OCCURRENCE OF METFORMIN AND GUANYLUREA IN WASTEWATERS IN GREECE

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ABSTRACT

Many pollutants such as pharmaceuticals and their transformation products (TPs) are not efficiently removed from wastewater treatment plants and enter into surface waters. However, antidiabetic drugs, which are among the most widely used pharmaceutical compounds, have received less attention. The number of people suffering from diabetes, a very common glucose-metabolic disease, accounts for more than 200 million on a worldwide scale. Hence, Antidiabetic drugs are widely used for the treatment of this chronic disease and consequently, they are continuously being released into the environment through wastewater discharges from wastewater treatment plants. Interestingly, despite their high prescription rates and consumption volumes, up to date, little work has been conducted on the presence of antidiabetics in waters and wastewaters.

The aim of this study was to investigate the occurrence and behavior of metformin, one of the most prescribed drugs worldwide, and its biological transformation product guanylurea, in eight wastewater treatment plants (WWTPs) of Greece. All WWTPs were equipped with conventional activated sludge treatment and the samples were taken from the influents and the effluents, over the four seasons of one year. The analytical method developed based on SPE followed by LC-UV/Vis-ESI/MS analysis, while positive findings were confirmed also by means of LTQ Orbitrap mass spectrometer. High polarity of both compounds led to the extraction with Oasis HLB and the use of the anionic surfactant SDS. The results showed that metformin dominated in the influents (bql-1167 ng/L), while guanylurea in the effluents (bql-627 ng/L) of the wastewater treatment plants, with Metformin/ Guanylurea ratio ranging between 0.88 and 81.3 in the influents and between 0.005 and 0.78 in the effluents. Lack of a clear seasonal tendency in the occurrence and removal or formation was observed. Finally, an ecotoxicological risk assessment of metformin in effluent wastewaters took place by calculating the ratio between the environmental concentrations (MEC) and the predicted no effect concentrations (PNEC). Despite the fact that metformin presented low risk in all cases, an environmental concern is suspected for guanylurea since it is continuously released into the aquatic environment.

Keywords: Metformin, guanylurea, occurrence, removal, wastewaters, risk assessment

1. Introduction

In recent years, there have been a number of research articles and reviews dealing with the environmental occurrence, distribution and transport at national, European and worldwide scale of a vast array of pharmaceuticals in aquatic environment. In this light, many high production volume medicines like b-blockers, analgesics, antibiotics, lipid regulators, anti-inflammatories, and X-ray contrasts etc have been increasingly documented in groundwater, surface waters and wastewaters (Gros *et al*., 2013; Kosma *et al*., 2014). However, antidiabetic drugs, which are among the most widely used pharmaceutical compounds, have received less attention. The number of people suffering from diabetes, a very common glucose-metabolic disease, accounts for more than 200 million on a worldwide scale. Hence, Antidiabetic drugs are widely used for the treatment of this chronic disease and consequently, they are continuously being released into the environment through wastewater discharges from wastewater treatment plants. Interestingly,

despite their high prescription rates and consumption volumes, up to date, little work has been conducted on the presence of antidiabetics in waters and wastewaters. Expectantly, the most attention has been given to metformin, which is the first line drug of choice used for the treatment of diabetes mellitus type II and is excreted non metabolized in the urine. In some countries metformin is in the top twenty list of prescribed, produced and environmentally loaded pharmaceutically active compounds and it is ranked number one in mass loading (1.10×10^6 kg/yr). Its consumption increased in Netherlands and in Western Europe, with 26% between 2008 and 2012 and is expected to grow in the near future (ter Laak and Baken, 2014; Kosma *et al*., 2015).

In view of the scarce data on the occurrence of antidiabetics in the aquatic environment, the aim of this work was: (1) to comprehensively investigate the simultaneous occurrence of metformin and its aerobic, bacterial dead-end TP, guanylurea, in eight WWTPs in Greece, by means of LC-UV/ Vis-ESI/MS and LC-MS/LTQ Orbitrap systems, during one year monitoring program, (2) to evaluate the removal efficiencies across various types of WWTPs, (3) to provide a risk analysis in order to assess and compare the potential environmental risk of various types of wastewaters (hospital and municipal effluents) towards different aquatic organisms, (algae, daphnids, fish) through the calculation of risk quotients. To the present state of knowledge this is the first time that the simultaneous occurrence of these substances, is investigated in Greek aquatic environment and especially in conventional wastewater treatment plants.

2. Materials and methods

2.1. Sampling and Sample Preparation

Influent and effluent samples were collected along 2010-2011. They were obtained from eight WWTPs (7 municipal WWTPs and 1 hospital WWTP), from various cities (Ioannina City, Ioannina Hospital, Arta, Preveza, Agrinio, Grevena, Kozani, Veroia), in N.W. Greece (Epirus, Macedonia, Aitoloakarnania). All municipal WWTPs investigated are equipped with primary treatment (grit removal) and conventional activated sludge secondary treatment with denitrification/ nitrification, removal of phosphorous and a final disinfection step. All samples were taken over a 24-h period (composite samples) (Kosma *et al*., 2014).

