Scientific paper

Ibuprofen as an Organic Pollutant in the Danube and Effects on Aquatic Organisms

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Abstract

The presence of emerging substances in surface water is of a great concern knowing they are the main source for community water supply needs. This study describes the development, optimization and application of an analytical method for the determination of ibuprofen in the Danube samples. Caffeine concentrations, as an indicator of human waste, were determined and maximum risk indexes for aquatic organisms were calculated. The Danube samples were collected from ten representative locations. A Solid-phase extraction was used for ibuprofen and caffeine separation and the analysis was performed by High-performance liquid chromatography method. Ibuprofen concentrations ranged (30.62–111.40) ng/L and caffeine (305.94–375.97) ng/L. Low risk on aquatic organisms was determined for ibuprofen and potential sublethal effect for caffeine was obtained. The results indicated that ibuprofen was effectively separated from other substances in the samples under defined chromatographic conditions for short period of time (4 minutes). Applied HPLC method showed good repeatability, accuracy, selectivity and robustness. Further studies including continuous monitoring of caffeine in the Danube are necessary in order to assess the real risks and possible prevention.

Keywords: Danube; ibuprofen; HPLC; maximum risk index

1. Introduction

Surface and groundwater are the main sources of drinking water worldwide, and purification processes that increase safety and quality of drinking water are of a crucial importance.[1] The presence of emerging substances (ESs) in the environment has become a subject of growing interest in recent decades. ESs are defined as materials present at low concentrations in the environment that have a potential or actual risk to the "One Health" trilogy – environment, human and animal.[2,3] Pharmaceuticals, as one of the major classes of ESs, reach the environment mostly as a result of incomplete removal from municipal wastewater. Surface and groundwater purification processes cannot completely remove these substances, so traces can also be found in drinking water. Although the concentrations of these medicines in water are extremely low (μg/L or ng/L), they are designed to have effects on human at low concentrations. Therefore, their continuous input into the environment must be monitored, as they can lead to long-term negative consequences for the health of humans and aquatic organisms.[2–5]

Ibuprofen, 2-(4-isobutylphenyl) propionic acid, is a non-steroidal anti-inflammatory drug (NSAID) that inhibits the synthesis of prostaglandins, compounds involved in inflammation, fever, blood pressure regulation, blood clotting, reproductive control and tissue growth by blocking cyclooxygenase (COX). As there are two isoforms of cyclooxygenase, it is important to note that ibuprofen is not a selective inhibitor of these isoenzymes, but inhibits both COX-1 and COX-2. It is believed that its positive therapeutic effects derived from the inhibition of COX-2, while the inhibition of COX-1 is responsible for its side effects and effects on the aggregation of platelets and mucous membranes of the digestive organs. It is also believed that antipyretic action is achieved by vasodilation and increased peripheral circulation.[6,7]

Caffeine, 1,3,7-trimethylxanthine, is an odorless, slightly bitter-tasting substance found in natural products such as coffee, cocoa and tea leaves, but is also added to certain industrial foods and pharmaceuticals. It is a natural psychostimulant that has a stimulating effect on the body by acting on the central nervous system. It leads to dilation of the coronary arteries and better blood supply to the brain and dilates the renal vessels, increasing diuresis. Caffeine improves respiration and acts as a general analeptic by stimulating the work of all organs.[8,9] It is often a part of combined analgoantipyretics. Scientific researchers showed that caffeine increases the effectiveness of these drugs by 40% when used together. Also, caffeine in such combinations not only increases the analgesic effect, but also eliminates possible sedative effects that certain analgesics can cause.[10–12]

It is very difficult to extract and detect different analytes from surface water with acceptable yields and detection limits. Therefore, there is still a need for new, reliable analytical methods, which enable fast, sensitive and selective determination of drug residues in environmental samples.[2] The aim of this paper is the development and application of a new, fast and sensitive High-performance liquid chromatography (HPLC) analytical method for ibuprofen determination in surface water. The paper includes the development of procedures for efficient extraction and preconcentration of analyte, optimization of chromatographic procedures and the establishment of protocols for the confirmation of the presence of ibuprofen. The developed method was applied to real samples of surface water, whereby a study on the state of water pollution was obtained. Also, the concentrations of caffeine, as an indicator of human pollution, in the same samples of the Danube were determined and the risk factors on aquatic organisms for both components were calculated. This is especially significant considering that the Western Balkans is a black box when it comes to the number of studies on the presence of drugs residues in the environment and data on the degree of water pollution.

