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DETERMINATION OF SOME BETA-BLOCKERS IN SURFACE WATER SAMPLES

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A SPE-LC/MS/MS liquid chromatography-mass spectrometry method was validated for the isolation and quantification of six beta-blockers (atenolol, propranolol, betaxolol, bisoprolol, nadolol and pindolol) and one metabolite (4-hidroxy propranolol) in surface water samples. Using entire procedure we obtained good recovery rates (74.8-102.5%) and low quantification limits (0.17-3.69 ng/L) for target compounds. The impact of two waste water-treatment plants (WWTPs) effluents on the Jiu and Danube rivers was evaluated by analyzing of surface water samples collected in upstream and downstream of WWTPs. It was found that Jiu river was more contaminated than Danube. For propranolol the increase concentration factors were 8.3 in Jiu river and 7.7 in Danube river.

Keywords: beta-blockers, liquid chromatography-mass spectrometry, surface water

1. Introduction

Among emerging contaminants, β-blockers (β-adrenergic receptor antagonists) are widely used in clinical pharmacology for the treatment of cardiovascular diseases such as hypertension, cardiac arrhythmias, tachycardia, thyrotoxicosis, hypertrophic subaortic stenosis, ischemic heart disease, etc. [1,2]. They are also used in treating ocular pressure in glaucoma for the prophylaxis of migraine, and for therapy acute panic symptoms in anxiety situations [3,4].

Large quantities of pharmaceuticals and their metabolites are discharged directly and continuously into the aquatic environment.

These pharmaceuticals compounds present in trace and ultra-trace concentrations are not completely removed during the treatment of sewage plants and, as a result, they could be observed in sewage treatment plants effluents,

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terrestrial run-off, surface waters, groundwater and drinking water all over the world [4,5].

The most commonly used β-blockers are atenolol, propranolol, metoprolol, bisoprolol, and betaxolol [6]. β-blockers are at the 4th position in the most prescribed medication in the USA [7]. In England, Germany, and Austria every year the beta-blockers products are consumed from 100 to 250 tons [5, 8]. After human consumption beta-blockers are absorbed, distributed, partially metabolized and finally excreted via urine in non-metabolized forms in large percentages, such as, for example, atenolol (>85%) and nadolol (100%). Betaxolol and propranolol are largely metabolized in percentages higher than 80%. Bisoprolol is metabolized both in urine and in feces in similar percentages, approx. 50% [9-11]. The most important sources of β-blockers compounds in the environment are APIs (active pharmaceutical ingredients) administered to the patients. These are excreted either as metabolites or as the unchanged parent compounds, through hospital wastewater (that represent major contributors of beta-blockers contamination), pharmaceutical industry waste, and rejected drugs from households, waste water treatment plants and intensive animal breeding farms [6,12,13].

The presence of beta blockers in the environment can affect the aquatic organisms and lead to chronic contamination and toxicological effects on non-target organisms. For example, some researchers pointed out that the beta-blockers belong to the class of Endocrine Disruptive Compounds, since they can disrupt testosterone levels in male organisms [14-16]. β-blockers compounds may have deleterious effects on different organisms such as fish (Japanese medaka, rainbow trout), invertebrates (Dapnia magna, Hyalella azteca, Daphnia lumholtzi, Ceriodaphnia dubia), and green algae (Pseudokirchneriella subcapitata) [16]. Propranolol indicated chronic toxicity not only on the cardiovascular system, but also on reproduction function. The growth of the fish Oryias latipes (medaka) was reduced and its levels of testosterone and plasma estradiol significantly changed after 14 days of exposure to 0.5 mg/L of propranolol containing water. A reduction of the number of laid eggs of fish was also observed after an exposure of 4 weeks to 0.5 μg/L and 1 μg/L of propranolol content in aquatic environment, but not at 50 and 100 μg/L [17]. Due to the lowest measured EC50 (EC50 is the concentration of a drug required to produce 50% of maximum effect and higher the potency), according the EU-Directive of Commission of the European Communities, 1996, the propranolol with an EC50 < 1 mg/L in the algal test would be classified as very toxic to aquatic organisms. Metoprolol (with lowest EC50 (7.9 mg/L) in the algal test) would be classified as toxic to aquatic organisms, while atenolol with a lowest EC50 of 313 mg/L would not be classified following this scheme [5].

