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A new method for macrolide antibiotics determination in wastewater from three different wastewater treatment plants

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Abstract: An effective and practical method for the determination of macrolide antibiotics azithromycin, clarithromycin, erythromycin and roxithromycin in wastewater samples has been developed. The analytical method combines solid phase extraction followed by a chromatographic separation by hydrophilic interaction liquid chromatography (HILIC) coupled with an ion trap mass spectrometer utilizing the electrospray ionization technique. Detection of positively charged ions was performed in full scan mode from 500 to 900 m/z. The method detection limits and method quantification limits obtained were in the range of 2.03—7.59 ng L^{-1} and 6.08—23.84 ng L^{-1}, respectively. Recoveries of solid phase extraction were obtained using Supel™-Select HLB cartridges ranging from 85.76 % to 92.54 %. All target antibiotics were detected in 100 % of the collected raw influent samples with concentrations varying from 15 ng L^{-1} to 1849 ng L^{-1}. Azithromycin, clarithromycin and erythromycin were also detected in 100 % of the treated water samples and roxithromycin was present in 96 % of the samples. The highest determined concentration in the treated water samples was 1404 ng L^{-1} of azithromycin. Based on the determined macrolide concentrations, removal efficiencies of individual wastewater treatment plants were calculated to range from 13 % to 100 %.

Keywords: high-performance liquid chromatography (HPLC), macrolide antibiotics, mass spectrometry (MS), solid phase extraction (SPE), wastewater

Introduction

Antibiotics are drugs used in human and veterinary practice to treat diseases caused by microorganisms. They may also serve as growth promoters for livestock in veterinary medicine (Ding et al., 2008). Macrolide antibiotics are widely used for the treatment of infections caused by gram-positive and gram-negative bacteria such as Mycoplasmas and Chlamydia (Horie et al., 2003). These antibiotics represent a good substitute for patients with penicillin allergy. Chemical structure of macrolides (Figure 1) includes a macrocyclic lactone ring (14, 15 or 16 atoms in the ring), units of sugar and an amino sugar linked to the macrocyclic ring via a glycosidic bond. They have basic character and exhibit lipophilicity (Carlson and Yang, 2004).

After administration, the drugs are excreted from the body in form of inactive metabolites or unchanged via urine or faeces. These substances are then discharged into wastewater and in most cases enter the wastewater treatment plants (McArdell et al., 2005). Municipal wastewater treatment plant technology is designed to reduce the concentrations of suspended solids, organic carbon, heavy metals and nutrients such as nitrogen and phosphorus using a combination of mechanical, chemical and biological processes (Forster, 2003). These processes are not primarily designed for antibiotics elimination. They can negatively affect biological treatment processes where microorganisms are essential for proper function of the wastewater treatment plant (WWTP). As it has already been reported many times (Loganathan et al., 2009; Ibáñez et al., 2017; Pugajeva et al., 2017), the efficiency of antibiotics removal is not sufficient; thus, WWTPs are considered as major point sources of environmental contamination by these compounds. Their subsequent occurrence in surface water can also have adverse effects on aquatic organisms (Smyth et al., 2014; Rossmann et al., 2014). In some cases, antibiotics were found also in drinking water (Chen et al., 2016). The emergence of bacterial resistance to antibiotics is a considerable problem nowadays. The life cycle of bacteria is relatively short (approximately 20 minutes) and therefore the development of mutations is a very rapid process which results in higher resistance towards specific antibiotics (Wong and McClure, 2007). On the other hand, the development and clinical testing of new antibiotics is a matter of many years or decades.

