Simultaneous Analysis of Selected Dissolved Pharmaceuticals in the Water of the Danube River and its Three Major Tributaries in Romania

JANA PETRE*, TOMA GALAON, VASILE ION IANCU, GABRIELA GEANINA VASILE, ELENA STANESCU, LUOANA FLORENTINA PASCU, MARIUS SIMION, LILIANA CRUCERU

National Research and Development Institute for Industrial Ecology, Department of Pollution Control, 71-73 Drumul Podul Dambovitei Str., 060652, Bucharest, Romania

Occurrence and fate of 18 pharmaceuticals belonging to various therapeutic classes were investigated in the aqueous phase of river water at 16 sampling locations along the Romanian side of the Danube and its three tributaries (Jiu, Olt, Arges). Analyses were performed by solid-phase extraction (SPE) followed by liquid chromatography-tandem mass spectrometry with electrospray ionization (LC-ESI-MS/MS). The target analytes belonged to the following therapeutic classes: analgesics, antiepileptics, stimulants and antibiotics. Out of 18 pharmaceuticals analysed, only 7 were detected and confirmed at concentration ranging from 1.2 ng/L to 128.0 ng/L. Caffeine and carbamazepine were the most ubiquitos compounds with detection frequencies of 100 % at a maximal concentration of 128 ng/L and 15.4 ng/L, respectively. Results revealed the presence of sulfamethoxazole in 87% water samples, clarithromycin in 75%, trimethoprim in 69%, cephalexin in 50% and ciprofloxacin in 12%. The obtained results are discussed in relation to relevant data available in literature.

Keywords: pharmaceuticals, surface water, LC-MS/MS, Danube river

Pharmaceuticals are a large group of compounds designed to prevent and treat diseases in human and veterinary medicine and have become indispensable to our modern society. Large quantities of pharmaceuticals are discharged directly and continuously into the rivers through untreated wastewaters, effluents from conventional wastewater treatment plants due to incomplete elimination, or terrestrial run-off [1-3]. The occurrence and distribution of pharmaceuticals in surface waters have been reported in the last decade in a number of studies [4-12]. This information is necessary to estimate the potential threat to aquatic biota as well as human health [6]. Such data might be relevant especially for large streams receiving high anthropogenic inputs such as Danube River. Growing concern for Danube water quality is mainly determined by the fact that it is an important source of drinking water for riparian population, in some cases without being subjected to any process of drinking water abstraction. The occurrence of pharmaceutical compounds in the Danube River has been poorly investigated up to now [5, 13-16]. Generally, the research concerning the pollution of the Danube surface water undergone in our country was focused on detecting priority organic compounds, toxic and persistent heavy metals and inorganic substances deriving from chemical fertilizers [16-18]. Therefore, there is a need of data on the presence of these contaminants in the Romanian part of Danube River basin. The aim of this work was to document the occurrence of selected pharmaceuticals along the Romanian part of the Danube River and assess the contribution by its main three tributaries: Jiu, Olt and Arges. A total of 18 pharmaceuticals belonging to different therapeutic classes were studied: an analgesic (acetaminophen), an anti-epileptic drug (carbamazepine), a nervous stimulant (caffeine) and 15 antibiotics (trimethoprim, sulfamethoxazole, ciprofloxacin, norfloxacin, azithromycin, erythromycin, roxithromycin, ampicillin, cephalexin, amoxycillin, penicillin G, penicillin V, oxacillin, ofloxacin, clarithromycin).

Experimental part

Sampling sites and sample collections

The collection of the samples was performed between 24th and 26th February 2014 under high flow. Mass flows have been increasing due to the combined effect of rainfalls within this period, the disposal of water from the snow and the propagation on the river courses. Mass flow for the Danube River was 6300 m^3/s in the Bazias sector [19]. Samples were collected at 10 locations along the Romanian part of the Danube River and at 2 locations from each of the main tributaries, one location being close to their confluence with Danube River, as shown in figure 1. All samples were grab samples and were taken from a depth of approximately 0.5 m at 2 m from the river bank with the exception of samples from sites S3, S7, S11 and S13 that were sampled at 10m from the bank being gathered from a bridge. Water samples were collected in 500 mL amber PET bottles, previously rinsed in the laboratory with methanol and with water sample at the sampling site. After collection, the samples were kept at 4°C until arrival to the laboratory and pretreated within 48h. Location data, GPS coordinates and description are given in table 1.