When the β-blockers enter a wastewater-treatment plant they are poorly removed and not usually completely degraded; they are either partially retained in the sludge, or transformed to a more hydrophilic but still persistent form. Therefore, they pass through the waste water-treatment plant (WWTP) and end up in the many environmental matrices (rivers, lakes, ground water and seas) worldwide at concentrations ranging from ng/L to µg/L [13,18]. Their removal in WWTPs is variable and depends on the properties of the substance and process conditions (sludge retention time, hydraulic retention time, temperature) [19].

In Romania, no data are available for beta-blockers concentrations in rivers and in WWTPs (waste water treatment plants). Some studies investigated the occurrence of antibiotics, hormones, anti-inflammatory drugs in rivers and WWTPs [20-21]. Thus, important analytical investigations are needed to evaluate the impact of two urban WWTP effluents (from Braila and Targu-Jiu cities) on rivers and to assess the potential contamination of surface water with beta-blockers. The present study aimed to validate a SPE-LC/MS/MS method and to assess the impact of some WWTPs on receiving rivers by determination of six beta-blockers and one metabolite (4-OH propranolol) concentrations in surface waters. Concentrations detected in rivers in upstream and downstream of the WWTP were compared to obtain information about potential influence of effluent on surface water. Beta-blocker contaminants from WWTP effluents are introduced into rives where they can have harmful effects on aquatic microorganisms. Then, the receiving rivers are used as sources of drinking water for urban and rural population. So, it is necessary to perform a quality control of surface water regarding the content of beta-blockers.

2. Experimental

2.1 *Materials and reagents*

Pure standards (>99%) of beta-blockers (nadolol, bisoprolol, betaxolol, propranolol, atenolol, pindolol, 4-hydroxy propranolol) were brought from Sigma-Aldrich. Stock calibration solutions of each compound (500 mg/L) were obtained by weighing of the standards in methanol. LC grade methanol (MeOH), acetonitrile, ammonium hydroxide (24% solution), formic acid (HCOOH, 98%), used to prepare standards and mobile phase, were purchased from Sigma-Aldrich. The intermediate standard solutions (5 ng/ml, 0.5 ng/mL) were obtained by dilution of stock solutions in MeOH:formic acid 0.1% mixture (50/50, v/v). The calibration standards (1-100 ng/mL) were prepared by diluting the intermediate standard solutions in formic acid 0.1%. Strata C18 (500 mg/6 mL), which is a silica-based end-capped *C18* solid phase extraction (SPE) sorbent, was acquired from Phenomenex (Torrance, USA). The ultrapure water was prepared with a Milli-Q water purification system (Milipore Bedford, USA). The Whatman glass microfilter (pore size 1.6 µm, 50 mm diameter) used to filtrate the samples was aquired from Sigma Aldrrich.

2.2 Apparatus

The SPE-LC-MS/MS method of analysis consists in a solid phase extraction using a reusable online trapping column combined with analytical separation on a C18 analytical column and detection by mass spectrometry. The 1260 HPLC system (Agilent Technologies, Germany) was equipped with a triple quadrupole mass spectrometer Model 6410 (Agilent Technologies, Germany). The mass spectrometer was provided with electro-spray ionization (ESI) source, operating in positive ion mode. For the separation of analytes, a Luna C18 (150 x 2 mm, 3 µm) column Thermo Fisher Scientific was used at 45 °C with a flow rate of 0.2 mL/min and 5 µL of standard solutions / sample extract was injected. The mobile phase was a gradient of MeOH and 0.1% HCOOH. The acquisition of data analysis was realized with Mass Hunter software in Multiple Reaction Monitoring (MRM) mode. According to recommendation of the EU Commission Decision 2002/675/EC, two ionization transitions were recorded for detection, corresponding from precursor (protonated molecule $[M+H]^+$) ion to the most intense (as signal) product ion for quantitation and the other from precursor to the second most intense ion for confirmation. The MRM chromatograms of standard solutions in acetonitrile:formic acid 0.2% mixture (90/10, v/v) (100 ng/mL) are presented in Fig. 1.

