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Determination of Hypoglycemic Agents in Surface Water Samples Using SPE-LC-MS/MS Method

VASILE-ION IANCU*, NICULESCU MARCELA, DIANA PUIU, TOMA GALAON, JANA PETRE, LUOANA FLORENTINA PASCU
National Research and Development Institute for Industrial Ecology-ECOIND, 71-73 Drumul Podu Dambovitei Str., 71-73, Bucharest, Romania

***Abstract**: Antidiabetic compounds are a class of emerging contaminants in environment, for which there are no regulations in the world environmental legislation. These compounds are among the most widely used drugs in the world due to the large number of patients with diabetic conditions. The presence of these pollutants in the environment is insufficiently studied, so efficient analytical methods are needed to allow their detection at trace levels (ng/L). For the simultaneously quantification of the five antidiabetics (glyburide, metformin, glipizide, gliclazide, glimepiride) and one bio-degradation product (guanyl urea) in surface water samples a SPE-LC-MS/MS (solid phase extraction -liquid chromatography coupled with mass spectrometry detection) method was validated using real river water samples. The compounds were separated on C18 LC column in 9 minutes at 30^0C using a gradient of mobile phase of 0.1% formic acid and acetonitrile. Good performance parameters were obtained using the method: low limits of quantification (LOQs 0.1-2.4 ng/L), precision (repeatability 3.5-7.2% and reproducibility 6.5-12.7%) and determination coefficients (higher than 0.99). The most contaminated river was represented by Ialomita, which had a total concentration of antidiabetics of 112.1 ng/L in the downstream point, followed by the Siret and Dambovita rivers, which had a total concentration of antidiabetics of 66.3 ng/L and 57.3 ng/L, respectively, also in the downstream points*.

***Keywords**: hypoglicemic agents (anditiabetics), surface water (river), SPE-LC-MS/MS*

1. Introduction

A large variety of pharmaceuticals such as antidiabetics, β-blockers, analgesics, antibiotics, antidepressants, lipid regulators, hormones have been monitored and detected in the environment, particularly in surface waters and wastewaters [1, 2]. A high number of administered pharmaceuticals passes the human body unchanged by excretion and enters into wastewater. The excreted and unchanged pharmaceuticals pass the sewage treatment plant (STP) and the incomplete removal contributes to environmental presence [3]. The presence of pharmaceuticals residues in the aquatic environment represent one of the most urgent emerging environmental issues [4]. The active substances used in obtaining pharmaceuticals from antidiabetic class were used in the treatment of diabetes mellitus or prediabetes treatment. Antidiabetics prescribed include the next classes: meglitinide (repaglinide), sulfonylurea derivatives (gliclazide, glibenclamide, glimepiride), biguanidine (metformin) [5**]**. These drugs were frequently detected in WWTP influents at ng/L concentration level, whereas in some cases comparable concentrations in the treated effluent were noticed. In 2019, the International Diabetes Federation reported the number of diabetic patients worldwide (20-79 years) as 463 million [6]. In Romania it was estimated in 2019 that the number of diabetes patients reached 900,000 [7].

Metformin (N, N-dimethyl-biguanide) is the most consumed antidiabetic for treat type 2 diabetes, but also it is prescribed as a cytostatic product [8, 9]. Because MET is consumed intensive by a large number of diabetic patients, it has a high polarity (low octanol water partition coefficient, log K_{ow} -2.6), is not metabolized by the human body and is eliminated unchanged by urine (90%) in 12 h

*email: vasileiancu10@gmail.com

and the rest by feces, is expected to be present in the influent treatment plants from where it is eliminated by effluent in the receiving rivers [10-12]. MET was determined with high concentrations of the order ng/L in the surface waters analyzed [13, 14].