Chemicals and reagents

All the pharmaceutical analytical standards were of purity higher than 98% and were purchased from Sigma-Aldrich (Seelze, Germany) with the exception of amoxicillin trihydrate (86%) that was from US Pharmacopeia (Rockville) and ampicillin anhydrous (86%) from European Pharmacopea (Strasbourg). Labeled internal standards were $^{13}C_3$-^{15}N-Ciprofloxacin, $13C_2$-^{15}N-Acetaminophen, $13C_3$-Caffeine, $13C_6$-Sulfamethoxazole, $13C_2$-Erythromycin, Carbamazepine-d_{10}, $13C_3$-Trimethoprim, from

* *email: janapetre@gmail.com; Tel: +4021.410.03.77*

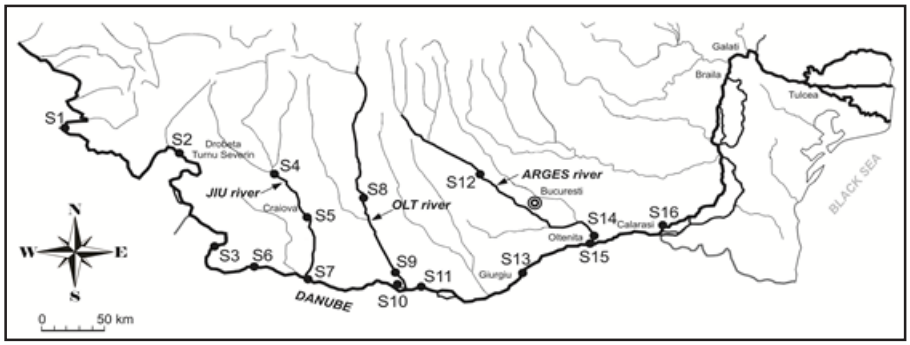

Fig. 1. Map of the locations sampled in the Danube River basin. S = sampling site

Sampling point code	Site name	GPS coordinates	Description
S1	Danube River	44°47'32.61"N 21°23'20.07"E	Bazias
S2	Danube River	44°40'7.40"N 22°33'10.74"E	Gura Vaii, upstream Drobeta Turnu Magurele
S3	Danube	43°57'50.94"N 22°54'15.72"E	Calafat
S4	Jiu river	44°34'8.32"N 23°27'18.14"E	Filiasi, upstream Craiova
S5	Jiu river	44°15'18.48"N 23°47'25.08"E	Podari, downstream Craiova
S6	Danube	43°51'24.84"N 23°17'18.79"E	Rast, upstream of the confluence with Jiu river
S7	Danube	43°45'11.32"N 23°56'30.69"E	Bechet, downstream of the confluence with Jiu river
S8	Olt river	44°23'29.63"N 24°21'4.84"E	Downstream Slatina
S9	Olt river	43°48'41.71"N 24°42'27.71"E	Izbiceni, upstream of the confluence with Danube
S10	Danube	43°42'22.72"N 24°44'4.01"E	Islaz, upstream of the confluence with Olt river
S11	Danube	43°43'2.69"N 24°48'56.65"E	Drobeta, downstream of the confluence with Olt river
S12	Arges river	44°28'45.25"N 25°40'47.87"E	36 km upstream Bucharest
S13	Danube	43°52'37.50"N 25°58'49.92"E	Giurgiu, upstream of the confluence with Arges river
S14	Arges river	44° 6'38.09"N 26°38'15.33"E	upstream of the confluence with Danube river
S15	Danube	44° 3'51.89"N 26°38'45.49"E	Oltenita, downstream of the confluence with Arges river
S16	Danube	44° 8'15.69"N 27°20'8.26"E	Calarasi