It is necessary to develop appropriate analytical methods for the determination of environmental contamination by these pollutants. In most cases,

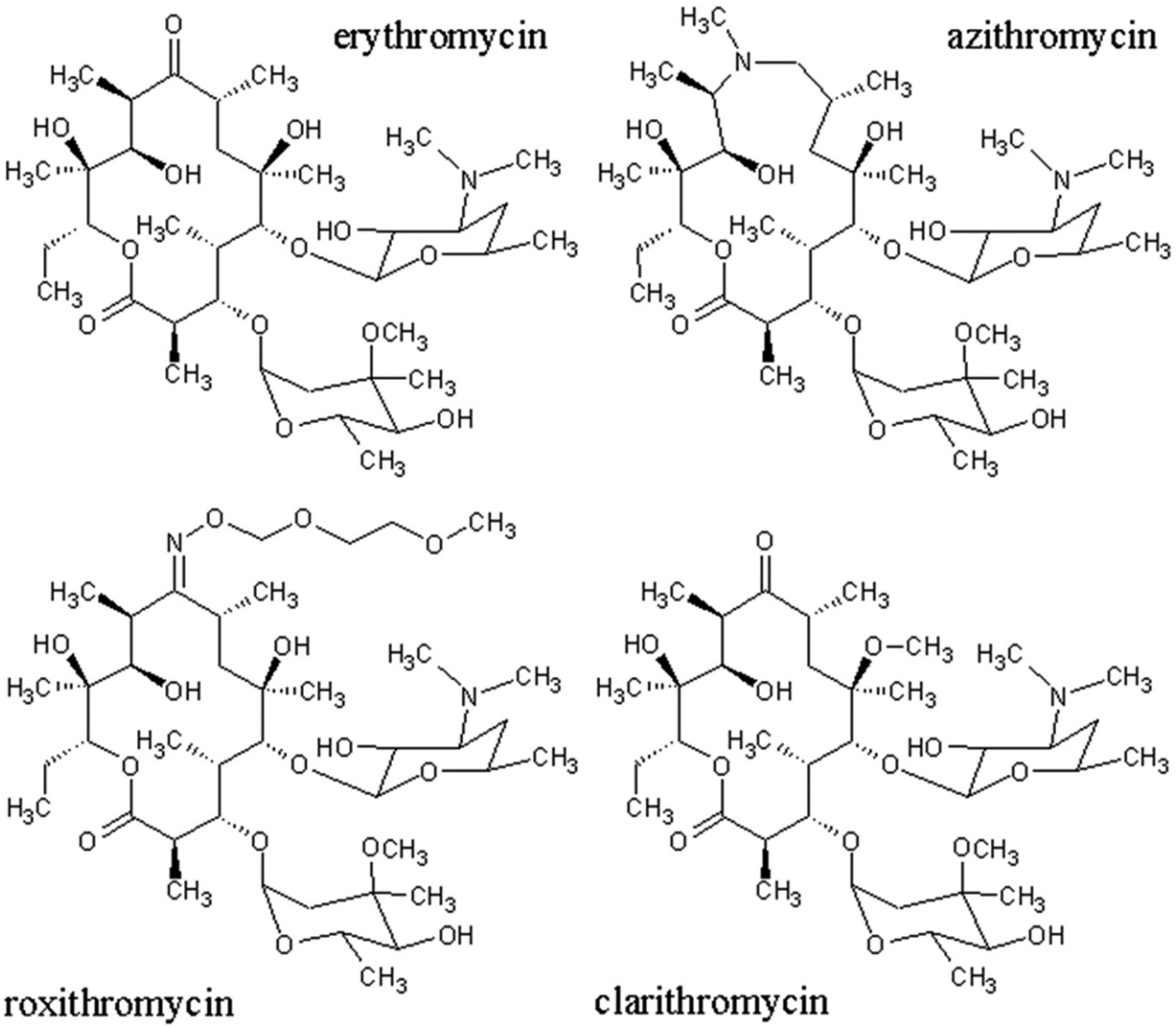

Fig. 1. Chemical structure of selected macrolide antibiotics.

high-performance liquid chromatography coupled with mass spectrometry with electrospray ionization is used for these purposes. However, low concentrations of these substances require a time-consuming sample preparation step such as solid phase extraction (SPE). SPE allows not only the pre-concentration of target analytes but also clean-up of the sample (Carlson and Yang, 2004; Compañó et al., 2006, Ding et al., 2009; Horie et al., 2003; Wong and McClure, 2007).