Scheurer et al., reported in 2009 the occurrence of metformin in Germany, in three WWTPs with a median of 110 μg/L in the influent and 11.4 μg/L in the effluent, respectively [15]. In German river waters, MET was detected in a range from 102 ng/L in the Lake Constance, 349 ng/L in the Weser river, 100 ng/L in the Rhine river, up to 1700 ng/L in the Elbe river [10, 16**]**. In Belgium, Metformin was detected in all WWTPs influent samples ranging from 20 μg/L to 94 μg/L with a medium concentration of 46 μg/L [17]. Kolpin *et al.* reported on the occurrence of metformin in United States surface waters and metformin was detected in 4.8% of 84 samples investigated with a maximum concentration of 0.15 μg/L and a medium concentration of 0.11 μg/L [18]. In China, metformin was detected in eleven Wastewater Treatment Plants (WWTPs) ranged from 1.7 μg/L to 239.0 μg/L, with an average value of 68.3 μg/L [19]. Glibenclamide (GLB), also known as glyburide is extensively metabolized, mainly by hydroxylation of the cyclohexyl moiety of the molecule, whereas its excretion rate as a parent compound are rather low, 35 and 42% in urine and feces, respectively. GLB was determined in surface waters at ng/L to μg/L levels [20]. Glimepiride (GMP) and gliclazide (GLZ) are metabolized in the human body, generating metabolites that exhibit pharmacological activity. Gliclazide was detected in river water at low concentrations at ng/L levels [20].

Ecotoxicological information for selected compounds is still too limited, particularly regarding chronic and behavioral data. For metformin a LC_{50} value of >982 mg/L for *Lepomis macrochirus* and an EC_{50} value of 130 mg/L for *Daphnia magna* were reported. Guanyl urea showed no toxic effects on the bacterial community in a manometric respiratory test at a concentration of 11.9 mg/L **[**21]. The physical/chemical properties of selected compounds are presented in Table 1 **[**22].

Table 1. Chemical properties of hypoglycemic agents

Compound	pKa*	Molar mass (g/mol)	Log K_{ow}
Glibenclamide/Glyburide (GLB)	6.8	494.0	4.79
Glimepiride (GMP)	5.1	490.6	3.5
Metformin (MET)	10.3; 12.3	129.16	-2.64
Glipizide (GLP)	5.9	445.4	1.9
Gliclazide (GLZ)	4.07;1.38	323.4	2.6
Guanyl urea (GUA)	8; 13.5	102.1	-1.2; -2.2

In Romania, data on the presence of antidiabetics in surface waters, intended for the production of drinking water, are not available. In general, environmental studies on pharmaceutical contaminants have focused on the following chemical classes: antibiotics (macrolides, sulfonamides, quinolones, penicillin's, tetracyclines), non-steroidal anti-inflammatory drugs (NSAIDs), antiepileptics, lipid regulators [23-30]. Also, multiple studies have been carried out for the emerging contaminants of the type of metallic elements present in the environmental components which are not regulated in the national environmental legislation [31, 32].

Thus, it is necessary to carry out analytical studies for the determination of the antidiabetic's concentrations (metformin, gliclazide, glimepiride, glyburide, glipizide and one degradation product: guanyl urea), from surface waters and for the evaluation of the potential impact of the effluents that they have on the quality of the receiving rivers. The main aim of the paper was to validate an SPE-LC-MS/MS method that would allow the quantification of hypoglycemic agents at traces (ng/L) levels in surface waters. Then, these concentration values were used for quantitative estimation of the potential impact of wastewater treatment plants that discharge waste water on the receiving inland waters. This was achieved by comparing the levels of antidiabetics from river samples taken downstream from those collected upstream from the treatment plants. Antidiabetic pollutants from WWTP effluents are continuously introduced into receiving rivers where they can irreversible affects the aquatic

microorganisms. Then, the surface water potentially contaminated with antidiabetics represents the source for obtaining drinking water for the resident population. Thus, rigorous analytical control is required regarding the occurrence of these compounds in surface water and in the drinking water.

2. Materials and methods

2.1. Materials and Reagents

Standards with purity higher than 99.1% of hypoglycemic compounds (glibenclamide/glyburide, glimepiride, metformin, glipizide, gliclazide) and one degradation product (guanyl) urea were brought from Sigma-Aldrich. Individual stock standard solutions (500 mg/L) were prepared by weighing of solid compounds and dissolution in methanol. LC grade methanol, acetonitrile, ammonium hydroxide (24%), formic acid (98%), were acquired from Sigma-Aldrich. The intermediate standard solutions (5ng/mL, 0.5ng/mL) were prepared by dilution of stock solutions in acetonitrile; the calibration standards (1-100 ng/mL) were obtained by diluting the intermediate standard solutions in mobile phase: 50/50 v/v, 0.1% formic acid/acetonitrile. The polymeric (styrene-divinyl benzene) cartridges Strata X (500mg/6mL, polymeric sorbent that contains N-Vinylpyrrolidone) were obtained from Phenomenex (Torrance, CA, USA). The Whatman glass microfilter (pore size 0.45 µm, 47mm diameter) used to filtrate the samples were aquired from Sigma Aldrrich.

