promote electrophilic substitution into the only free position. Obviously, due to the high reactivity of organic sulfides, the initial stage of umifenovir transformation involves two reaction pathways leading to the formation of

![Fig. 3. HPLC-ESI-HRMS (total ion current) and HPLC-ICP-MS (selected ion monitoring at m/z 79) chromatograms of umifenovir/sodium hypochlorite reaction mixture (pH 5.0, active chlorine dosage 40%, reaction time 1 min).](image-url)
two related types of products: sulfoxide VII (Fig. S2) arises due to the oxidation of the thioether group to the sulfoxide moiety, while C–S bond cleavage leads to product III (Fig. S3) with terminal hydroxy group. The latter is the major transformation product (Fig. 3) and acts as a precursor for the formation of numerous further products. Elimination of the whole methylthiophenol moiety results in formation of compound II (Fig. S4) with hydroxyl group in position 2 of the indole ring.

Compound VII participates as a precursor in several reactions. Oxidation leads to the formation of product VIII (Fig. S5). Quite specific process involves demethylation of alkylamine group (Product VI, Fig. S6). It is rather difficult to propose a mechanism of that transformation, however accurate mass measurements and fragmentation pattern in MS/MS mode do not leave any doubts concerning the structure of that product. Similar reaction of the deethylation of diethylamine moiety was recently observed in the study of aqueous chlorination of hexyl 2-[4-(diethylamino)-2-hydroxybenzoyl]-benzoate UV-filter (Grbović et al., 2013). Elimination of the whole trimethylamine fragment results in the formation of Product XIV (Fig. S7). That process may be accompanied by simultaneous penetration of hydroxyl group (Product XV, Fig. S8) or chlorine atom (Product XIII, Fig. S9) into the aromatic cycle.

Compound III demonstrates the ability to hydroxylation (Product I, Fig. S10), although the corresponding chlorinated product was not detected. Similar to the mentioned above process of elimination of trimethylamine fragment leads to the corresponding hydroxyl (Product XII, Fig. S11), and chlorine (Product X, Fig. S12) substituted compounds. Chlorinated species XIII and X may be treated as compounds formed by electrophilic substitution of the trimethylamine moiety for chlorine as a result of ipso-attack. Hydroxyl group in ortho-position strongly benefits to the realization of that process. Similar reactions of substitution of halogen for halogen in water disinfection conditions were studied recently (Detenchuk et al., 2021).

Product IX, Fig.S13, has the following elemental composition: C_{13}H_{11}BrClNO_3. Its formation involves elimination of water molecule from compound X. Interestingly, in CID spectrum of X the loss of water molecule is also quite pronounced. If benzylic hydroxyl represents an easily leaving group, the source of hydrogen is not obvious. It might come from the neighboring N-CH_3 group. The structure of the forming compound remains unknown however one may propose the formation of 7-member azepin ring (Fig. S13).

It should be specially noted that the reactive benzylic hydroxy group of compound III also participates in side reactions with phosphate anions (a component of a buffer solution) and methanol (a solvent of umifenovir) resulting in the formation of phosphorylated and methylated derivatives IV (Fig. S14) and V (Fig. S15), respectively.
Based on the foregoing, it is obvious that the oxidation processes significantly prevail over electrophilic substitution during aqueous chlorination of umifenovir. In total, only three chlorine-containing compounds (IX, X and XIII) were detected in the reaction mixtures as minor components. It is noteworthy that all of them were formed by replacing the trimethylamine group with chlorine atom, while hydrogen atoms in indole core (ortho-position towards Br), aliphatic groups and benzene ring of thiophenol moiety remained unsubstituted with chlorine.

The list of the detected chlorination products differs from that for umifenovir metabolites formed in the human body (Balakin et al., 2018; Wang et al., 2008; Deng et al., 2013). Instead of numerous conjugates with carbohydrates, sulfated and acetylated derivatives, aqueous chlorination leads to the formation of a significant array of compounds without thiophenol moiety. Nevertheless, four compounds (III, VI, VII, VIII), including the major detected transformation products III and VII, are formed by both chlorination and the action of body enzymes and can enter natural waters from these two sources.