Only a small number of publications on the application of an HILIC column in the macrolide antibiotics determination are available. This technique works for high organic content in the mobile phase, which makes it more than suitable to be combined with mass spectrometry. Using bonded phase silica (for example C_{18}) in the analysis of basic compounds can be problematic mainly because of peak tailing or insufficient retention of these compounds. The use of solid core particles has also its advantages, especially higher separation efficiency and lower back pressure; e.g., a column with 2.7 µm solid core particles can be used in conventional HPLC systems and its performance is comparable to UHPLC systems equipped with a column with 1.8 µm fully porous particles.

Materials and methods

Chemicals and materials

Analytical standards of azithromycin dihydrate (97 %) and clarithromycin (99 %) were purchased from Dr. Ehrenstorfer GmbH (Germany). Standards of erythromycin (≥ 90 %) and roxithromycin (≥ 90 %) were obtained from Sigma-Aldrich (USA). Standard stock solutions with the concentration of 2 mg mL^{-1} were prepared by dissolving respective amounts of standards in acetonitrile. The prepared solutions were then stored in a refrigerator (not longer than for six months).

Acetonitrile LC-MS Chromasolv® (≥ 99.9 %) was purchased from Sigma-Aldrich (Germany), ammonium acetate p.a. (≥ 99 %) was provided by Fluka (Netherlands). Deionized water (MQ water) was produced by Milli-Q® Academic devices from Millipore (France). All other reagents were of analytical reagent grade. Macherey-Nagel GF-1 glass fiber filters (Germany) were used for water samples filtration and Cronus PTFE 0.45 µm syringe filters from Chromservis (Czech Republic) were used for SPE extracts filtration. Supel™-Select HLB 200-mg cartridges were purchased from Sigma-Aldrich (Germany).

Sample extraction

First, the samples were filtered through glass fiber filters and pH was adjusted to 7 using diluted solutions of ammonia or hydrochloric acid. Supel™-Select HLB columns with a 200 mg sorbent bed and cartridge volume of 6 ml were used for the extractions.

The columns were conditioned with 5 mL of acetonitrile followed by 5 mL of 10 mM CH_3COONH_4 (pH = 7). After the conditioning, 200 ml of the water sample were passed through the cartridge at the flow rate of 7.5 mL min^{-1}, the cartridge was washed with 3 ml of MQ water (pH = 7). The column was air-dried (15 minutes) and macrolides were eluted with two aliquots (2 mL) of acetonitrile. Then, the SPE extract was evaporated to dryness under a stream of nitrogen and finally dissolved in 1 mL of acetonitrile. Samples were then filtered through 0.45-µm syringe filters, transported to 2-ml glass vials and finally injected into the chromatographic system.

HPLC/MS analysis

The final analysis was carried out using a liquid chromatograph Agilent 1100 Series coupled with a mass spectrometer Agilent Ion Trap 6320 LC/MS from Agilent Technologies (USA) applying the electrospray ionization technique.

For chromatographic separation, column Ascentis® Express HILIC from Sigma-Aldrich (Germany) with the length of 150 mm, inner diameter of 2.1 mm and particle size of 2.7 µm was used. The column temperature was maintained at 50 °C and the injection volume was adjusted to 2.5 µL. Mobile phase contained an ammonia acetate buffer with pH = 6.7 (A) and acetonitrile (B), the flow rate was set to 0.6 mL min^{-1}. Initial composition of the mobile phase was set to 80 % of B, the composition linearly changed to 50 % of B from minute 3.0 to 5.0 and then to minute 6.0, the composition changed back to 80 % of B. The total analysis time was 11 minutes (6 minutes analysis and 5 minutes column equilibration). Mass spectrometric conditions were as follows: nebulizer pressure of 241.3 kPa (N_2); drying gas (N_2) flow rate of 12 L min^{-1}; drying gas temperature of 350 °C; positive ionization mode; full scan mode from 500 to 900 m/z.