Table 1
LOCATION DATA, GPS COORDINATES AND DESCRIPTION

Cambridge Isotope Laboratories (Andover, MA, USA). HPLC-grade methanol, acetonitrile and sodium hydroxide were supplied by Sigma-Aldrich (Steinheim, Germany). EDTA disodium salt (Na2-EDTA) and hydrochloric acid were purchased from Merck (Darmstadt, Germany). Reagent grade formic acid (99,9% purity) was supplied by Agilent Technologies (CA, USA). HPLC-grade water was obtained with a Simplicity UV ultrapure water system from Millipore (Molsheim, France). All solutions used in HPLC system were passed through a 20 μm pore size nylon filter, before use. Individual stock standard solutions at around 500mgL^{-1} were prepared in methanol with the exception of the fluoroquinolones which were prepared in a 1:1 (v/v) mixture of methanol and 0.01 M HCl. These solutions were stored in amber glass vials at -18°C. A mixture of all pharmaceuticals was prepared through appropriate dilution of individual stock solutions in methanol and was renewed before each analytical run. A separate mixture of labelled internal standards, used for internal standard quantification, was prepared in methanol. A binary mobile phase with a gradient elution was used. Mobile phase A was a mixture of water with 0.1 % formic acid and acetonitrile was mobile phase B. The cartridges used for solid phase extraction were Oasis® HLB (500 mg, 6 mL) from Waters Corporation (Milford, MA, USA).

Sample treatment and analysis of the pharmaceuticals

Analytical procedure was based on a previously reported validated method with some modifications [20]. Sample treatment was based on solid phase extraction with an enrichment factor of 500, using the protocol described in EPA Method 1694 for extraction of acid fractions of aqueous samples [21]. Prior to extraction a mixed internal standard was added to 500 mL water sample at a concentration of 100 ngL^{-1} for each internal labeled compound. Separation of the pharmaceuticals was carried out in a HPLC system (1290 Infinity Series Agilent Technologies, Santa Clara, CA, USA). The separation of the analytes was performed at 40°C on a Zorbax SB-C18 Rapid Resolution HT column 50 mm x 2.1mm i.d., 1.8 μm particle size, at a flow rate of 0.3

Compound	Retention time (min)	Precursor ion (m/z)	MRM transitions (*m/z*)	Fragmentor voltage (V)	Collision energy (eV)	Dwell time (msec)
13C2-15N Acetaminophen	1.681	155.1	155.1 → 93 155.1 →111	90 90	15 25	120
Acetaminophen	1.694	152.1	152.1→65 152.1→110	90 90	15 15	120
Ampicillin	5.084	350	350→106 350→160	70 70	10 15	10
13C3-Caffeine	5,111	198.1	198.1→112 198.1→140	110 110	15	10
Caffeine	5.112	195.1	195.1→110 195.1→138	110 110	15 25	10
Trimethoprim	5.116	291.2	291.2→230 291.2→261	110 110	25 25	10
13C3-Trimethoprim	5.121	294.2	294.2→233 294.2→264	110 110	25 25	10
CEF	5.137	348	348→158 348→174	120 120	19 15	10
NOR	5.356	320.1	320.1→276,1 320.1→302	70 70	15 15	10
OFL	5.377	362.2	362.2→261,2 362.2→318,2	110 110	15 25	10
13C3-15N CIP	5.455	336.1	336.1→235 336.1→318	110 110	15 35	10
CIP	5.455	332.1	332.1→231 332.1→314	110 110	20 35	10
AZI	6.141	749.4	749.4→158 749.4→591,3	130 130	30 35	10
13C6-SMX	6.180	260.1	260.1→98 260.1→162	110 110	15 25	10
SMX	6.181	254.1	254.1→92 254.1→155,9	110 110	15 25	10
AMX	7.480	366	366→114 366→208	110 110	5 15	10
13C2-ERY	7.482	736.4	736.4→160 736.4→578	90 90	15 25	10
ERY	7.486	734.4	734.4→158 734.4→576	90 90	15 35	10
PEN G	7.720	335	335→160 335→176	90 90	5 5	10
d10-CBZ	7.869	247	247→202 247→204	110 110	15 35	10
CBZ	7.960	237	237→194 237→179	110 110	15 35	10
PEN V	8.349	383.5	383.5→114 383.5→160	70 70	5 25	10
OXA	9.727	402	402→160 402→243	70 70	5 15	100
CLA	9.994	748.5	748.5→158 748.5→590	110 110	15 25	100
ROX	10.526	837.5	837.5→158 837.5→679	130 130	15 35	100