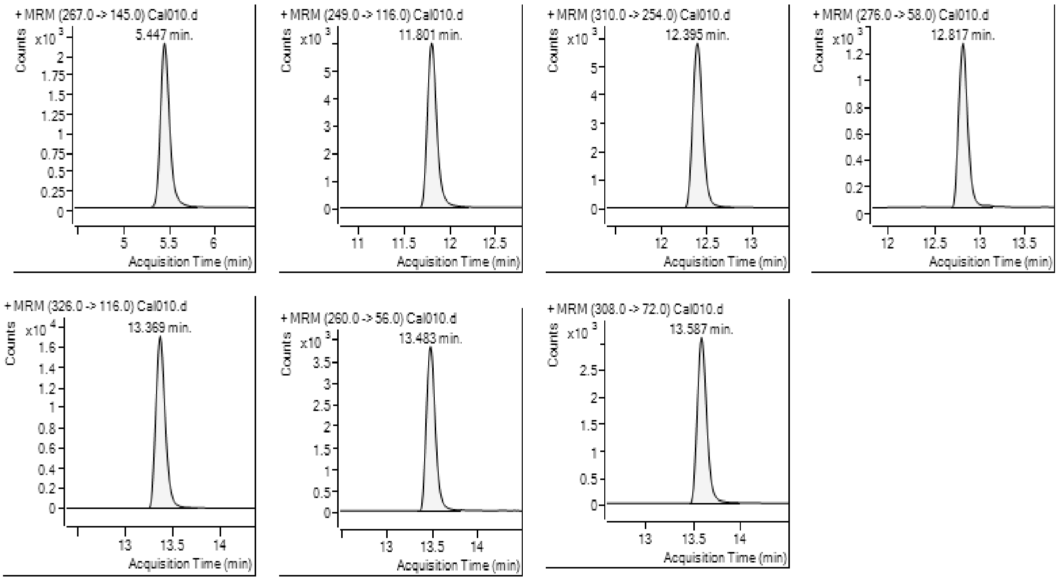


Fig. 1. MRM chromatograms (transitions) obtained for a standard solution of six beta-blockers and a metabolite (from left to right in order: atenolol, pindolol, nadolol, 4-hidroxy propranolol, bisoprolol, propranolol, betaxolol)

2.3 Procedure

2.3.1 Samples treatment and analysis of the beta-blockers

For surface water the SPE-LC-MS/MS method of analysis was validated using river water. Analytical procedure was based on our previously developed method for waste water. Thus, for SPE extraction we used 500 mL of river water sample which was filtrated by microfiber glass filter 0.45 µm (4.7 mm diameter). Then the pH of samples was adjusted to pH 10 using NH_4OH 0.2% solution. The samples were extracted with Dionex Autotrace 280 automated SPE apparatus (Thermo Fisher Scientific). The cartridges (Strata C18, 500 mg/6mL) were conditioned with 8 mL of MeOH and 8 mL of ammonium hydroxide 0.2% at pH 10. Then, interferences were eliminated from sorbent material by washing with 20 mL MilliQ water -NH_4OH solution at pH 10. The sorbent was air dried 20 minute and the analytes were eluted with 6 mL of MeOH. The organic extracts were concentrated by evaporation in a gentle nitrogen stream at 40 ^oC. The residue was reconstituted with 1 mL of MeOH: HCOOH 0.1% mixture (95/5, v/v), of which 5 µL volume was filtrated and automated injected in LC using autosampler.

