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Ecotoxicological effects, water quality standards and risk assessment for the anti-diabetic metformin☆,☆☆

Aline Andrade Godoy [a, b], Inês Domingues [c], António José Arsénia Nogueira [c], Fábio Kummrow [a, d, *]

[a] Department of Clinical and Toxicological Analysis, Faculty of Pharmaceutical Sciences, University of São Paulo (USP), Avenida Professor Lineu Prestes, 580, São Paulo, SP, 05508-000, Brazil
[b] Science and Technology Institute, Federal University of Alfenas (Unifal-MG), Rodovia José Aurélio Vilela, 11,999, Poços de Caldas, MG, 37715-400, Brazil
[c] Department of Biology & CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193, Aveiro, Portugal
[d] Institute of Environmental, Chemical and Pharmaceutical Sciences, Federal University of São Paulo (Unifesp), Rua São Nicolau, 210, 09972-270, Diadema, SP, Brazil

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ABSTRACT

Metformin (MET) is among the most consumed pharmaceuticals worldwide. This compound has been frequently detected in fresh surface water. However, ecotoxicological information for MET is still too limited, particularly regarding chronic and behavioral data. This study aimed to help filling these knowledge gaps, by carrying out both acute and chronic studies with four different test organisms from three different trophic levels. We assessed different endpoints, including the swimming behavior of *Danio rerio* larvae. We also derived both short-term and long-term environmental quality standards (EQS) for the protection of freshwater pelagic biota towards MET adverse effects. A risk quotient (RQ) was calculated for MET in fresh surface water, considering a worst-case scenario. *Daphnia similis* was by far the most sensitive species evaluated. An EC_{10} of 4.4 mg L^{-1} was obtained from the reproduction test with *D. similis*. A long-term EQS of 88 μg L^{-1} was derived and a RQ of 0.38 was obtained. An ecological risk is not expected for the chronic exposure of pelagic freshwater species to MET, considering the endpoints and the standard bioassays usually recommended in standard protocols. However, endocrine disruptive effects and potential interactive effects of MET with other co-occurring contaminants cannot be ruled out. To the best of our knowledge, this study presents the first data related with MET effects on population endpoints of *D. similis* and *Hydra attenuata*, as well as on the locomotor activity of *D. rerio*.

1. Introduction

Active Pharmaceutical Ingredients (APIs) are among the contaminants considered of emerging concern by the scientific community due to their potential environmental risk (Godoy and Kummrow, 2017). Of special environmental relevance are the pharmaceuticals sharing the properties of (1) high production volume; (2) environmental persistence; and (3) biological activity, especially considering long-term exposure (Fent et al., 2006). The anti-diabetic metformin (MET) appears to be a potential candidate to meet these requirements.

The biguanide MET is the first-line oral therapy and the most widely used oral agent prescribed for type 2 diabetes (Foretz et al., 2014; Rena et al., 2013). This drug also presents one of the highest consumption rates of all pharmaceuticals worldwide (Scheurer et al., 2012). Rena et al. (2013) estimate that over 100 million patients are prescribed MET annually worldwide. Moreover, it is considered to be one of the APIs with the largest emissions into the environment on a mass basis from wastewater treatment plants (WWTP) (Crago et al., 2016; Dong et al., 2013; Kosma et al., 2015; Scheurer et al., 2009). These findings are partly due to the increasing number of people affected by diabetes mellitus.

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☆☆ The ecotoxicity data for metformin presented in this study contribute to fill an important knowledge gap and allowed us to derive water quality standards for the protection of freshwater pelagic biota.

* Corresponding author. Institute of Environmental, Chemical and Pharmaceutical Sciences, Federal University of São Paulo (Unifesp), Rua São Nicolau, 210, 09972-270, Diadema, SP, Brazil.
E-mail address: fkummrow@unifesp.br (F. Kummrow).

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Projection from the International Diabetes Federation points out that the number of people with diabetes worldwide will increase from 415 million in 2015 to 642 million by 2040 (IDF, 2015).

It must be also highlighted the large quantities of MET required for therapeutic effects, with daily dosage varying from 500 to up to 2500 mg L^{-1} (Rena et al., 2013; Trautwein and Kümmerer, 2011). In addition to its high consumption, MET is excreted unaltered in the urine (Bailey and Turner, 1996), which makes the emissions of this pharmaceutical after consumption relevant. Trautwein et al. (2014) highlighted the high rates of removal of MET in WWTP (93–97%), mainly due to its microbiological transformation into guanylurea. Despite of these high removal rates, this compound has been detected at relatively high concentrations in effluent and surface waters due to its high influent load (Oosterhuis et al., 2013). Moreover, since MET lacks functional groups that hydrolyze under environmental conditions, hydrolysis is not likely to occur with this compound (ter Laak and Baken, 2014). As a consequence, the aquatic organisms may be exposed to considerable concentrations of this API. In fact, unexpected high concentrations of this hydrophilic pharmaceutical (27.8 ng g^{-1}) were observed in sculpin fishes (*Leptocottus armatus*) from the Nisqually estuary (Meador et al., 2016).