Table 2
MRM TRANSITIONS AND MS/MS OPERATING PARAMETERS SELECTED FOR THE ANALYSIS OF TARGET PHARMACEUTICALS

$mLmin^{-1}$. A gradient programme was used with the mobile phase, combining solvent A (with 0.1 % formic acid in ultrapure water) and solvent B (acetonitrile) as follows: from 2 to 30% B in 2.5 min, isocratically till 6 min followed by a gradient change to 58% B in 4 min and to 61.5% B in 2 min. Then the system was equilibrated for 6 min prior to the next injection. The column temperature was maintained at 40^0C and the injected sample volume was 2 µL for all the compounds and all the analytes were eluted within 11 min. The HPLC system was connected to a triple quadrupole mass spectrometer Model 6410 Agilent (Agilent Technologies, Waldbronn, Germany) equipped with electrospray Jet Stream technology operating in positive and negative ion mode. Nitrogen gas was used as collision and nebulising gas (9.0 $Lmin^{-1}$, nebuliser pressure 40 psi).The analyses were done in the positive ion mode. The source temperature was 300°C. The capillary voltage was 4000V. Positive mode electrospray ionization was used for all pharmaceutical compounds with multiple reaction monitoring (MRM) mode. For each compound, two signals were monitored, corresponding to the transition between the precursor ion and the two most abundant product ions. The most abundant one was used for quantification while the other one was used for confirmation. Agilent Technologies MassHunter software was used for data acquisition and quantitative determinations. MRM transitions, the optimum collision energies and cone voltages selected for each transition are indicated in table 2.

Quality assurance protocol

Prior to its application, the analytical procedure was validated for river water. Linearity was studied for all selected compounds. Six point calibration curves, with a wide range of concentrations from 1 to 500 μgL^{-1} were generated by injecting mixed standard solutions. A mixed solution of labeled standards was added to all the calibration points at a fixed concentration of 50 igL^{-1} as internal standards. High linearity was observed in all cases ($R^2 > 0.99$). In order to evaluate the method accuracy,

Compound	Range (ngL^{-1})	Median ± IQR (ngL^{-1})[a]	Frequency (%)[b]
Caffeine	28.3-128.0	76.4±35.6	100
Carbamazepine	5.4-15.4	9.65±6.1	100
Sulfamethoxazole	3.2-15.7	7.9±2.4	87
Clarithromycin	1.2-23.2	3.0±2.7	75
Trimethoprim	1.3-11.1	2.6±1.2	69
Cephalexin	5.6-17.8	9.9±4.6	50
Ciprofloxacin	3.6-4.8	3.7±0.6	19

Table 3
RANGE AND MEDIAN OF PHARMACEUTICAL CONCENTRATIONS AND DETECTION FREQUENCY OF INDIVIDUAL PHARMACEUTICALS FROM SURFACE WATER SAMPLES

[a] *IQR-interquartile range;* [b]*Percentage of particular pharmaceuticals in 16 sampling sites*

extraction recoveries for target compounds were determined by spiking river water samples (n=3) at 50 ngL^{-1}. Recoveries were determined by comparing the concentrations obtained after the whole SPE procedure, calculated by internal standard calibration, with the initial spiking level. Unspiked river water samples were analyzed in order to determine their concentrations and to subtract those obtained from the spiked waters. Precision of the overall analytical method was determined by calculating the relative standard deviation (%RSD) of five replicates surface water samples spiked at 50 ngL^{-1}. Recoveries achieved for the most target compounds were higher than 50 %. Only penicillin G and ampicillin showed lower recovery rates (42 and 40%, respectively) but with satisfactory RSD. Limits of detection (LOD) and limits of quantification (LOQ) were determined as the minimum detectable amount of analyte giving peaks for which the signal-to-noise ratio was 3 and 10, respectively.