2.3.2 Validation study

The performance parameters evaluated for river water were: recovery rate, precision, limits of quantification, linearity. Linear calibration range was obtained for each compound by injecting of five standard beta-blockers mixture solutions in the interval 1-100 ng/mL. Calibration graphs were obtained by linear regression analysis. The correlation coefficients of linearity were good, being higher than 0.99. The recovery was calculated by using of a river water spiked with 100 ng/L beta-blocker mixture. The accuracy was accepted if the recovery value was >70%.

First, the sample was analyzed to determine the potential beta-blocker presence from spiked samples. Repeatability of method was calculated by analyzing replicates of three 0.5 L surface waters spiked before extraction in the same day. Reproducibility was obtained by replicates of the same sample spiked in three days. The limit of quantification was calculated for each compound assuming that a minimum amount of analyte gives a signal to noise ratio of 10. Another condition to accept the method to be reproducible and repeatable was the relative standard deviation (RSD) of the precision experiment to be under 15%.

2.3.4 Sample collections

The collection of the samples was performed in September 2018, for a period of 5 consecutive days (Monday through Friday). To analyze the influence of WWTP effluents, water samples were taken from Danube and Jiu rivers in upstream and downstream of Braila and Targu-Jiu WWTPs. The surface water samples were taken in every hour for 24 hours. The upstream collection points were situated at 100 m and the dowstream points were located at 50 m. Surface waters were sampled in 1 L amber glass bottles, previously rinsed with ultrapure water. After collection, the samples were stored at 4 °C until arrival to the laboratory and pretreated by solid phase extraction within 48 h. Table 1 presents some information about the WWTP and received surface water.

Table 1

Information about WWTP and river from which samples were taken for analysis

WWTP name	**Population**	**Flow rate (m^3/day)**	**River**
Braila	200 000	132 000	Danube
Targu-Jiu	80 000	100 000	Jiu

3. Results and discussion

3.1 Results of validation study

The obtained correlation coefficients (R^2 > 0.99) show that the calibration range 1-100 ng/mL is linear and the procedure could be used for detection of beta-blockers in water samples (Table 2). Fig. 2 gives an example for bisoprolol.

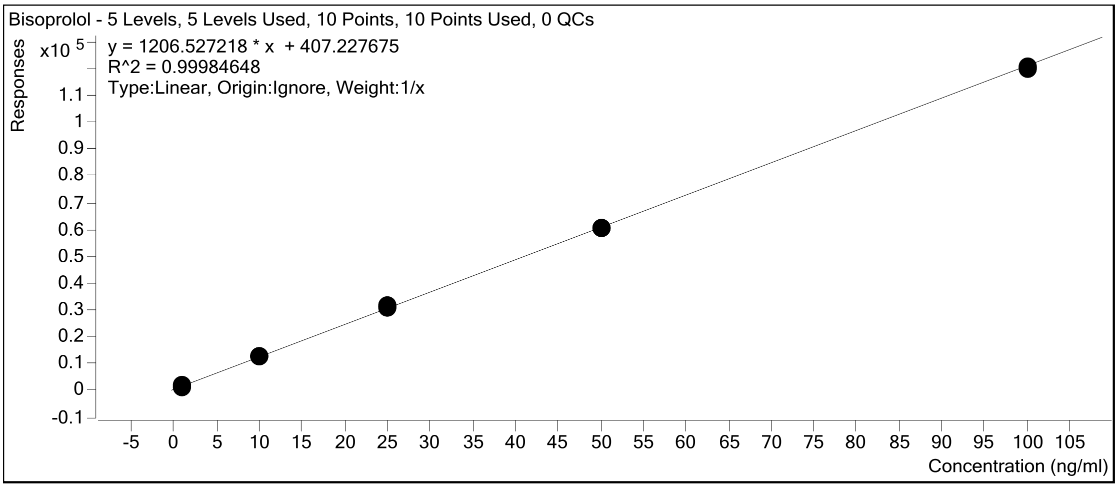


Fig. 2. Linear regression obtained for bisoprolol in the range of 1-100 ng/mL using the LC-MS/MS method