Regarding the biological activity, MET acts by inhibiting complex I in the mitochondrial electron transport chain in humans, leading to adenosine triphosphate (ATP) depletion and an increase in adenosine monophosphate (AMP) levels (Foretz et al., 2014; Rena et al., 2013). Although these same mechanisms remain relatively unexplored on non-target organisms, the crucial involvement of mitochondria in the molecular mechanism of action of MET could raise an alert on its potential effects also on this organelle of aquatic invertebrates and vertebrates, following the concept of evolutionary conserved molecular drug targets. In fact, Pinho et al. (2013) showed that zebrafish and mammalian mitochondria display high genetic and functional homology and proved that mitochondrial inhibitors, such as antimycin, myxothiazol, rotenone and oligomycin, induced developmental and cardiovascular dysfunctions in this fish species. Therefore, the possible adverse effects of MET on non-target organisms deserve special investigation.

However, despite its high production, prescription, environmental load and persistence, information on ecotoxic effects of MET is still scarce, especially taking into account long-term effects, which hampers interpretation of its risk (ter Laak and Baken, 2014). Therefore, ter Laak and Baken (2014) recommend filling this knowledge gap in order to allow that proper interpretation of monitoring results and environmental risk assessment (ERA) for this pharmaceutical can be achieved.

In this sense, the present study aimed to assess the ecotoxicological effects of MET, in short and long-term studies with organisms of different trophic levels and including different endpoints of proved population relevance. The swimming behavior was also considered in our study, by monitoring the effects of MET on the locomotor activity of *Danio rerio* (zebrafish). Behavior has been demonstrated to give rise to very sensitive measures of stress exposure (Andrade et al., 2016; Henriques et al., 2016). Moreover, locomotor behavior has a relevant connection with survival of populations (Scott and Sloman, 2004). In the *D. rerio* case, we hypothesized that possible physiological dysfunctions via ATP depletion, already shown to be induced by mitochondrial complex I inhibitors such as rotenone (Pinho et al., 2013), could ultimately impact locomotor activity of zebrafish exposed to MET.

Moreover, by assessing several endpoints from standard (*Lemna minor*, *Daphnia similis* and *D. rerio*) as well as a non-standard species (*Hydra attenuata*), we aimed to enlarge the ecotoxicological database regarding MET adverse effects. Based on our results and on reports from the literature, we also aimed to derive environmental quality standards (EQS) for the protection of freshwater pelagic biota towards MET, based on relevant and reliable ecotoxicity tests. We finally aimed to assess the environmental risk posed by MET, considering a worst-case scenario.

2. Materials and methods

2.1. Chemicals

Metformin hydrochloride (1,1-Dimethylbiguanide hydrochloride; CAS number 115-70-4), was provided by Abhilasha Pharma (India), with 99.2% purity. Stock solutions and tested concentrations were achieved by dissolving MET in the appropriate test medium for each organism without using any solvent. All compounds used for composition of the respective test medium were of high purity (>98%), supplied by Sigma-Aldrich (Brazil) or by Merck (Germany).

2.2. Analytical determination of MET in the test medium

In order to confirm the nominal concentrations of MET used in the chronic tests, chemical analyses were performed using a double beam UV-visible spectrophotometer (Cintra 6, GBC scientific equipment). The methodology used for the analyses was in accordance with the procedure described in the U.S. Pharmacopoeia (USP, 2015). Prior to the analysis, spectral scans were performed at the concentration of 10 mg L^{-1} of MET, dissolved in each culture medium as well as in distilled water, in order to confirm the maximum absorption peak established by the U.S. Pharmacopoeia for distilled water. The peak of absorbance of 232 nm for MET was used for quantification, using the respective test medium as a blank. The limit of detection (LOD) and limit of quantification (LOQ) for the analyses of MET were determined, respectively, by the following mathematical formulas:

$$\mathrm{LOD} = (3.3\,s)/\mathrm{S} \quad (1)$$

$$\mathrm{LOQ} = (10\,s)/S \quad (2)$$

in which s = the estimate of the standard deviation of the blank samples (n = 10) and S = the slope of the calibration curve.

The parameters obtained for the calibration curves (linearity, determination coefficient and equations) as well as the analytical results are described in Appendix A of Supplementary material. The quantification of MET in Steinberg medium was not possible, due to the noise of the baseline observed for the analysis of the Steinberg medium at the 232 nm. However, previous stability tests were carried out for the MET in distilled water, at the same conditions of exposure time, temperature, and luminosity as those used in the *L. minor* toxicity tests (Table A.5 of Appendix A of Supplementary material) and proved the stability of this pharmaceutical during the 7-d exposure time.

2.3. Test organisms

L. minor, *D. similis* and *H. attenuata* test organisms were provided by the Laboratory of Ecotoxicology and Genotoxicity (LAEG), State University of Campinas, Unicamp (Brazil). *L. minor* plants were maintained in Steinberg medium (OECD, 2006), pH 5.5 ± 0.2, conductivity 900 ± 50 μS cm^{-1}, at 24 ± 2 °C and under continuous cool white fluorescent lighting with light intensity of 6500 lux. Young plants without visible lesions or discoloration (chlorosis) were selected for the tests using a magnifying glass.

D. similis were cultivated in MS medium (ABNT NBR 12713, 2016), hardness 40–48 mg L^{-1} $CaCO_3$, conductivity 200 ± 20 μS