SIMULTANEOUS DETERMINATION OF B-LACTAMS ANTIBIOTICS IN WASTEWATER SAMPLES BY SOLID PHASE EXTRACTION FOLLOWED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY

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Abstract

In the present work, an analytical method for the simultaneous determination of six β-lactam antibiotics (ampicillin, amoxicillin, penicillin V, penicillin G, oxacillin and cephalexin) is proposed for the determination of these compounds in wastewater treatment plants (WWPTs) influents and effluents. The β-lactams were extracted from water samples using Oasis HLB cartridges with pre-concentration factors up to 250. The compounds have been separated using a Zorbax SB-C18 (50 mm x 2.1 mm, 1.8 µm) HPLC column and gradient elution with mobile phase consisting of aqueous formic acid and acetonitrile. Detection was performed by mass spectrometry with a triple quadrupole using an electrospray interface. The linear range of the standard curve was from 1.0 to 600 $ngmL^{-1}$ (R^2>0.99). Average recoveries of β-lactams in fortified samples were generally above 74% with relative standard deviations (RSDs) lower than 11 %. Limits of detection were in the range 20-150 ngL^{-1} and 8-60 ngL^{-1} for influent and effluent wastewater samples, respectively. The described method was applied to the determination of the β-lactams in wastewater samples from a municipal WWTP.

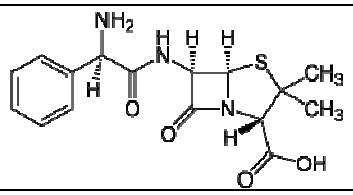
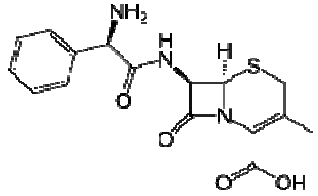
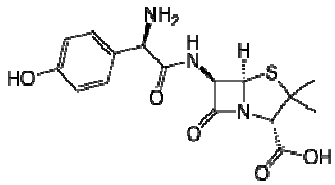
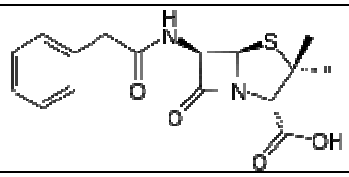
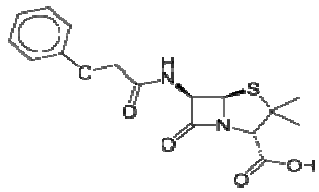
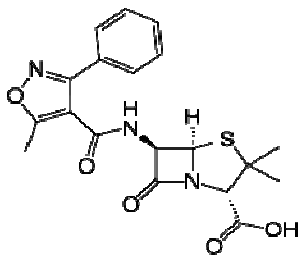
Keywords: antibiotics, β-lactams, wastewater, WWTPs

Introduction

The increasing use of drugs during the last decades has produced resistence genes in bacteria leading to substantial scientific efforts to evaluate the occurrence and fate of these compounds in the environment and to identify the pathways of their release into the ecosystem. After the administration to humans and animals, pharmaceutical compounds are eliminated in a considerable amount in unchanged form via urine and faeces into the sewage. Medicinal factories effluents and disposal of unused and expired drugs in sewage systems are other potential source of pharmaceuticals in the environment /1/. Removal of antibiotics during conventional wastewater treatment processes was found to be incomplete. Therefore, the municipal WWTPs are the main route of entrance of the pharmaceuticals in the aquatic environment. Many papers have reported the occurrence of various pharmaceutical compounds in WWTP effluents as well as in the receiving surface waters /2-7/. The β-lactams antibiotics are widely used antimicrobial drugs against both gram-positive and gram-negative organisms in human and veterinary medicine practices to prevent and treat infections of skin, ear, respiratory tract, and urinary tract /8/. The aim of this work was to develop a

sensitive method for the analysis of 6 β-lactam antibiotics at trace level in WWPTs influent and effluent samples. The chemical structures and the physical properties of the studied compound are shown in Figure 1 /9/. For this purpose an analytical strategy based on based on solid-phase extraction coupled to liquid chromatography/electrospray tandem mass spectrometry has been optimized. The optimized methodology has been applied to the analysis of the selected antibiotics in a WWTP influent and efluent samples.