2.2. Instrument/Equipment and Operating Parameters

The analytical determinations of antidiabetic contaminants were conducted on a 1260 UHPLC system (Agilent Technologies, Germany) which was equipped with a triple quadrupole mass spectrometer (QQQ) Model 6410 Agilent (Agilent Technologies, Waldron, Germany). The compounds were separated on C18 LC column, in 9 minutes, at 30^0C using a gradient of mobile phase of 0.1% formic acid and acetonitrile (0.2mL/min flow rate). The injection volume for calibration standard and for sample extract was 10 µL. The ionization of compounds was realized by positive electrospray ionization in MS source using the optimal parameters shown in Table 2, 3. The system was controled by Mass Hunter software from Agilent Technologies. Formic acid was used in mobile phase to obtain good peak shape and for the production of the precursor ion $[M-H]^+$. Ionization of compounds was performed using the next optimized settings: gas temperature 300^0C, capillary voltage, 3000 V, nitrogen nebulizer gas flow rate (10 L/min), nebulizer pressure 50 psi, the cell acceleration voltage (CAV) 4 V, collision energy 10-25V, fragmentor voltage 80-120V.

Table 2. Separation parameters of the liquid chromatograph (LC) and detection parameters of the triple quadrupole mass spectrometer (QQQ, MS/MS) for analytes

No. crt.	UHPLC	MS/MS
1	Column: Eclipse C18, 100 x 2 mm d_p of 3.4 µm	Ionization mode: ESI+ (positive)
2	Column temperature: 30 ^{0}C	Gas temperature: 300 ^{0}C
3	Injection volume: 10µl	Drying gas flow rate: 10 L / min
4	Mobile phase: 0.1% Formic acid: Acetonitrile	Gas pressure in Nebulizer: 50 psi
5	Mobile flow rate: 0.2 mL / min;	Voltage applied to the capillary: 3000 V
6	Solvent samples: 0.1% formic acid / ACN mixture = 50/50 (v/v)	Cell acceleration voltage: 4 V
7	Running time of sample 14 min	Fragmentation voltage: 80-120 V Collision energy: 10-25 V
8	Elution: in gradient	MRM transitions: 2 transitions for each compound

Table 3. The mobile phase gradient used to separate hypoglycemic agents from environmental samples

Time (min)	0.1% Formic Acid (%)	Acetonitrile (%)	Flow rate (mL/min)
0	50	50	0.2
6	50	50	0.2

9.5	40	60	0.2
9.51	50	50	0.4
13.5	50	50	0.4
13.51	50	50	0.2

The two most intense product ions were selected for the analysis. Table 4 presents the optimized mass spectrometer /QQQ parameters for the determination of antidiabetic drugs in the environment samples. The adduct $[M-H]^+$ was used as the precursor ion for MS determinations in the positive ionization mode. The first product ion as abundance was used for quantification and the second ion as abundance for confirmation.

Table 4. Mass spectrometric parameters such as time segments, MRM transitions, fragmentation voltage (frag), collision energy (CE) (all in V), Dwell time

Compound	Time Segments (min)	MRM transitions (m/z)	Fragmentor voltage (V)	Collision energy (eV)	Dwell time (ms)
Metformin	0-4	130→71	80	25	40
		130→60	80	10	40
Guanul urea		103→60	80	15	40
		103→43	80	10	40
Glipizide	4-6	446→321	80	10	40
		446→167	80	20	40
Gliclazide		324→127	120	15	70
		324→110	120	20	70
Gliburide	6-9.5	494→369	100	15	40
		494→169	100	20	40
Glimperide		491→352	100	20	40
		491→126	100	10	40

Acquisition of spectra and chromatograms were realized in Multiple Reaction Monitoring (MRM) mode. Thus, for detection of each compound were recorded two ionization transitions corresponding from precursor ion (protonated molecule $[M-H]^+$ to the most intense product ion for quantitation and the other from precursor to the second most intense ion for confirmation. The MRM Chromatogram of mixture standard solution in mix acetonitrile: formic acid 0.1% (50/50, v/v) (100ng/mL) is presented in Figure 1.