The levels of umifenovir transformation products in the reaction mixtures depend on two most important parameters – pH of the solution and the dosage of hypochlorite (umifenovir/active chlorine ratio). The first factor significantly affects the reactivity of the antiviral drug determining both the equilibrium between molecular chlorine and hypochlorite ion as well as the protolytic equilibria with participation of the acidic phenolic group and alkylamine substituent with high proton affinity in the structure of umifenovir. The results of quantitative analyses of reaction mixtures by HPLC-ICP-MS (Table S1, Fig. 5) demonstrate rapid increase in the concentration of inorganic bromine with an increase in pH due to a further decay of two primary products III and VII. The concentrations of products XIII, XIV, and XV, formed with the elimination of the alkylamine group from compound VII, remain unchanged or even decrease. A similar tendency is observed with an increase of the hypochlorite dose as the concentration of inorganic bromine rapidly increases and reaches 70% of the total content of all transformation products when the active chlorine/umifenovir ratio reaches 100% (pH 5). In parallel, there is an increase in the levels of the secondary degradation products (XIII, XIV, and XV) until the dosage of active chlorine reaches 60% to umifenovir, while the content of the parent substance becomes insignificant even at the ratio of 40%.

To study the chemical composition of umifenovir deep degradation products, not detected by HPLC-ESI-HRMS due to their low molecular weight, inability to be ionized under ESI conditions, and poor retention on a reversed stationary phase, an HSSPME-GC-HRMS technique was used (Section 2.4). Fourteen identified compounds were represented by trihalomethanes, dihalogenated acetonitriles, chloro- and bromobenzene, haloacetic acid esters, and methylphenylsulfone (Fig. S16, Table 2). Most of these compounds are classic disinfection by-products (Richardson et al., 2007). Four dominant products were (in order of decreasing signal intensity on chromatograms) tribromomethane, ethyl dibromacetate, dibromochloromethane, and ethyl chlorobromoacetate. The high levels of polybrominated compounds witness that the formation of disinfection by-products at the advanced stages of the reaction involves numerous mechanisms with participation of bromide-ions and hypobromite ions forming due to oxidation of bromides with hypochlorite species. Bromine-containing compounds from this list were not detected by HPLC-ICP-MS tool and probably eluted with close to dead volume simultaneously with bromide ion. To establish their contribution to the peak intensity, the concentration of bromide anion in the studied reaction mixture (pH 5, active chlorine/umifenovir ratio 100%) was determined by an independent ion chromatography method and amounted to 1.8 mg L$^{-1}$, which is more than half of the total bromine amount introduced into the reaction mixture with umifenovir (3.3 mg L$^{-1}$). This means that the total concentration of bromine-containing organic products of umifenovir deep degradation does not exceed 20% of the bromide anion content. The latter can be considered the main final bromine-containing product.

**3.2. Aqueous oxidation of umifenovir by dissolved oxygen**

Considering the high antioxidant activity of umifenovir, one could expect its efficient oxidation under the influence of dissolved oxygen. However, analysis of umifenovir aqueous solutions at different pH, which were in contact with air, showed a low transformation rate of the drug. Even after ten days the reaction mixture contained only two primary transformation products (VII and III), also characteristic for the treatment with active chlorine (Section 3.1). The oxidation rate increases with increasing acidity of the medium and at pH 5 the content of each product reaches 3–4% of the initial amount of umifenovir. Longer contact with atmospheric oxygen (90 days) did not lead to a significant change in the concentrations of compounds VII and III, however, a new product XVI with an elemental composition of $C_{16}H_{12}BrN_{2}O_{3}$ ($-3.9$ ppm) and retention time 9.06 min was found in the solution in rather significant amounts (1.4 mg L$^{-1}$ or 7% of the initial umifenovir). MS/MS spectrum (Fig. S17) allowed its tentative identification as metabolite with a structure similar to product III with CH$_2$OH moiety replaced for CH$_3$. That conclusion may be supported by no loss of CH$_2$OH fragment in CID (as opposed to compound III). It is, most likely, formed under conditions of significant excess of umifenovir in relation to the oxidizing agent. In summary, umifenovir transformations in the reactions with dissolved oxygen can be expressed by a simple scheme (Fig. 6) involving the oxidation of thioether to sulfoxide, as well as...
elaboration of thiophenol moiety with formation of terminal methyl or hydroxymethyl groups.

3.3. Analyses of environmental samples

The high selectivity of HPLC-ESI-HRMS and HPLC-ICP-MS (with respect to bromine-containing compounds) made it possible to reliably detect, identify and quantify umifenovir and its transformation products in real environmental samples with complex chemical composition and high content of organic matter (Fig. S18) without noticeable matrix interferences. The results of the analyses of SL, SED, WW-1, WW-2, and RW samples (Table 3) demonstrated the presence of the parent umifenovir, as well as three products of its transformation (III, VI, VII), identified in model experiments involving interactions of the antiviral drug with active chlorine and dissolved oxygen.