The estimated values of LODs were in the range from 1.0 to 30 ngL^{-1}, whereas corresponding LOQ values were in the range 3.3-96 ngL^{-1}. Laboratory blanks made every day from ultrapure water were analyzed to assess potential sample contamination. The reported concentrations are corrected with the recoveries of the compounds.

Results and discussions

The optimized analytical method was applied to characterize the presence and concentration of target pharmaceuticals in the surface water of Danube River and three of its tributaries. The study has shown that all investigated samples contained pharmaceuticals compounds. From all pharmaceuticals investigated in analyzed surface water samples were detected caffeine, carbamazepine, trimethoprim, ciprofloxacin, sulfamethoxazole, clarithromycin and cephalexin (table 3).

The lowest concentration obtained corresponded to clarithromycin (1.2 ngL^{-1}) and the highest to caffeine (128 ngL^{-1}). Fig 2 shows the scatter plots of individual pharmaceutical concentration versus location along Danube River (a) and its Jiu, Olt and Arges tributaries (b). Points are connected with lines for visual clarity only. Pharmaceutical concentration levels detected in the surface water samples collected from Danube River were relatively low. The most contaminated samples were collected at tributary rivers, downstream of the big cities Craiova (Jiu River), Slatina (Olt River) and Bucharest (Arges River).

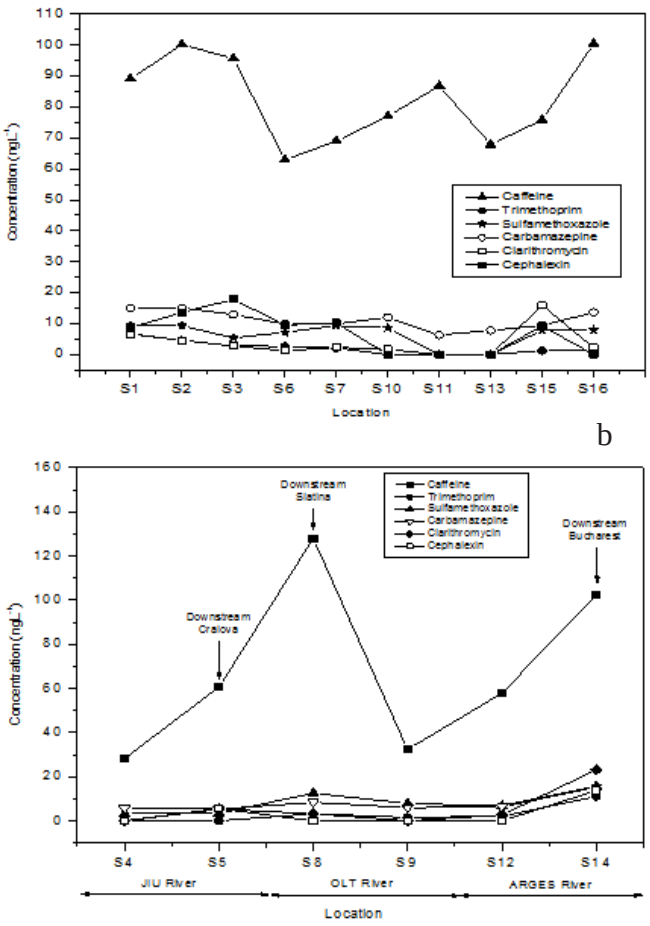

Fig. 2. Concentrations of pharmaceuticals detected along the Danube River (a) and tributaries (b)

Caffeine and carbamazepine were detected in all surface water samples in concentrations which varied from 28.3 to 128 ngL^{-1} and from 5.4 to 15.1ngL^{-1}, respectively. The monitoring results are consistent with those determined within the Joint Danube Survey 2 in 2007 [13]. Previous publication on the levels of caffeine in surface waters has reported its omnipresence in the samples collected [4]. The occurrence of caffeine in the River waters is a proof of their pollution by wastewaters, this compound being an indicator for wastewater pollution. Levels of carbamazepine detected in surface waters of Romanian Danube River basin were similar to those reported for surface waters in Serbia (6-130 ngL^{-1}) [5]. The very low removal rate of carbamazepine in wastewater treatment and its high resistance in aquatic environment