Sampling campaign

For the study of antibiotics removal efficiency, the samples of the WWTPs influent and effluent were collected by a competent person. Samples were collected into clean dark glass bottles, stored in a refrigerator and processed within 24 hours. Grab samples were taken from the WWTP of the University of Veterinary and Pharmaceutical Sciences (UVPS), Brno, on April 7, 2016. Composite samples (24 hours) were taken from WWTP Mikulov on April 20 and 27, 2016. The ten days sampling period of composite samples (24 hours) was performed in WWTP Brno-Modřice from April 19 to April 29, 2016.

Technological specifications of monitored wastewater treatments plants

In this brief description, only basic characteristics of the selected WWTPs are presented, as well as the main differences between them.

WWTP of the University of Veterinary and Pharmaceutical Sciences, Brno

This plant is used for the pretreatment and disinfection of raw wastewater from the entire university campus. The facility consists of mechanical (primary) treatment and biological (secondary) treatment, including an activation unit with aeration. Before water enters the sewer system of the city of Brno, it is chemically disinfected by chlorine.

WWTP Mikulov

This plant works on mechanical-biological principles with the maximal projected capacity of 24 850 EI (equivalent inhabitant) and a hydraulic load of 5 184 m^3 day^{-1}. The biological treatment consists of an anoxic selector and an activation tank with fine bubble aeration. Microfiltration membrane system is used for tertiary treatment.

WWTP Brno-Modřice

This plant works also on mechanical-biological principles but the maximum capacity is much higher than that of the WWTP Mikulov (515 000 EI and 137 000 m^3 day^{-1}). The biological treatment consists of an activation tank with aeration, predenitrification and anaerobic dephosphatation.

Results and discussion

HPLC/MS method optimization

Parameters of the detection were optimized for every compound by direct infusion of a standard

solution (10 mg L^{-1}) in acetonitrile. Direct infusion was performed under the following conditions: flow rate of the sample solution of 5 µL min^{-1}; nebulizer pressure of 48.3 kPa (N_2); drying gas (N_2) flow rate of 15 L min^{-1}; drying gas temperature of 350 °C; positive or negative ionization mode; full scan mode from 100 to 1 000 m/z. Results showed that macrolides have a very good response in the positive ionization mode. Therefore, this mode was chosen as optimal. Protonated molecular ions ($[M+H]^+$) were most abundant in each macrolide mass spectrum; for azithromycin (AZI) it was 749.6 m/z, for clarithromycin (CLA) 748.5 m/z, for erythromycin (ERY) 734.5 m/z and for roxithromycin (ROX) 837.6 m/z.

The use of an HILIC column for the separation of these compounds is not as typical as the use of reverse phase columns such as C_{18} or C_8 bonded silica. However, these columns are appropriate especially for the separation of basic compounds because they can solve possible problems occurring when reverse phase columns are used, such as the lack of retention or peak tailing. Reverse phase column (C_{18} bonded silica) was also tested but because of bad peak shapes and insufficient limits of detection, the HILIC column was the best choice.

Under appropriate conditions, macrolides exhibit good peak shape, height and width; also total analysis time of 11 minutes makes the HPLC separation very short which is a benefit. Characteristic retention times (R_t) for each macrolide are listed in Table 1.

Tab. 1. Retention times of monitored analytes.

Compound	R_t [min]
AZI	2.8
CLA	2.8
ERY	3.0
ROX	2.7

Quantitative evaluation

For quantitative evaluation, a calibration line with the peak area and analyte concentration was plotted. The linear dynamic range was observed from 2.5 ng mL^{-1} to 400 ng mL^{-1}. Detection limits (DL) were calculated for every analyte from the lowest concentration point of calibration using equation (1) and quantification limits (QL) were obtained according to equation (2).

$$DL = 3 \cdot \frac{c}{S/N} \quad (1)$$

$$QL = 10 \cdot \frac{c}{S/N} \quad (2)$$

Where c is the concentration (in this case it was 2.5 ng mL^{-1}); S is the peak height for this concentration and N is the height of noise (evaluated as the average height of ten peaks near the analyte peak). Theoretically calculated values of the limits of detection and the limits of quantification were verified by an analysis of the prepared standard solutions with concentrations close to the calculated limits, RSD (n = 6) of detection limits were close to 12 % for every macrolide and those of quantification limits were close to 5 % for every macrolide. These values can be considered as instrument detection limits (IDL) and instrument quantification limits (IQL); they are presented together with regression equations and coefficients of determination in Table 2.