2. Material and Methods

2. 1. Chemicals

The following HPLC grade compounds were used as standards for analysis by HPLC-DAD: ibuprofen (> 99%) and caffeine (> 99%) from Fluka. HPLC grade acetonitrile, methanol, chloroform and tetrahydrofuran were obtained from J.T. Baker. Sodium hydroxide, potassium dihydrogen phosphate, dipotassium hydrogen phosphate and tetrabutylammonium chloride were purchased from Sigma Aldrich. Distilled deionised water (dd H_2O) was used throughout the experiments.

2. 2. Sample Preparation

The Danube samples were collected during September 2020 from 10 locations in Novi Sad, Serbia, and stored at 4 °C in dark bottles in a place protected from light until the beginning of the analysis. The samples were purified on an SPE column (Supelco, supelclean™ LC-18 SPE Tubes 6 ml (0,5 g)). Ibuprofen was eluted with 3 ml of methanol with TBACl (tetrabutylammonium chloride). The solution was evaporated to dryness and reconstituted in a mixture of water and methanol 30:70 (v/v). It was filtered through a 0.45 μm nylon membrane filter directly into the vial and 20 μl was injected into the HPLC system. Caffeine was extracted from the SPE column with chloroform (10 ml), and the solvent was removed by evaporation under reduced pressure. The dry residue was reconstituted in water pH = 8.0 (2.0 ml) and caffeine was analyzed by injecting 20 μl of the solution into the HPLC.[13]

2. 3. HPLC Analysis

The HPLC method[13], using HPLC-DAD model Agilent HP 1100 system with autosempler injector (Waldbron, Germany) was applied for caffeine analysis.

For the analysis of ibuprofen in the Danube samples, the method of HPLC (HPLC-DAD model Agilent HP 1100, Waldbron, Germany) was developed and validated. The mobile phase was acetonitrile: phosphate buffer = 60:40 (v / v, pH 7.0) with flow rate 0.8 mL / min. Detection was performed at 260 nm, and the run time was 5 minutes.

3. Results

3. 1. HPLC method for Ibuprofen Determination in Surface Water

The standard stock solution of ibuprofen was prepared by dissolving 5 mg of the standard substance in 10 mL of the mobile phase (acetonitrile: phosphate buffer = 60:40 (v/v, pH 7.0)). The solution was stable approximately three days under refrigeration (4 °C). Working standard solutions were obtained by taking aliquots of (0.4–4) mL from the standard solution and dilution to 10 mL with mobile phase in a measuring flask. 20 μL of working solutions were injected into the HPLC system and the peak area responses were obtained. A method of the external standard calibration was used. Linear standard curve for ibuprofen was determined by plotting concentrations versus area responses and each calibration point was obtained as an average of three injections. The procedures used to validate the HPLC method for the determination of ibuprofen in surface water have been described in the literature[14–16] and the results are presented in Tables 1-2. The linearity between ibuprofen concentrations and the areas under the curve was tested for concentration levels in the range (0.020–200) mg/L. Under defined HPLC conditions, working standard ibuprofen solutions were injected into the HPLC system and based on the areas under the curves, a regression equation was obtained. The high value of the

correlation coefficient r = 0.99 indicated that there is a good correlation between the concentrations and the areas under the curves. The limit of detection (LOD-3.3 * σ / S) and quantification (LOQ-10 * σ / S) were calculated using the standard deviation of the signal and slope, where S is the slope and σ is the standard deviation of the regression line. Calculations for accuracy and repeatability of this method are presented in Table 1.

Table 1. Accuracy and repeatability of analytical method

Accuracy		
Theoretical concentrations (μg/mL)	**Experimental concentrations (μg/mL)**	**Recovery R (%)**
20.08	19.98 ± 0.09	98.50
30.11	30.00 ± 0.14	99.63
50.20	51.08 ± 0.16	101.75
100.40	100.95 ± 0.22	100.54
200.80	201.33 ± 0.09	100.26
	R ± 2SD (%)	100.14 ± 2.39
Repeatability		
Theoretical concentrations (μg/mL)	**Retention time (min)**	**Area under a curve (AUC)**
20.08	3.551 ± 0.009	49.85 ± 0.154
50.20	3.525 ± 0.006	62.93 ± 0.228
200.80	3.515 ± 0.009	117.13 ± 0.435
	RSD (0.185–0.256) %	RSD (0.309–0.371) %

Data are presented as mean value of triplicate measurements ± standard deviation.