Table.1 Chemical structures and physical properties of the chosen β-lactams

Compound	Structure	Molecular formula /molecular weight (g/mol)	CAS number	pK_a	$logK_{ow}$
Ampicillin		$C_{16}H_{19}N_3O_4S$ 349,405	69-53-4	2.7 7.3	1.35
Cephalexin		$C_{16}H_{17}N_3O_4S$ 347,39	15686-71-2	4.5	0.65
Amoxicillin		$C_{16}H_{19}N_3O_5S$ 365.404	26787-78-0	2.8 7.2	0.87
Penicillin G		$C_{16}H_{18}N_2O_4S$ 334.4	61-33-6	2.8	1.83
Penicillin V		$C_{16}H_{18}N_2O_5S$ 350,39	87-08-1	2.7	2.09
Oxacillin		$C_{19}H_{19}N_3O_5S$ 401.436	66-79-5	2.7	2.38

Experimental part

Reagents and materials

Penicillin G potassium salt (PEN G) (98.9%), penicillin V potassium salt (PEN V) (99%), cephalexin (99.8%) and oxacillin sodium salt monohydrate (99.2%) were purchased from Sigma-Aldrich (Seelze, Germany). Amoxicillin trihydrate (AMX) (86%) was from US Pharmacopeia (Rockville) and ampicillin anhydrous (AMP) (86%) from European Pharmacopea (Strasbourg). $^{13}C_3$-Trimethoprim internal standard was obtained from Cambridge Isotope laboratories (Andover, MA, USA). HPLC-grade methanol and acetonitrile and sodium hydroxide were supplied by Sigma-Aldrich (Steinheim, Germany). EDTA disodium salt (Na2-EDTA) and hydrochloric acid 37 % were purchased from Merck (Darmstadt, Germany). Reagent grade formic acid (99.9% purity) was supplied by Agilent Technologies (CA, USA). HPLC-grade water was obtained with a Simplicity UV ultrapure water system from Millipore (Molsheim, France). All solutions used in HPLC system were passed through a 20 µm pore size nylon filter before use. Individual stock standard solutions at around 500mgL^{-1} were prepared in methanol. These solutions were stored in amber glass vials at -18 °C. Working solutions containing a mixture of all analytes were freshly prepared in methanol and stored at 4°C in the dark. A binary mobile phase with a gradient elution was used. Mobile phase A was a mixture of water with 0.1 % formic acid and acetonitrile was mobile phase B. Oasis HLB {[poly(divinylbenzene-co-*N*-vinylpyrrolidone)]} 60 mg cartridges used for solid phase extraction were purchased from Waters (Milford, MA, USA).

HPLC-MS/MS instrumentation

The pH of the samples was adjusted with a Multimeter 340i from WTW GmbH (Weilheim, Germany). Solid phase extraction was carried out on a Chromabond® SPE vacuum manifold for 10 cartridges from Macherey-Nagel GmbH (Düren, Germany). The chromatographic system consisted of an Agilent 1290 Infinity series HPLC equipped with a quaternary pump, on-line degasser, autosampler, automatic injector, column thermostat. Separation was achieved using an Agilent Zorbax Eclipse SB-C18 Rapid Resolution HT column 50 mm x 2.1mm i.d., 1.8 µm particle size, at a flow rate of 0.3 mL/min. A gradient programme was used with the mobile phase, combining solvent A (with 0.1 % formic acid in ultrapure water) and solvent B (acetonitrile) as follows: from 2 to 30% B in 2.5 min, isocratically till 6 min followed by a gradient change to 58% B in 4 min and to 61.5% B in 2 min. Then the system was equilibrated for 6 min prior to the next injection. The column temperature was maintained at 40^{0}C and the injected sample volume was 2 µL for all the compounds and all the analytes were eluted within 10 min. The HPLC system was connected to a triple quadrupole mass spectrometer Model 6410 Agilent (Agilent Technologies, Waldbronn, Germany) equipped with electrospray Jet Stream technology operating in positive and negative ion mode. Nitrogen gas was used as collision and nebulising gas.The analyses were done in the positive ion mode. MS/MS signal acquisition was performed in Multiple Reaction Monitoring (MRM) mode.