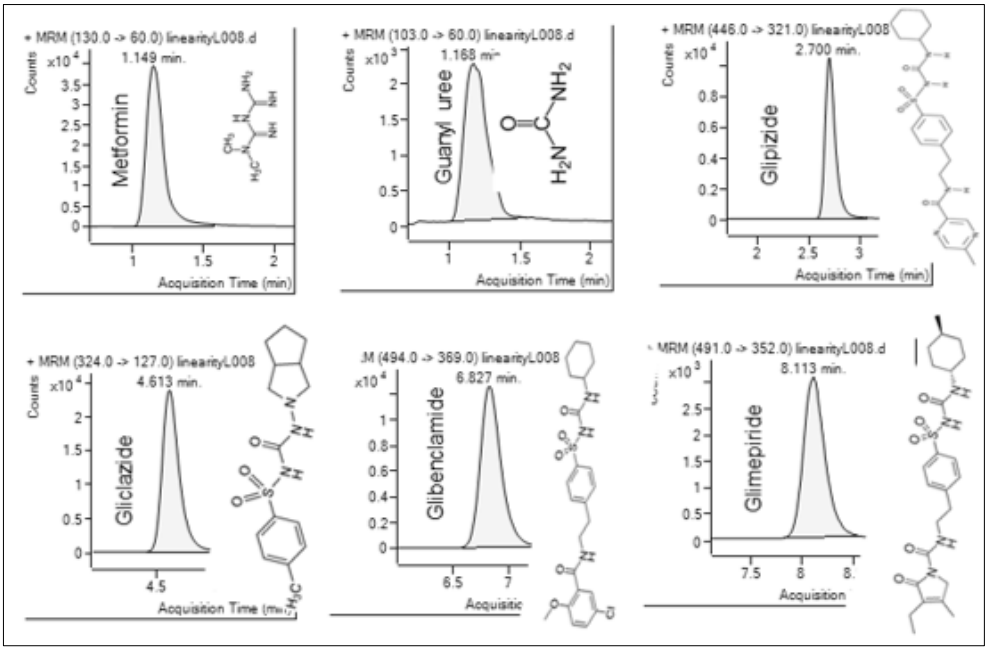

Figure 1. The ESI MRM chromatogram of hypoglicemic compounds obtained by LC-MS/MS (metformin guanyl urea, glipizide, gliclazide, glyburide, glimepiride in mobile phase, each, 100 ng/mL)

2.3. Samples Treatment and Analysis of the Antidiabetics

The SPE-LC-MS / MS method previously developed for wastewater samples (effluent and influent treatment plants) was validated using surface water. Thus, the method used a volume of 500 mL samples of river water that was subjected to the entire procedure without addition of standard and with known addition of standard. It was used an enrichment factor of 500 for each water sample. First the sample was filtered on a 0.45 µm glass filter (4.7 mm diameter) to remove the suspended matter that can block the SPE extraction material. Then, the *p*H of the sample was adjusted to 10 with 0.24% ammonium hydroxide, after which the entire volume of water was passed through SPE cartridge, pre-conditioned with 2x4 mL methanol and 2x4 mL ultrapure water with *p*H 10. The extraction was performed on an automatic SPE equipment type Dionex Autotrace 280 (Thermo Scientific) using cartridges Strata X (500mg / 6mL, Phenomenex). To remove the interfering matrix, the adsorbent material was washed with ultrapure water, after that the cartridge was air dried (20 min.) in order to remove traces of water. The compounds of interest were eluted from the SPE cartridge with 2x3 mL methanol. To change the extraction solvent, the obtained extracts were evaporated in a water bath (50^0C) under a dry nitrogen stream, after which the organic residue was taken up with 1 mL of the mobile phase (0.1 formic acid: acetonitrile, 50/50).