Tab. 2. Method validation parameters.

Compound	Regression equation	Coefficient of determination R^2	IDL [ng mL^{-1}]	IQL [ng mL^{-1}]
AZI	y = 359 619 x – 317 740	0.9992	0.70	2.20
CLA	y = 867 798 x – 25 942	0.9999	0.40	1.20
ERY	y = 701 534 x – 56 754	0.9999	0.60	2.00
ROX	y = 838 944 x – 765 101	0.9999	0.40	1.20

Tab. 3. Average SPE recoveries and RSD at both concentration levels.

Compound	75 ng L^{-1}		1 500 ng L^{-1}	
	Average recovery [%]	RSD [%]	Average recovery [%]	RSD [%]
AZI	88.13	3.29	90.13	2.11
CLA	86.87	4.74	89.58	2.72
ERY	85.76	2.97	86.18	1.17
ROX	92.54	2.84	90.79	2.33

However, the analytical procedure is not included in IDL and IQL (SPE and matrix effect evaluation). Method detection limits (MDL) and method quantification limits (MQL) after the SPE recovery evaluation and matrix effects assessment are summarized in Table 5.

SPE recoveries

In order to evaluate SPE recoveries, extractions of spiked MQ water were performed. The recoveries were tested at two different concentration levels: 75 and 1 500 ng L^{-1}.

Six repetitions were carried out for each concentration level in order to obtain validated values. Average recoveries and relative standard deviations (RSD) for both concentration levels are listed in Table 3. At both concentration levels, the obtained recoveries are very similar, for the results evaluation, average recovery values from the lower concentration level were used.

Matrix effect

Substances present in water can negatively or positively affect SPE and especially ionization in the ion source of the mass spectrometer. Therefore it is necessary to evaluate the matrix effect.

Matrix effect was examined in wastewater from influent and also in treated water from the effluent of the WWTP of UVPS Brno. Water was spiked with a small volume of the prepared standard solution of macrolides with the concentration of 3 mg L^{-1} of each compound in order to obtain its concentration in water of 1 500 ng L^{-1}. Spiked samples were processed under the same conditions as real samples and spiked MQ water samples. Water without spike was also analyzed. Matrix effect (*ME*), in %, was then calculated according to equation (3).

$$ME = 100 \cdot \frac{A_S - A_{US}}{A_{SPE}} \qquad (3)$$

Where A_S represents the analyte peak area in the spiked sample, A_{US} is the peak area in the sample of real water without spike and A_{SPE} is the peak area in the spiked MQ water sample.

The lower the obtained number, the higher is the matrix effect (values below 100 % indicate a negative influence of the matrix). For each analyte, the matrix effect was found to have a negative influence on the extraction and ionization processes. The highest matrix effect was observed in the samples from influent, especially for AZI and CLA; calculated values are given in Table 4.

Tab. 4. Matrix effects with RSD (n = 3).

Compound	Matrix effect [%]			
	Influent	RSD [%]	Effluent	RSD [%]
AZI	46.14	4.12	92.15	3.45
CLA	47.53	4.91	91.25	4.14
ERY	55.35	3.55	95.87	2.87
ROX	62.44	2.98	98.75	2.63

Because of significant differences in the matrix effects in influents and effluents from the WWTPs, it was necessary to calculate method detection limits and method quantification limits separately. Values of IDL and IQL were recalculated in respect to matrix effects and multiplied with a factor of 5 (because of the SPE procedure where 200 mL of water were used — see Sample extraction). Obtained values are shown in Table 5.