Municipal wastewater entering the wastewater treatment plant in Arkhangelsk in March 2021 (WW-1) contained about 0.8 μg L⁻¹ of umifenovir and ten-fold lower level (0.07 μg L⁻¹) of the primary product of its transformation VII. In addition, the second most important transformation product III was found in trace amounts. These levels correspond to a daily intake of about 70 g of antiviral drug into the sewage system and are in a good agreement with the number of registered new cases of COVID-19 during the sampling period (~30 people/day). It should be noted that at the peak of the incidence in December 2020, the number of cases exceeded 200 people/day and, accordingly, the amount of umifenovir entering the wastewater was also significantly higher. The study of the treated wastewater (sample WW-2) showed that the used purification technology removed approximately 40% of the incoming umifenovir. The degree of removal of its metabolites did not exceed 20–25%, as they permanently form during the transformation of the antiviral drug. A sharp drop in pollution levels occurs in the natural water reservoir – 2 km downstream from the treated wastewater discharge point (sample RW). The concentration of umifenovir was dramatically lower (35-fold), while its transformation products were not detected. This result may be rationalized by both a significant dilution of wastewater and the possibility of binding and transformation of the drug in natural water ecosystems.

Of greatest interest are the results of studying samples of biological sludge and bottom sediments. As was already stated in the introduction, the umifenovir content in SL sample was extremely high (1.3 mg kg⁻¹), more than 3 orders of magnitude higher than its concentration in the water contacting with the sludge. That issue indicates a high sorption capacity of biological sludge in relation to the antiviral drug and the possibility of accumulation of the latter with the achievement of rather unsafe levels. An important consequence of this accumulation involves the problem of disposal of the sludge – the practice of placing it on a landfill is fraught with leaching of umifenovir into groundwater and secondary pollution of water bodies. It is natural that, in addition to umifenovir, its sulfoxide derivative VII was found in the sludge in large quantities (20% relative to umifenovir), as well as degradation product III, the content of which was 2 orders of magnitude lower. In contrast to the water samples, product VI forming during demethylation of compound VII was also found in SL sample in significant amount (13 μg kg⁻¹). Very high content of umifenovir (3.2 μg kg⁻¹) was found in the bottom sediments of the Khatortisa River (SED sample) even though the levels of the drug and its main derivatives III and VII were 2–3 orders of magnitude lower than in SL sample. Contamination of bottom sediments with a compound with high biological activity can have a negative impact on the bentic fauna, as well as create a secondary source of river water pollution.

4. Conclusion

Umifenovir, due to its high consumption during the COVID-19 pandemic, is found in municipal wastewater at a level of 1 μg L⁻¹ and is characterized by incomplete removal (~40%) during biological wastewater treatment. When interacting with dissolved oxygen and active chlorine, at the initial stages of wastewater treatment umifenovir forms a wide range of bromine-containing transformation products, fifteen of which were identified by mass spectrometry. Reaction of oxidation with electron transfer appeared to be the dominant transformation process triggered by sodium hypochlorite. At the advanced stages of aqueous chlorination fourteen classic disinfection by-products with bromoform as the dominant one is formed. Chlorinated derivatives are minor disinfection by-products forming by electrolytic ipso-substitution of alkylamine group in the aromatic ring. In addition to umifenovir itself, two primary transformation products formed by oxidation of the thioether group to sulfoxide, as well as elimination of thiophenol were detected in noticeable concentrations in the wastewater.

An important feature of umifenovir and its transformation products involves their ability to accumulate in biological sludge at wastewater treatment plant and bottom sediments of natural reservoirs. The levels

Table 3

Concentrations of umifenovir and its transformation products in environmental samples.

| Compound | Concentration, μg kg⁻¹ | Concentration, μg kg⁻¹ | Concentration, μg kg⁻¹ | Concentration, μg kg⁻¹ |
|----------|------------------------|------------------------|------------------------|------------------------|
| SL       | SED                    | WW-1                   | WW-2                   | RW                     |
| Product III | 1.3 ± 0.1             | 0.027 ± 0.004          | 8 ± 2                  | 6 ± 1                  |
| Product VI  | 13 ± 1                | –                      | –                      | –                      |
| Product VII | 270 ± 20              | 0.53 ± 0.06            | 65 ± 10                | 52 ± 8                 |
| Umifenovir | 1260 ± 80             | 3.2 ± 0.3              | 780 ± 50               | 460 ± 30               | 13 ± 2               |
Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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