The accuracy was tested by comparing measured and theoretical ibuprofen concentrations. According to the obtained values (98.50–101.75)%, the method showed good accuracy. The repeatability of the method was tested by analyzing three different concentrations of ibuprofen standards in 6 replicates. The relative standard deviation (RSD) ranged from (0.185–0.256)% for retention time and (0.309–0.371)% for peak area, confirming excellent repeatability. By comparing the chromatograms obtained for the ibuprofen standard and the chromatograms for the samples, as well as checking the spectra of the obtained signals, it was determined that there are no signals in the samples whose retention time corresponds to the retention time of ibuprofen, which indicates that the method is selective. The yield (recovery) for the purification procedure was calculated by adding 300 μL of standard ibuprofen solution to the Danube samples and acceptable value for recovery was obtained (92.25 ± 1.55)%. Validation parameters and chromatogram of working standard solutions and UV spectrum of ibuprofen are presented in Table 2 and Figure 1.

Table 2. Validation parameters of analytical method for the determination of ibuprofen in surface water

Number	**Parameters**	**Acceptable values**	**Results**
1	System convenience test		
	Asymmetry	< 2	1.11
	Theoretical plateaus	> 1800	11538.53
2	Limit of detection	–	0.007 mg/L
3	Limit of quantification	–	0.02 mg/L
4	Linearity	r^2 = 0.995 to 1	0.999
5	Repeatability	RSD < 2 %	(0.015–0.65) %
6	Selectivity	Without interference	Acceptable
7	Accuracy	Recovery: (98–102)%	(98.33–100.22) %
8	Robustness (reliability)	< 1%	(0.5–0.9) %

3 . 2. Ibuprofen and Caffeine Determination in the Danube and Assessment of Potential Risk on Aquatic Organisms

Ibuprofen and caffeine amounts for each sampling site are presented in Table 3 and representative chromato-

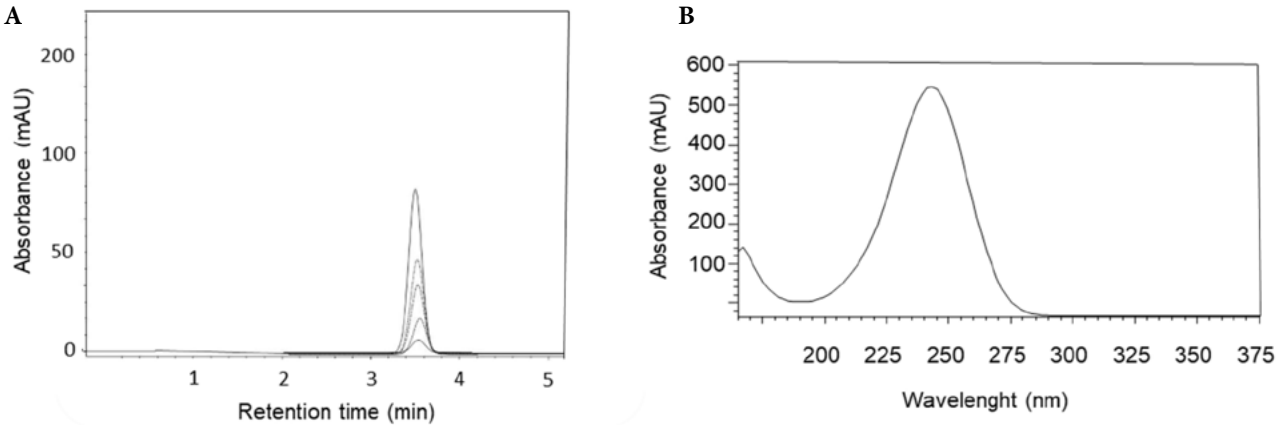

Figure 1. A) chromatogram of working standard solutions of ibuprofen; **B)** UV spectrum of ibuprofen