The lowest point on calibration curve (limit of quantification, LOQ) ranged in the interval 0.16-3.8 ng/L which allows detection of beta-blockers present in traces in surface waters. The recovery experiments performed on spiked surface water samples with known amounts of beta-blockers and also on un-spiked samples, show that the SPE-LC-MS/MS method is accurate with recovery values in the range of 74.8-102.5%. Repeatability (intra-day precision) determined varies between 3.3 and 7.3% and reproducibility (inter-day precisions) had values in the range 7.2-12.5% for surface water. The validation parameter proves that the method is sensitive, exact and precise.

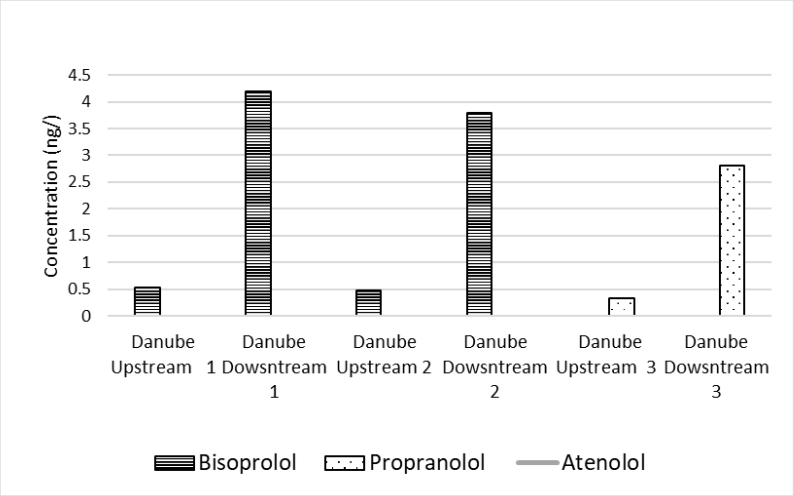
Table 2

Validation parameters

Compound	**Correlation coefficient**	**Regression equation**	**Limit of quantification (ng/L)**	**Recovery rate (%)**	**Precision**	
					Intra-day (% RSD_r)	**Inter-day (% RSD_r)**
Atenolol	0.9998	153x+46.67	0.40	102.5	3.7	8.3
Bisoprolol	0.9998	1206.5x+407.2	0.16	86.9	3.3	7.2
Nadolol	0.9999	436.96x-24.54	1.0	94.3	4.2	7.3
Betaxolol	0.9943	207.06x+17.8	0.5	82.4	4.2	9.7
4-OH propranolol	0.9907	77.84x+56.05	3.8	74.8	5.3	9.1
Propranolol	0.9986	258.1x+447.6	0.42	88.5	7.3	8.3
Pindolol	0.9996	441.2x+36.5	0.61	83.2	4.9	12.5

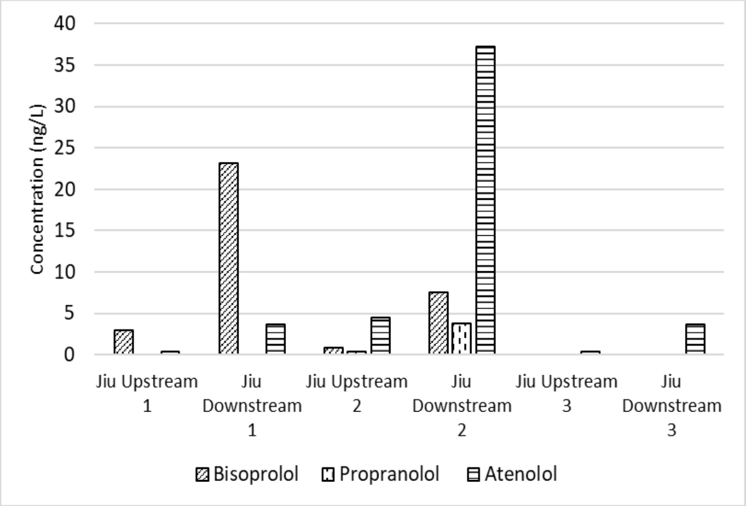
3.2 Beta-blockers occurrence in Danube and Jiu rivers

