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Comprehensive determination of macrolide antibiotics, their synthesis intermediates and transformation products in wastewater effluents and ambient waters by liquid chromatography–tandem mass spectrometry

Ivan Senta, Ivona Krizman-Matasic, Senka Terzic*, Marijan Ahel

Division for Marine and Environmental Research, Rudjer Boskovic Institute, Bijenicka c. 54, 10000 Zagreb, Croatia

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ABSTRACT

Macrolide antibiotics are a prominent group of emerging contaminants frequently found in wastewater effluents and wastewater-impacted aquatic environments. In this work, a novel analytical method for simultaneous determination of parent macrolide antibiotics (azithromycin, erythromycin, clarithromycin and roxithromycin), along with their synthesis intermediates, byproducts, metabolites and transformation products in wastewater and surface water was developed and validated. Samples were enriched using solid-phase extraction on Oasis HLB cartridges and analyzed by reversed-phase liquid chromatography coupled to electrospray ionization tandem mass spectrometry. The target macrolide compounds were separated on an ACE C18 PFP column and detected using multiple reaction monitoring in positive ionization polarity. The optimized method, which included an additional extract clean-up on strong anion-exchange cartridges (SAX), resulted in high recoveries and accuracies, low matrix effects and improved chromatographic separation of the target compounds, even in highly complex matrices, such as raw wastewater. The developed method was applied to the analysis of macrolide compounds in wastewater and river water samples from Croatia. In addition to parent antibiotics, several previously unreported macrolide transformation products and/or synthesis intermediates were detected in municipal wastewater, some of them reaching μg/L levels. Moreover, extremely high concentrations of macrolides up to mg/L level were found in pharmaceutical industry effluents, indicating possible importance of this source to the total loads into ambient waters. The results revealed a significant contribution of synthesis intermediates and transformation products to the overall mass balance of macrolides in the aquatic environment.

1. Introduction

In the last 20 years, a large number of studies [1–5] reported on widespread occurrence of antimicrobial compounds in wastewater and in the aquatic environment. Since the spread of antibiotic resistance is considered to be one of the most serious global threats to human health in the upcoming decades [6], the ubiquitous presence of antibiotic residues in the environment raised a great concern about possible contribution of the aquatic route to proliferation of the resistant bacterial strains [7]. In order to meet the needs of reliable and sensitive assessment of antibiotic exposure concentrations in different matrices, a number of analytical methods for the determination of antimicrobials in environmental samples have been described in the literature over the past decade. Many of the published methods are multiresidual and multiple-class methods, which typically include a smaller number of selected representatives from each antimicrobial class [8,9]. Moreover, the majority of the published methods have focused exclusively on the parent compounds [5], or, at best, include some major metabolites [10,11]. As a consequence, the contribution of transformation products (TPs), as well as synthesis intermediates and byproducts, to the total mass loads of antimicrobial-related compounds in the environment is much less understood.

Macrolide antibiotics represent one of the most prominent classes of antimicrobial agents, with widespread usage in both human and veterinary medicine. In the global antibiotic consumption in 2010, macrolides were ranked third [12], and their usage in human medicine in Croatia and other European countries [13] is in accordance with the global figures. Moreover, together with

* Corresponding author.
E-mail address: terzic@irb.hr (S. Terzic).

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penicilins, macrolides have reached the highest global sales among veterinary antimicrobials ($600 million in 2009) [14].

After therapeutic use, the macrolide antibiotics are released from the human body via urine and feces as a mixture of unchanged parent compound and various metabolites [15]. In addition, several literature reports [16–21] indicated that macrolides can be transformed in the environment either via microbial or photochemical degradation, yielding a number of TPs, mainly by *N*-demethylation, *O*-demethylation and hydrolytic cleavage of sugar residues. Nevertheless, most of the existing exposure data refer only to the parent macrolides, while the information on possible synthesis intermediates and/or TPs in the aquatic environment are still very scarce. In fact, to the best of our knowledge, none of the published methods for the quantitative determination of macrolide compounds in environmental samples tackled the problem of synthesis intermediates and byproducts. Our previous study, which focused on comprehensive analysis of freshwater sediments chronically polluted by wastewaters from the pharmaceutical industry [22], indicated that azithromycin synthesis intermediates can be found in significant concentrations along with high concentrations of the parent compound. Moreover, TPs of azithromycin and roxithromycin, formed by microbial phosphorylation, were identified in wastewater effluent of the membrane bioreactor [23]. However, due to the lack of methods for quantitative determination of macrolide synthesis intermediates and TPs, their contribution to the overall macrolide loads in wastewater effluents and in the aquatic environment could not be properly assessed.

The aim of this work was therefore to develop and validate a novel analytical method for comprehensive analysis of macrolide compounds in aqueous samples, with a special emphasis on azithromycin- and erythromycin-derived compounds. The relevance of this analytical approach will be demonstrated by the analysis of municipal wastewater, pharmaceutical industry effluent and receiving river water. Thus, the method is expected to provide a necessary tool for a more detailed assessment of the occurrence and fate of macrolide compounds in wastewater treatment and in the freshwater environment.