Oasis HLB cartridges were preconditioned with 5 mL of methanol, 5 mL of HPLC-grade water and 5 mL of aqueous 2 mM SDS solution. Next, 400 mL of the sample, without pH adjustment, were loaded into the cartridge, at a flow rate of 10 mL/min, washed with 5 ml of HPLC-grade water and dried under vacuum for 10 min. The analytes were eluted with 2 x 5 mL of methanol at 1 mL/min. The extracts were dried over anhydrous sodium sulphate and then under a gentle stream of nitrogen until dryness and finally, reconstituted with 0.5 mL of methanol:water, 50:50 (v/v) and stored at -20 ºC until being analyzed.

2.2. Instrumentation

An LC-UV/Vis-ESI/MS system from Shimadzu (Kyoto, Japan), was used for the determination of metformin and guanylurea in wastewaters. For the analysis of the samples, ESI interface in positive ionization (PI) mode was used and the mobile phase contained of solvent (A) water with 0.1% formic acid and solvent (B) acetonitrile with 0.1% formic acid using gradient as follows: Initial conditions 70% A, kept constant for 1 min, decreased to 40% in 4 min, decreased to 0% in 4 min, returns to the initial conditions after 1 min and re-equilibration time was set at 2 min. The flow rate was 0.5 mL/min and the column temperature was set at 40 ºC. Quantitative analysis was performed in selected ion monitoring (SIM) mode and for each compound the precursor molecular ion [M + H], and at least one confirming ion was acquired (Kosma *et al*., 2014).

Next, for further confirmation of positive findings of metformin and guanylurea, liquid chromatography coupled to a high resolution Orbitrap mass spectrometer was used (Thermo Fischer Scientific, Bremen, Germany). For the identification of the compounds, their retention time, relative to that of the standards, as well as their accurate mass (theoretical mass was matched with the observed mass, with mass window <5 ppm) were taken into consideration. For the analysis in PI mode the mobile phase contained of solvent (A) water with 0.1% formic acid

and solvent (B) methanol with 0.1% formic acid using gradient as follows: Initial conditions 100% A, decreased to 90% in 2 min, decreased to 60% in 5 min, then decreased to 0% in 2 min, remain 0% for 1 min and finally returns to the initial conditions after 2 min with the re-equilibration of the column set at 3 min. The flow rate was set at 0.45 mL/min (Kosma *et al*., 2014). MS/MS experiments were carried out at 35 eV.

2.3. Validation studies

All the validation and quality assurance/quality control studies performed according to Kosma *et al*. (2014). Matrix matched calibration curves were used for quantification, using influent and effluent SPE extracts. Firstly, blank samples of effluent and influent wastewaters were analysed and positive findings were subtracted from the spiked samples. LODs were 3.9 ng/L and 4.6 ng/L in distilled water, 7.8 ng/L and 6.2 ng/L in influent wastewater and 5.1 ng/L and 9.8 ng/L, in effluent wastewater, for metformin and guanylurea, respectively. Both compounds presented signal suppression, which means that the signal intensity of the analytes decreased due to the ionization of various coeluting substances. Recoveries of the target compounds were determined for different matrixes (distilled, influent and effluent wastewaters) by spiking the samples with the analytes at concentrations of 0.2 and 2 mg/L. As influents and effluents might contain already the target analytes, concentrations of the respective unspiked samples were subtracted from concentrations of the spiked samples and then divided by the spiked level. Mean recoveries ranged between 38.3% in distilled water for metformin and 59.4% in effluent wastewater for guanylurea, when spiking with 0.2 mg/ L and between 40.7% in distilled water for metformin and 62.2% in influent wastewater for metformin, when spiking with 2 mg/L.

3. Results and discussion

Both compounds were present in the influents. Metformin was a very abundant compound in the influents found in the 97% of the samples analyzed. Influent concentrations of metformin ranged in municipal WWTPs from bql to 573 ng/L, presenting maximum concentrations in the WWTPs of Veroia (496 ng/L) and Arta (357 ng/L), while the lowest concentrations were observed in the WWTPs of Preveza (38 ng/L) and Agrinio (54 ng/L). In the Hospital WWTP concentrations were found between bql and 1167 ng/L demostrating that hospitals can be considered hotspot sources for pharmaceutical emission in the aquatic environment.

On the other hand, guanylurea was present in the 69% of the influent samples, at relatively low concentrations ranging in municipal WWTPs between bql and 59 ng/L. This may indicate, in most of the cases, the fact that the long distance between the cities and the WWTPs results in the transformation of metformin by bacteria that already exist in the sewer system (Trautwein and Kümmerer, 2011). The occurrence of metformin and guanylurea in the influents was also confirmed by the Orbitrap mass analyzer (Figure 1).