2.4. Validation Study

The method has been validated in terms of linearity, limit of quantification, efficiency of recovery and precision (repeatability, reproducibility). Linear regressions (1-100 ng/mL) were obtained for each antidiabetic compound by injecting of 5 calibration solutions with increasing concentrations. Regressions were accepted if the coefficients of determination were over 0.99. The limit of quantification was calculated as the minimum analyte concentration that can be determined from a spiked surface water sample with a concentration of compound for which the signal to noise (S/N) ratio is 10, following the entire extraction and analysis process. The recovery was experimentally determined from a river water sample with the addition of 50 ng/L calibration mix solution. Also, the water sample was analyzed without addition, and the determined antidiabetic drugs were subtracted from the sample with addition. A recovery of 70-120% was considered good for accuracy experiments. To calculate the precision, to 4 sub-samples of surface water (500 mL) were added 1 mL calibration solution, 50 ng/L mixture of antidiabetics in the mobile phase. The samples were extracted and analyzed in the same day, determining the repeatability, expressed as RSD (residual standard deviation), and on different four days, calculating the reproducibility. Precision was accepted if the values of repeatability and internal reproducibility were below 15%.

2.5. Sample Collections

Surface water samples were collected in November 2019 in a single day, from 5 rivers (Siret, Bahlui, Ialomita, Dambovita, Somes) (Table 5). Thus, samples were collected from downstream and upstream of the municipal wastewater treatment plant (WWTP). Sampling points were located 100m before the station (upstream) and 50m after the station (downstream). The sample was collected in a 1L volume glass falcon, then stored at 4^0C during transport to the laboratory and extracted within 48 h.

Table 5. Data about the WWTP and the receiving rivers

Receiving river	WWTP	Population	Flow rate (m^3/day)
Bahlui	Iasi	791210	362880
Siret	Galati	509471	66300
Ialomita	Targoviste	124000	25130
Dambovita	Glina (Bucharest)	2400000	1036800
Somesul Mic	Cluj-Napoca	440000	111000

3. Results and discussions

3.1. Validation of method

The mass spectrometer detector response was linear in the range of 1-100 ng/mL for all compounds except Gua (5-100ng/mL), yielding coefficients of determination between 0.99-0.998 (Table 6, Figure 2). The limit of quantification had low values (0.10-2.45 ng/L) allowing the simultaneous determination of antidiabetics from surface water samples using the LC-MS/MS method.

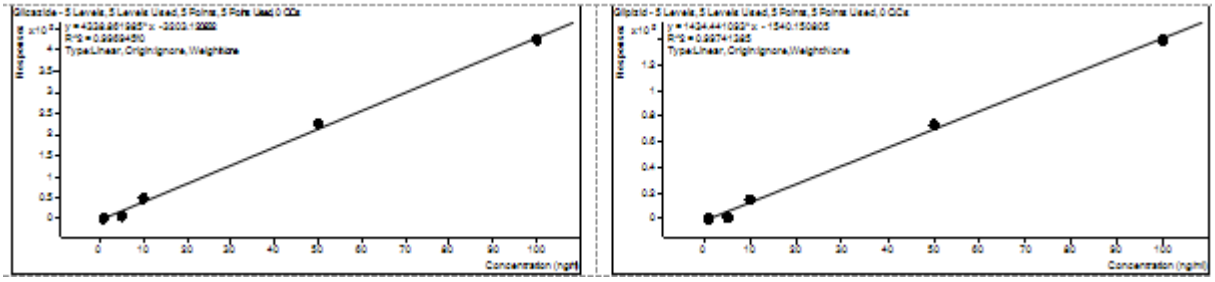

Figure 2. Linear regression obtained for gliclazide and glipizide in the range 1-100 ng/mL using LC-MS/MS method

The efficiency of the solid phase extraction step has been demonstrated by obtaining acceptable recoveries (57.4-105.2%) for all hypoglycemic agents in a single step by adjusting the *p*H to 10 with NH_4OH 0.24%. The experiments were made on real surface water contaminated with known antidiabetic concentrations (standard calibration mix solution, 50 ng/L, in mobile phase).