2. Experimental

2.1. Chemicals and materials

All analytes and internal standards, together with their abbreviations, are listed in Table 1, while their structures and properties can be found in Supplementary material (Table S1). AZI, EIE, EOX and CLA were kindly supplied by Pliva (Zagreb, Croatia). ROX was purchased from Sigma-Aldrich (Germany), while all other reference materials of macrolide compounds were supplied by Toronto Research Chemicals (Canada). AZI-PO_4 was prepared in our laboratory by microbial transformation of AZI (details can be found in Supplementary material with Fig. S1). The reference standard of CLA-OH was isolated from the urine of a patient treated with CLA (details can be found in Supplementary material with Fig. S2) and was used only as a qualitative standard.

Individual stock solutions of the target analytes, except AZI-PO_4, were prepared in methanol, at the concentration level of either 1 mg/mL or 2 mg/mL and stored at −20 °C. Working standard solutions, containing all analytes at 10, 1 and 0.1 μg/mL, were prepared by diluting the stock solutions with methanol and stored at 4 °C. The mixture of internal (surrogate) standards (AZI-d_3 and CLA-d_3) was also prepared in methanol at the concentration level of 5 μg/mL. HPLC grade methanol (MeOH) and acetonitrile (ACN) were delivered by BDH Prolabo (UK). Formic acid and ammonium formate were purchased from Sigma-Aldrich (Germany), while 25% ammonia solution in water was supplied by Merck (Darmstadt, Germany). Ultrapure water was produced using Elix-Milli-Q system (Millipore, Bedford, MA, USA).

Solid-phase extraction (SPE) cartridges Oasis HLB (200 mg/6 mL) and Oasis MCX (150 mg/6 mL) were supplied by Waters (Milford, MA, USA), while SPE cartridges for an additional extract clean-up, Strata SAX (100 mg/3 mL), were purchased from Phenomenex (Torrance, CA, USA). Glass-fiber filters (GF/D) were delivered by Whatman (UK).

2.2. Sample collection and preparation

All raw wastewater (RW) and secondary effluent (SE) samples for the method development and validation were collected at the central wastewater treatment plant (WWTP) of the city of Zagreb. Industrial effluent was collected at the discharge point of the pharmaceutical industry into the Sava River, near the city of Zapresic. River water sample for the method validation was collected from the Sava River upstream from the discharge point, while the samples for the preliminary survey were collected at two additional locations downstream of the discharge point. All samples were processed within 24 h after collection. Typical sample volumes were 100 mL, 200 mL, 250 mL and 1 mL for RW, SE, river water and industrial effluent samples, respectively. After the filtration through glass-fiber filters, the mixture of surrogate standards (100 ng of each) was added and pH was adjusted with formic acid if necessary. In the preliminary experiments, two types of SPE cartridges were tested: Oasis HLB and Oasis MCX. In the final procedure, samples were extracted at the original pH (7–7.5) using HLB cartridges, previously conditioned with 6 mL of methanol, 6 mL of ultrapure water and 6 mL of spring water, at the flow rate of approximately 5 mL/min. Cartridges were then washed with 6 mL of ultrapure water and 2 mL of H_2O/MeOH (80/20, *v*/*v*), dried under the nitrogen stream and eluted with 4 mL of methanol at the flow rate of ≤1 mL/min. If the extracts were not analyzed on the same day, they were stored at −20 °C. The SPE procedures tested in the preliminary experiments, using either HLB cartridges or MCX cartridges, are described in Supplementary material.

Before the instrumental analysis, extracts were additionally cleaned up using strong anion-exchange cartridges Strata SAX. Extracts were loaded onto the cartridges previously conditioned with 3 mL of MeOH and eluates were collected in the glass tubes. The cartridges were additionally eluted with 4 mL of MeOH. The eluates were combined and evaporated to dryness under N_2 using a TurboVap evaporator (Caliper Life Sciences, Hopkinton, MA, USA). The residue was re-dissolved in 0.5 mL of 100 mM ammonium formate/MeOH (1/1, *v*/*v*) for instrumental analysis.

2.3. Instrumental analysis

Samples were analyzed using liquid chromatography–tandem mass spectrometry (LC–MS/MS) on a Thermo Electron TSQ AM (San Jose, CA, USA) LC–MS/MS system, which consisted of HPLC pump and autosampler (Surveyor) interfaced to a triple quadrupole mass spectrometer equipped with an electrospray ionization source.

Four HPLC columns were tested for the separation of target compounds during the method development: YMC Pro C18 (150 mm × 2,1 mm; 3 μm), Gemini C18 (150 × 3 mm; 3 μm), Synergi Hydro RP 80 Å (150 × 3 mm; 4 μm) and ACE C18 PFP (150 × 3 mm; 3 μm). Optimal separation was achieved on ACE C18 PFP column, by using gradient elution with 0.1% formic acid in water (*v*/*v*, eluent A) and acetonitrile (eluent B) at a flow rate of 0.4 mL/min. The gradient was as follows: 0–10 min from 15% to 30% B; 10–30 min from 30% to 45% B; 30–31 min from 45% to 95% B; 31–34 min 95% B (isocratic hold), 34–35 min from 95% B to 15% B; 35–45 min from 95% to 15% B (reconditioning to initial conditions). The injection volume was 20 μL.