From the results obtained in the present study, in most of the cases, the increase of guanylurea concentrations in the effluents, matches the decrease of metformin concentration in the effluents. Guanylurea was found to be very stable in photolysis and as a consequence in sunlight or technical irradiation in advanced water treatment, indicating that is not expected to be eliminated in wastewater and advanced water treatment (Trautwein and Kümmerer, 2011).

Removal efficiencies of metformin ranged between 78% and 99%. The WWTPs were operating normally during all sampling events, and generally comparable removal efficiencies were observed in almost all of them, indicating small variations with respect to different capabilities of the treatment technologies (clarification, activated sludge, P removal etc) and hydraulic retention times (HRT) (1.5-39 h depending on the technology).

On contrary to removals of metformin, noticeable differences were observed for the formation of guanylurea through the tested WWTPs. Formation ranged between 17 and 95%, observing the highest in hospital of Ioannina city, Grevena and Veroia WWTPs. In general, the high abundance of guanylurea in effluent samples demonstrates the high degree of metformin biotransformation in WWTPs.

Risk Quotient (RQ) values calculated for metformin for acute toxicity, in three trophic levels, showed that in all effluents RQ < 0.001 which means that low risk is suspected for the three trophic levels in the receiving water bodies of the eight WWTPs. This was due to the fact that low effluent concentrations of metformin were detected in all cases since its removal rates were very high. To the authors knowledge this was the first time that ecotoxicology of metformin in the effluents of WWTPs was assessed in Greek aquatic ecosystem.

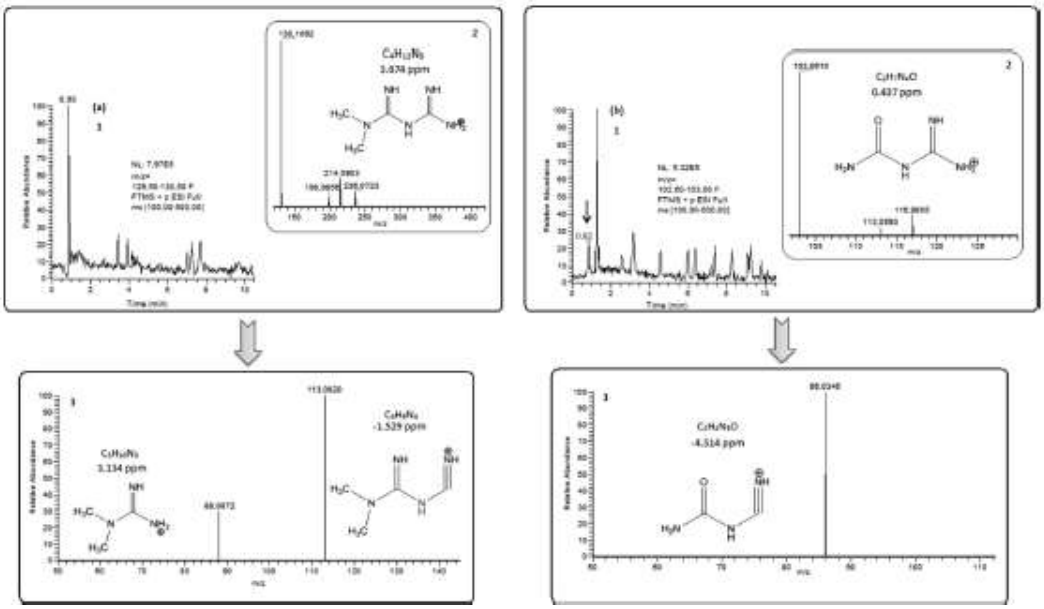

Figure 1: (1) Chromatogram, (2) full scan accurate mass product ion spectrum and (3) MS/MS data obtained using Orbitrap MS targeting the corresponding ions, for (a) metformin and (b) guanylurea, respectively, found in the influent of Ioannina hospital WWTP in winter.

4. Conclusions

According to the results the following conclusions can be reached:

- ✓ The extensive work demonstrated the occurrence of metformin and guanylurea in the influents and the effluents of all WWTPs.
- ✓ Concentrations ranged between bql and 1167 ng/L in the influents and between bql and 627 ng/L, in the effluents.
- ✓ High concentrations of metformin in the influents resulted in high concentrations of guanylurea in the effluents.
- ✓ Removal efficiencies of metformin were up to 99%, while formation of guanylurea reached 95%.
- ✓ Elimination of metformin can be mainly related to its biological formation to guanylurea but also some other abiotic elimination pathways may exist such as the faculty of metformin to stable complexes with heavy metals.
- ✓ Ecotoxicology assessment of metformin in the effluents took place and the results indicated low acute risk for water bodies.

AKNOWLEDGEMENTS

This research has been co-financed by the European Union (European Social Fund e ESF) (MIS 346819) and Greek national funds through the Operational Program “Education and Lifelong Learning” of the National Strategic Reference Framework (NSRF) e Research Funding Program: Heracleitus II. Investing in knowledge society through the European Social Fund. Furthermore, the authors would like to thank the Unit of Environmental, Organic and Biochemical high resolution analysis- ORBITRAP-LC-MS of the University of Ioannina for providing access to the facilities.

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