The method presented corresponding performance characteristics as repeatability, generating intra-day precision within the range of 3.5-7.2% and internal reproducibility (inter-days precision) in the range of 6.5-12.7%. The validation parameter proves that the method is sensitive, accurate and precise. The validation parameters of the method and data obtained by the external standard calibration methodology for all antidiabetic compounds in surface water are presented in Table 3.

Table 6. Determination coefficients (R^2), recovery rate, limits of quantification (LOQs), and intra-day and inter-day precision

Compound	Calibration range (ng/mL)	Determination Coefficients	Limits of quantitation (ng/L)	Recovery rate (%)	Precision	
					Intra-day (% RSD_r)	Inter-day (% RSD_R)
Metformin	1-100	0.9904	0.20	68.7	7.2	12.7
Guanyl uree	5-100	0.9982	2.45	57.4	5.4	9.8
Glipizide	1-100	0.9974	0.15	99.7	3.5	6.5
Gliclazide	1-100	0.9969	0.10	96.3	6.2	11.1
Glyburide	1-100	0.9977	0.20	84.6	4.5	8.6
Glimperide	1-100	0.9982	0.15	105.2	5.7	10.5

3.2. Antidiabetics Occurrence in Surface Water

A total of 10 surface water samples (from 5 receiving rivers) taken in the downstream and in the upstream points of the wastewater treatment plants of some municipalities were analyzed in order to determine the concentrations of antidiabetics. At the same time, the aim of this study was to evaluate the chemical quality of surface water used in the production of drinking water. Compounds that were ubiquitous (100% detection frequency) in all samples analyzed were metformin and guanyl urea being detected in all 5 rivers both upstream and downstream of the treatment plants. The compound detected with the highest frequency (90%) was gliclazide followed by glipizide which was determined only in 50% of the samples. The glibenclamide and glimepiride were never detected in the analyzed surface waters.

The highest concentrations were determined in Ialomita river (MET 65.6 ng/L, GUA 30.8 ng/L), in downstream of the Targoviste WWTP (Figure 3). Also, high concentrations were observed in Siret river, in downstream of the Galati WWTP (GLZ 26.9 ng/L, MET 16.6 ng/L, GUA 22.8 ng/L), followed by the Dambovita river which had high concentrations of GLZ 20.9 ng/L, GUA 18.4 ng/L, MET 11.2 ng/L), in downstream of Glina (Bucharest) WWTP.

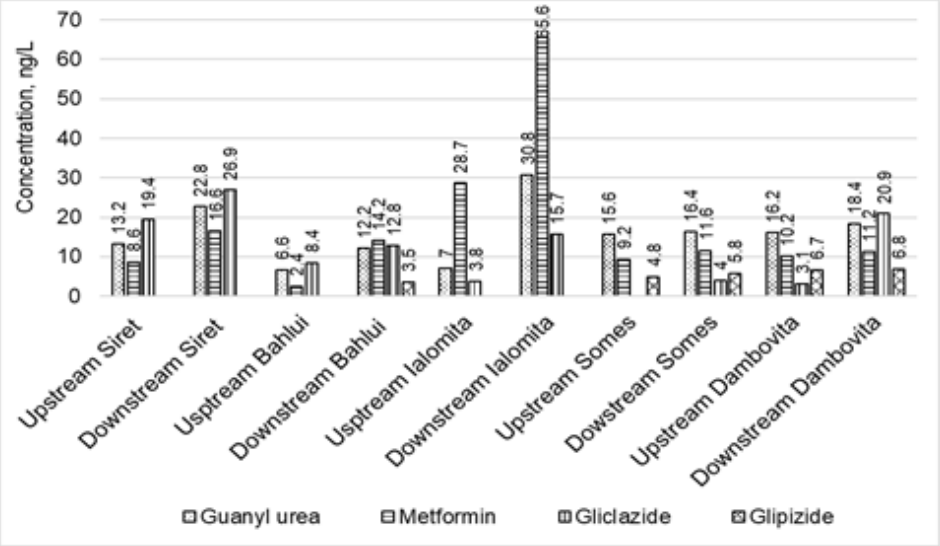

Figure 3. Antidiabetic concentrations in next rivers: Siret, Bahlui, Ialomita, Somes, Dambovita (upstream and downstream of WWTP)

In order to assess the potential impact of WWTP effluents discharged in river, we calculated an increase factor (IF) for each antidiabetic compounds with equation (1):

$$IF = \frac{c_{dw} - c_{up}}{c_{up}} \quad (1)$$

were, c_{dw} is the antidiabetic concentration in surface water sampled from downstream of the WWTP and c_{up} is the antidiabetic concentration in surface water taken from river in the upstream of WWTP.