explains the high frequency detection of this pharmaceutical in river waters [5]. The next most frequently detected pharmaceutical was sulfamethoxazole (87%) with a median concentration of 7.9 ± 2.4 ngL^{-1} and a maximum concentration of 15.7 ngL^{-1}. The levels detected in this study were lower than levels reported for Seine River, of above 40 ngL^{-1} [6]. Trimethoprim, usually in combination with sulfamethoxazole, is among the antibiotics most frequently detected (69%), but with a relatively low maximum concentration of 11.1ngL^{-1} on Arges River, downstream of Bucharest. The fairly regular occurrence of sulfamethoxazole and trimethoprim in the sample collected is consistent with the compounds occurrence in the upper Tennessee River basin [7] and Douro River estuary (Portugal) [8]. Trimethoprim and sulfamethoxazole with low values of log K_{ow} of 0.91 and 0.89 are already known as having a high degree of persistence in the surface water [9]. Maximum concentration of sulfamethoxazole determined in this study is in the same domain with that determined in surface water of Canal Danube-Tisa-Danube from Serbia (35.5 ngL^{-1}) [15], ngL^{-1} and very closed to that found in a monitoring study within the Joint Danube Survey 2 [13]. Similar frequency was reported for sulfamethoxazole (90%) and trimethoprim (68%) with median concentration of 6.9 and 12.0 ngL^{-1} in surface waters of the Henares-Jarama-Tajo River system (Madrid, Spain) [12]. Clarithromycin was detected in 75% samples from 1.2 to 23.2 ngL^{-1}. Similar concentrations (up to 44.76 ngL^{-1}) were determined in Arno River (Italia) [11] and in Ebro river basin (Spain) with average concentrations ranging from 1.09 to 36.9 ngL^{-1} [12]. Cephalexin, another largely used β-lactam antibiotic was detected in half of analyzed water samples in concentrations ranging from 5.6 to 17.8 ngL^{-1}, lower than those found in Begej River (Serbia) with a maximum of 283 ngL^{-1} [15]. Between fluoroquinolone antibiotics, only ciprofloxacin was detected in three samples collected from Jiu River, downstream of Slatina city (3.7 ngL^{-1}) and from Arges River, upstream (3.6 ngL^{-1}) and downstream (4.8 ngL^{-1}) of Bucharest. These results are comparable with reported data from Po River (2.24 ngL^{-1}).

Samples S7, S11 and S15 were collected downstream of the confluence with the three tributaries in order to assess the impact of their discharges on Danube River waters. The concentrations of the pharmaceuticals in the Danube waters after the confluence had different fluctuations. Significant changes in the pharmaceuticals concentrations in Danube waters were observed downstream of the Arges River discharges, the most contaminated of tributaries. Trimethoprim, sulfamethoxazole, clarithromycin and cephalexin which were not detected upstream of the Arges discharge occurred downstream of the confluence in concentrations of 1.3 ngL^{-1}, 7.8 ngL^{-1}, 15.9 ngL^{-1} and 9.2 ngL^{-1}, respectively. Caffeine and carbamazepine were detected in Arges River before its discharge in Danube River, in relatively high concentrations of 102.6 ngL^{-1} and 15.4 ngL^{-1}, respectively. Consequently, comparisons between the caffeine and carbamazepine analyzed in Danube River waters, upstream and downstream of Arges river discharge, show an increase of caffeine from 67.9 ngL^{-1} to 75.8 ngL^{-1} and of carbamazepine from 7.8 ngL^{-1} to 9.4 ngL^{-1}.

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Conclusions

An optimized analytical method was applied to analyze simultaneously 18 pharmaceuticals belonging to different therapeutic classes in surface waters collected from the Romanian part of Danube River basin. In all analyzed samples, a nervous stimulant and a psychiatric drug, caffeine and carbamazepine respectively, were found in all surface water samples. Occurrences with frequency higher than 50% in samples collected were observed for sulfamethoxazole, trimethoprim, clarithromycin and cephalexin. The present study contributes to the identification of "hot points" from the studied hydrological basin with a high concentration of pharmaceutical compounds. Considering that the river waters are source of drinking water, the study of the concentration dynamics is necessary in order to protect the health of the aquatic ecosystem and implicitly that of the riparian population.

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