Determination of macrolide antibiotics in real samples from three wastewater treatments plants

The total occurrence of macrolides in 26 collected samples was 100 % for azithromycin, clarithromycin and erythromycin and 96 % for roxithromycin. In general, the highest concentrations were observed in WWTP Brno-Modřice and the lowest in WWTP of UVPS. A comparison of the concentrations of individual substances shows that the concentrations of azithromycin and clarithromycin were one or two orders of magnitude higher than the concentrations of erythromycin and roxithromycin. Concentrations determined in WWTP Brno-Modřice are listed in Table 6, those determined in WWTP Mikulov and

Tab. 5. Method detection limits and method quantification limits in influents (MDL_i and MQL_i) and effluents (MDL_e and MQL_e).

Compound	Influent		Effluent	
	MDL_i [µg L^{-1}]	MQL_i [µg L^{-1}]	MDL_e [µg L^{-1}]	MQL_e [µg L^{-1}]
AZI	7.59	23.84	3.80	11.94
CLA	4.21	12.62	2.19	6.58
ERY	5.42	18.07	3.13	10.43
ROX	3.20	9.61	2.03	6.08

Tab. 6. Determined macrolides concentrations (ng L^{-1}) in WWTP Brno-Modřice and average removal efficiencies (%) of the treatment process for monitored analytes.

Compound	Influent (n = 10)			Effluent (n = 10)			Average
	Min[1]	Median	Max[2]	Min	Median	Max	efficiency
AZI	1291	1585	1737	926.5	1122	1404	26.07
CLA	1283	1724	1849	935.3	1150	1394	27.22
ERY	50.88	71.44	148.7	45.72	68.09	82.55	12.91
ROX	34.56	45.40	98.46	18.20	30.68	41.32	33.05

[1]Minimum value.
[2]Maximum value.

Tab. 7. Determined macrolides concentrations (ng L^{-1}) in WWTP Mikulov and WWTP of UVPS and removal efficiencies (%) of the treatment process.

Compound	WWTP of Mikulov			WWTP of UVPS		
	Influent	Effluent	Removal efficiency	Influent	Effluent	Removal efficiency
AZI	720.6	192.8	73.24	172.0	39.69	76.92
	495.1	101.1	79.58			
CLA	714.8	207.5	70.97	180.3	49.78	72.39
	466.3	111.8	76.02			
ERY	78.19	25.35	67.58	32.27	16.20	49.80
	33.34	25.22	24.36			
ROX	19.22	10.30	46.41	38.55	ND [a]	~100.0
	15.17	7.878	48.07			

[a]Not detected.

UVPS are listed in Table 7. Based on their concentrations in influents and effluents, removal efficiencies were calculated and are summarized together with obtained results in Tables 6 and 7.

The highest concentrations were observed for azithromycin and clarithromycin, which correlates with their higher consumption in the Czech Republic compared to erythromycin and roxithromycin. Variations in the determined concentrations during ten days can be caused by different dilutions day by day (precipitation) or, simply, by variations in pharmaceuticals consumption.

A comparison of removal efficiencies between the WWTPs is not reasonable mainly because the sample types vary. The WWTP Brno-Modřice represents statistically more valuable results due to the ten day sampling campaign. In general, these measurements show that macrolides antibiotics can easily leak into the environment through WWTPs because of insufficient treatment. This problem is probably caused by high persistence of these pharmaceuticals to biological treatment.

Conclusion

A suitable method for macrolides antibiotics determination in wastewater and treated water was developed and successfully used for the analysis of real samples. High-efficiency solid phase extraction method (85.76—92.54 %) together with rapid and sensitive HPLC/MS allowed measuring low concentrations of macrolides present in wastewater. The method detection limits and method quantification limits obtained were in the range of 2.03—7.59 ng L^{-1} and 6.08—23.84 ng L^{-1}, respectively. The presence of macrolides has been proven in both the influents and the effluents from the WWTPs on the concentration scale of ng L^{-1} to μg L^{-1}. Removal efficiencies ranged from 13 % to 100 %.

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