Table 3. Ibuprofen and caffeine concentrations with MaxRIs

Sample	Sampling site	GPS coordinate	Ibuprofen			Caffeine		
			Concentrations (ng/L)	MaxRI	Level of risk	Concentrations (ng/L)	MaxRI	Level of risk
1	VB us	4525498N	63.45±0.05	273.22	Class III	362.1±0.22	24.39	Class II
2	VB ds	1985616E	30.62±0.03	476.19	Class III	305.94±0.11	28.57	Class II
3	Beogradski kej us	4528327N 1981049E	81.57±0.04	212.77	Class III	341.63±0.45	25.60	Class II
4	Beogradski kej ds		111.40±0.12	156.25	Class III	325.97±0.62	25.64	Class II
5	Cepelin us	4525069N	48.26±0.04	357.10	Class III	321.34±0.77	27.03	Class II
6	Cepelin ds	1985648E	76.01±0.03	227.27	Class III	375.97±0.82	23.26	Class II
7	Štrand us	4523491N	53.62±0.04	322.58	Class III	345.62±0.55	25.00	Class II
8	Štrand ds	1984722E	80.31±0.02	217.39	Class III	358.84±0.77	24.39	Class II
9	DTD us	4528327N	66.62±0.04	263.16	Class III	349.29±0.33	25.00	Class II
10	DTD ds	1981049E	n.d.	–	–	354.45±0.45	25.00	Class II

Data are presented as mean value of triplicate measurements ± standard deviation; us – upsteam; ds – downstream; n.d. not detected; MaxRI – maximum risk index; Class II (10<MaxRI<100) – sublethal effect on fish; Class III (MaxRI>100) – low risk on fish.

grams are shown in Figure 2. Sampling site locations are highlighted in Figure 3.

Ibuprofen is considered to be one of the most frequently detected drugs in surface waters although the highest percentage is removed in the purification process.[2,17,18] The significant presence in natural waters can be explained by the high consumption of ibuprofen and persistence in the aquatic environment.[19] In our study ibuprofen concentrations ranged (30.62–111.40) ng/L with the highest value determined at sampling site No.4 and the lowest at No.2. These results can be explained by the fact that through the sewage system in Novi Sad, Serbia, all waste water without purification ends up in the Danube via two main pumping stations – GC1, which is located near Cepelin and GC2, located near Beogradski kej. Sampling site No.4 (Beogradski kej), where the highest concentration of ibuprofen was detected, is the closest to the GC2 discharge and it is located downstream. The other sampling site closest to GC2 (No.5) is upstream so lower ibuprofen concentrations were found which is in an agreement with the literature.[6] Compared to the research conducted in Madrid, Spain, on Henares-Jarama-Tajo river system (2784 ng/L)[20] a significantly lower value for ibuprofen was obtained in our study. Also, higher concentrations were found in the research analyzing Lis river (Portugal) samples (723 ng/L).[6] Lower results in our work can be explained by the fact that more sensitive methods have been used in previous researches (HPLC-electrospray tandem mass spectrometry). The results of our study are similar to results obtained for Msunduzi river (South Africa) ranging 0.28–85 ng/L.[21]

Caffeine is found in relatively large quantities in surface waters and, along with carbamazepine and sucralose, has often been used as a detector of human pollution.[22,23] According to our work, caffeine concentrations ranged (305.94–375.97) ng/L (Table 3). Highest value was determined at sampling site No.6 and the lowest at No.2. Sampling site No.6 (Cepelin) is near the place of discharge of wastewater (GC1) and it is located downstream which may explain the high concentration of caffeine. These results