The most frequent detected beta-blocker compound was bisoprolol (66.6%) followed by atenolol (50%) and propranolol (28.6%). Beta-blockers concentrations calculated in Jiu river (0.41-23.1 ng/L) were higher than values obtained in Danube river (0.32-4.2 ng/L). The highest concentrations were determined in Jiu river for atenolol 37.2 ng/L followed by bisoprolol 23.1 ng/L (Fig. 3). Propranolol was quantified in the range of 0.32-3.8 ng/L in both rivers. Jiu river was most contaminated with atenolol which was detected only in this river in the concentrations of 0.43-37.2 ng/L. Also, the Jiu river samples contained propranolol (0.41-3.8 ng/L) and bisoprolol (0.89-23.1 ng/L).



a)

Fig. 3.a. Beta-blocker concentrations in Danube river



b)

Fig. 3.b. Beta-blocker concentrations in Jiu river

The nadolol, betaxalol, pindolol and 4-hydroxi propranolol were never detected in the analyzed surface waters. In order to assess the impact of WWTP effluents discharged in rivers, we calculated an increase factor for each beta-blocker detected with equation:

$$Increase\ factor = (C_{downstream} - C_{upstream}) / C_{upstream} \quad (1)$$

where: $C_{downstream}$ is the concentration in surface water sampled from downstream of the WWTP, and $C_{upstream}$ is the concentration in surface water taken from river in the upstream of WWTP.

The impact of WWTP was strong for both rivers, but the most pronounced was the WWTP influence on Jiu river. As it can be seen from Table 3, the mean concentration increased after the effluent discharge with factors of 7.3 (from 1.8 to 14.9 ng/L) for atenolol, 6.9 (from 1.9 to 15.3 ng/L) for bisoprolol and 8.3 (from 0.4 to 3.8 ng/L) for propranolol, respectively.

Table 3

Increase factor for beta-blocker concentrations in surface samples taken from upstream and downstream of the WWTPs

Parameter	WWTP	Surface water	Atenolol	Bisoprolol	Propranolol
Increase factor	Braila	Danube	-	1.5	7.7
	Targu-Jiu	Jiu	7.3	6.9	8.3
Detection frequency, %	-	-	50	66.7	28.6

In Danube river the highest increase was for propranolol (factor of 7.7) whose mean concentration increased from 0.32 to 2.8 ng/L, followed by bisoprolol which had an increase of 1.5 with concentration increasing from 1.6 to

4 ng/L. Higher increasing factors calculated in Jiu river are due probably to the low dilution of this river which is smaller in comparison with Danube water.

4. Conclusions

A SPE-LC/MS/MS method was validated in order to quantify six beta-blockers and one metabolite (6-hidroxy propranolol) in surface water samples. The limit of quantification (LOQ) ranged in the interval of 0.16-3.8 ng/L. The recovery rates obtained for spiked samples were between 74.8% and 102.5%, proving that the method is accurate. The linear regressions (1-100 ng/L) used to calibrate the LC-MS system had correlation coefficients higher than 0.99. The method was precise having good intra-day precision (3.3-7.3%) and inter-day precision (7.2-12.5%).

In Danube river, the highest increase was for propranolol (factor of 7.7) whose mean concentration increased from 0.32 to 2.8 ng/L, followed by bisoprolol which had an increase of 1.5 with concentration increase from 1.6 to 4 ng/L. Higher increasing factors calculated in Jiu river are due probably to the low dilution of water which is smaller in comparison with Danube water.

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