Regarding the potential impact of the WWTPs on the surface water quality, it was observed that the Bahlui river presented the highest increase factor (22) for the concentration of glipizide, probably due to Galati WWTP, followed by the Dambovita river which had an increase factor of 5.7 in the case of gliclazide, generated probably by Glina (Bucharest) WWTP (Figure 4). The next degree of potential impact corresponded to GUA (3.4) and MET (3.1) in the Ialomita river probably due to the effluent discharged by the Targoviste WWTP. In the case of Somes river, for the gliclazide, an increase factor of 3 it was obtained, probably due to the discharge of the Cluj-Napoca WWTP effluent in the Ialomita river in the downstream area.

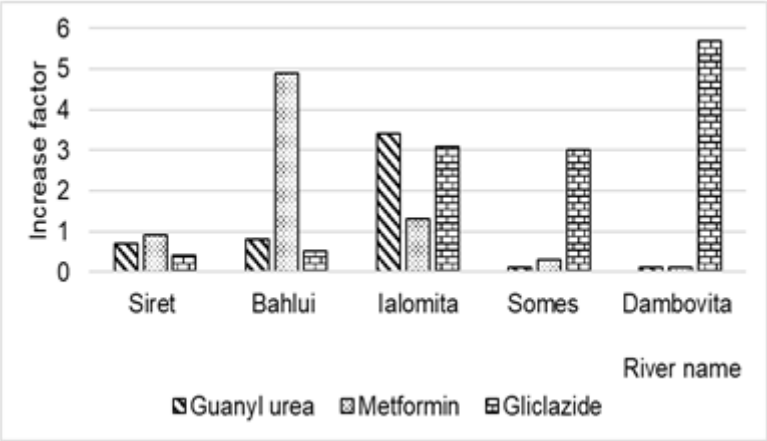

Figure 4. Increase factor for antidiabetic concentrations in surface samples taken from upstream and downstream of the WWTPs

The potential impact of the 5 WWTPs was strong for four rivers (Bahlui, Dambovita, Ialomita, Somes), but the most pronounced was the WWTP Iasi effluent on Bahlui river, followed by WWTP Bucharest for Dambovita river. The antidiabetic concentration increased, after the effluent discharge, with factors of 22 from <LOQ to 3.5 ng/L for glipizide (Bahlui), 5.7 from 3.1 to 20.9ng/L for gliclazide (Dambovita) and 4.9 from 2.4 to 14.2ng/L for metformin in Bahlui.

These values are similar or lower than the concentrations reported by other paper in Germany rivers (Lake Constance MET 102 ng/L, Rhine river MET 100ng/l), or in USA rivers (MET 150ng/L) [10, 14, 18]. The most contaminated river was represented by Ialomita, which had a total concentration of antidiabetics of 112.1 ng/L in the downstream point, followed by the Siret and Dambovita rivers, which had a total concentration of antidiabetics of 66.3 ng/L and 57.3 ng/L, respectively, also in the downstream points.

4. Conclusions

A SPE-LC/MS/MS method was validated in order to quantify 5 antidiabetic compounds (metformin, glimepiride, glyburide, gliclazide, glipizide) and one degradation (guanyl ure) in surface water samples. The limit of quantification (LOQ) ranged in the interval of 0.1-2.45 ng/L. The recovery rates obtained for spiked samples were between 57.4 and 105.2%, proving that the method is accurate. The linear regressions (1-100ng/L) used to calibrate the LC-MS/MS system had determination coefficients higher than 0.99. The method was precise having good intra-day precision (3.5-7.2%) and inter-day precision (6.5-12.7%). The most contaminated river was represented by Ialomita, which had a total concentration of antidiabetics of 112.1 ng/L in the downstream point, followed by the Siret and Dambovita rivers, which had a total concentration of antidiabetics of 66.3 ng/L and 57.3 ng/L, respectively, also in the downstream points.

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