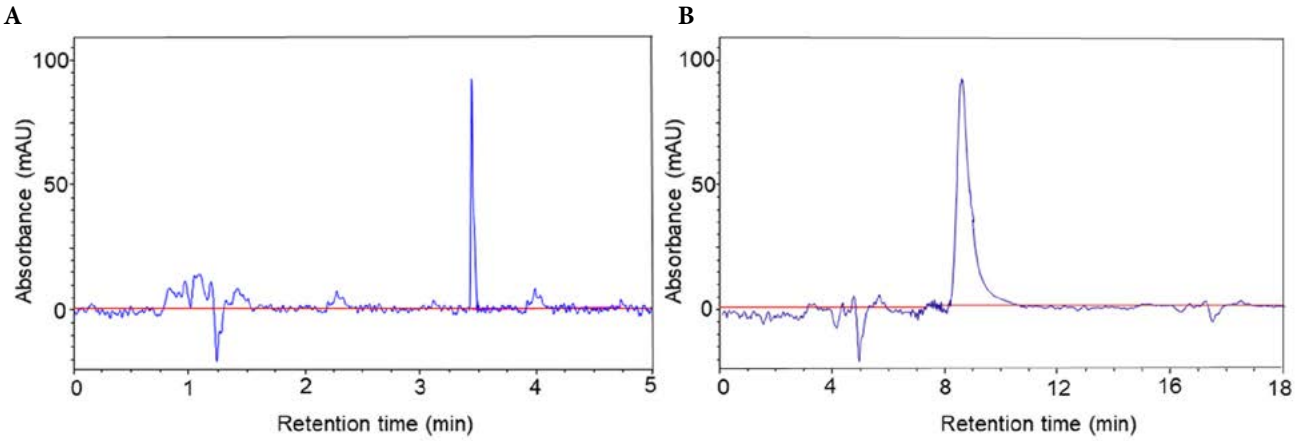

Figure 2. Representative chromatograms of samples: **A)** ibuprofen sample No. 4; **B)** caffeine sample No. 6

Figure 3. Sampling site locations

are slightly higher than in study analyzing the Danube samples during 2014[13] where caffeine concentrations varied (15.91–306.12) ng/L, and lower than one obtained in Paquequer River, Brazil (0.16–47.25 μg/L) using analytical method (SPE-HPLC) with the same sensitivity.[24]

MaxRIs of sublethal effects on fish for ibuprofen and caffeine are calculated as described in the literature[20] and presented in Table 3. According to previous study[20], there are three levels of risks: Class I or high risk of sublethal effects on aquatic organisms, with MaxRI<10; Class II or risk of sublethal effects on aquatic organisms, with 10<MaxRI<100 and Class III or low risk of sublethal effects on aquatic organisms with MaxRI>100. The results for ibuprofen showed that all sampling sites have low risk (Class III) on aquatic organisms with MaxRIs ranging 156.25–476.19 (MaxRI>100). MaxRIs for caffeine varied from 23.26–28.57 and belonged to Class II (10<MaxRI<100) where risk of sublethal effects on aquatic organisms exists. The results are similar to those obtained for caffeine in the Danube in 2014[13], which confirms the fact that in wastewater treatment pharmaceuticals are not removed efficiently and end up in natural waters.

4. Conclusion

The analytical method for determining the presence of ibuprofen in surface water developed and optimized in this work is fast, sensitive, precise and reliable. Traces of ibuprofen and caffeine were detected in most samples of the Danube. Low risk (Class III) on aquatic organisms with MaxRIs ranging 156.25–476.19 (MaxRI>100) was established for ibuprofen. MaxRIs for caffeine (23.26–28.57) showed that the risk of sublethal effects on aquatic organisms exists (Class II). These findings indicate the need for frequent collection of information for caffeine presence in the Danube in order to determine the real risk for resident organisms. Future researches should also be more focused on determining the origin, chemical stability and persistence of caffeine in aquatic environments.

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Povzetek

Prisotnost različnih snovi v površinski vodi je zelo zaskrbljujoča, saj so površinske vode ponekod glavni vir oskrbe z vodo. Raziskava opisuje razvoj, optimizacijo in uporabo analizne metode za določanje ibuprofena v vzorcih Donave. Določene so bile tudi koncentracije kofeina kot indikatorja človeških izločkov in izračunani maksimalni indeksi tveganja za vodne organizme. Vzorci Donave so bili zbrani na desetih reprezentativnih lokacijah. Za ločevanje ibuprofena in kofeina je bila uporabljena ekstrakcija v trdni fazi, analiza pa je bila izvedena z metodo tekočinske kromatografije visoke ločljivosti (HPLC). Koncentracije ibuprofena so bile (30,62–111,40) ng/L, kofeina pa (305,94–375,97) ng/L. Za ibuprofen je bilo ugotovljeno nizko tveganje za vodne organizme, za kofein pa je bil dosežen potencialni subletalni učinek. Rezultati so pokazali, da je bil ibuprofen učinkovito ločen od drugih snovi v vzorcih pod določenimi kromatografskimi pogoji za kratek čas (4 minute). Uporabljena metoda HPLC je pokazala dobro ponovljivost, natančnost, selektivnost in robustnost. Potrebne so nadaljnje študije, vključno s stalnim spremljanjem kofeina v Donavi, da bi ocenili dejanska tveganja in možno preprečevanje.

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