For each analyte, two signals were monitored, corresponding to the transition between the precursor ion and the two most abundant product ions. The most abundant one was used for quantification while the other one was used for confirmation. Instrument control and data processing were carried out by means of MassHunter software from Agilent Technologies. Quantification was carried out by calculating the response factor of each analyte relative to $^{13}C_3$-Trimethoprim used as internal standard and concentrations were determined using a least-square linear regression analysis of the peak area ratio versus the concentration ratio. The use of internal standard as surrogate in the water sample before extraction accounts for matrix effects for all of the analytes and prevents slight variations in the signal and possible enhancement or suppression of the signal /10,11/.

Sample preparation and SPE procedure

Before extraction water samples were filtered through 1.6 µm glass fiber filters (Whatman, Maidstone, UK). In the filtered aliquots of wastewater (100 mL for influent and 250 mL for effluent) Na_2EDTA chelating agent was added to achieve a final concentration of 0.1 % in the samples. The measured volumes were subsequently enriched onto Oasis HLB cartridges using the protocol described in EPA Method 1694 /12/ for extraction of acid fractions of aqueous samples. However, due to the fact that β-lactams are readily degraded under strong acidic and basic conditions as a result of the hydrolysis of the nucleophilic β-lactam ring, samples were neutralized at pH 7 ± 0.5 /8/. HLB cartridges were conditioned with 20 mL methanol and 6 mL water at pH 7 ± 0.5. The cartridges were loaded with 100 mL influent and 250 mL effluent respectively, at a constant flow rate of 5-6 mL/min. After sample preconcentration, cartridges were rinsed with 10 mL HPLC water to remove the EDTA and dried under vacuum for aproximately 5 min to remove excess water. The target analytes were eluted with 12 mL methanol. Using a gentle stream of nitrogen gas, the extract volumes were reduced to near dryness in a water bath held at 50 ± 5°C, and reconstituted in 1 mL of methanol.

Quality assurance protocol

The method was evaluated in terms of linearity, repeatability, accuracy and sensitivity. Linearity was evaluated by constructing six point calibration curves within a wide range of concentrations from 1 to 500µgL^{-1}. Linearity was assumed if the R^2 value was higher than 97.5%, and the residuals lower than 30% for each calibration point /13/. Precision was defined as the relative standard deviation (RSD) of a five replicates analysis of wastewater samples spiked at 200ngL^{-1} concentration. Accuracy was evaluated by analysing wastewater (influent and effluent) samples spiked with antibiotics and an internal standard. The method was considerated accurate if recoveries were in the 70-120% range, and precision was satisfactory if the RSD was lower than 15% /13/. The limit of detection (LOD) and the limit of quantification (LOQ) were estimated for each analyte from the chromatograms at the lowest analyte concentration assayed, as the concentrations giving a signal to noise ratio of 3

and 10, respectively. Laboratory blanks made every day from ultrapure water were analyzed to assess potential sample contamination.

Results and discussion

Method optimisation

β-Lactam antibiotics were firstly identified in full-scan mode (m/z 100-1000) by direct infusion of individual standard solutions (500μgL^{-1}) at a flow rate of 0.3 mL/min, using acetonitrile as mobile phase. The protonated molecule $[M+H]^+$ produced the most intense ion and was selected as the precursor ion. Once the $[M+H]^+$ signal was optimized, different collision energies were tested in order to obtain the maximum sensitivity with the highest number of product ions available. The fragmentor voltage and collision energies were optimized for each compound and ranged from 70 to 130 V and from 10 to 30 eV, respectively. The following ESI-MS/MS parameters were also optimized: capillary voltage was set at 4000V; nebulizer pressure was set at 40 psig; drying gas was set at 9L/min and source temperature was set at 300°C. HPLC-MS/MS parameters for collision energy, fragmentor voltage, MRM transitions, dwell time and retention times are shown in Table 2. In order to improve the sensitivity, each run was divided into four acquisition windows and dwell time was set at 10-100 ms, depending on the time segment. The optimum setting for column temperature giving the highest resolution and the strongest analyte response was found to be 40°C. As regard the optimal flow rate, 0.3 mL/min was observed to provide best analyte separation and maximum sensitivity.

Table 2. Retention times and MS/MS parameters for the analysis of target analytes by MRM positive ionization mode

Compound	t_R (min)	MRM transitions (*m/z*)	Fragmentor voltage (V)	Collision energy (eV)	Dwell time (ms)
Ampicillin	5,084	350→106	70	10	10
		350→160	70	15	
Cephalexin	5,137	348→158	120	19	10
		348→174	120	15	
Amoxicillin	7,480	366→114	110	5	10
		366→208	110	15	
Penicillin G	7,720	335→160	90	5	10
		335→176	90	5	
Penicillin V	8,349	383.5→114	70	5	10
		383.5→160	70	25	
Oxacillin	9,727	402→160	70	5	100
		402→243	70	15	

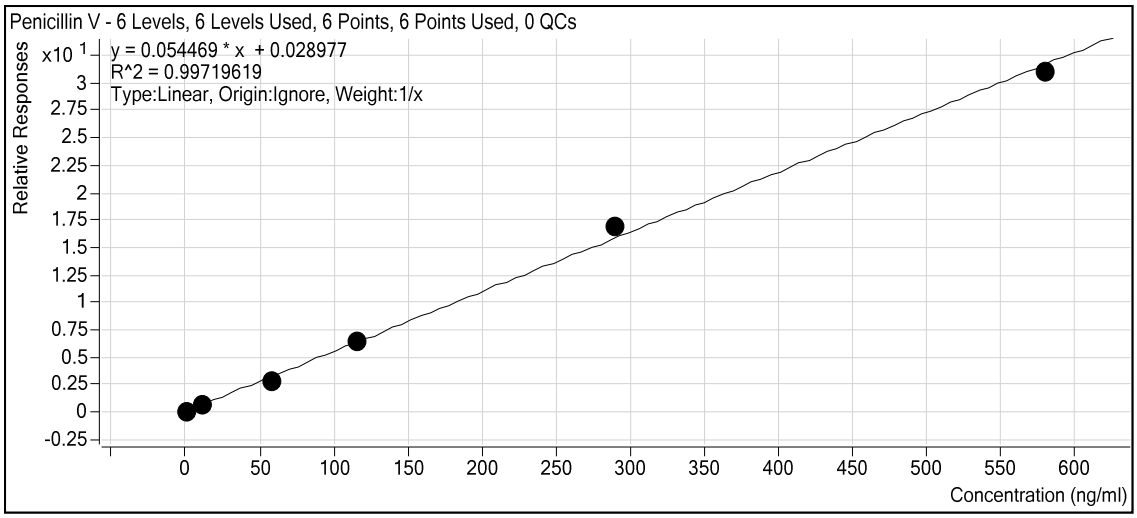


Fig. 2. Calibration curve for penicillin V usig a six point curve from 1.16 to 580µgL^{-1} using a linear fit with no origin treatment

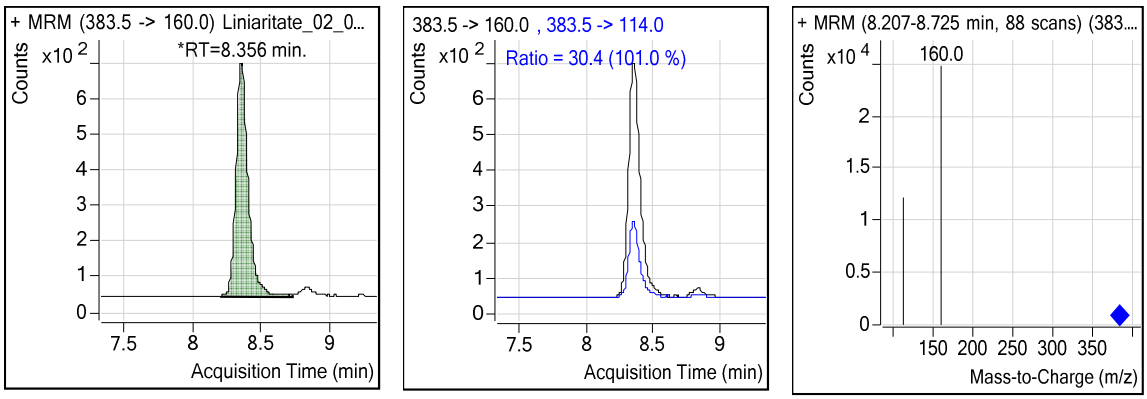


Fig. 3. MRM chromatograms of $[M+H]^+$ obtained for penicillin V at a concentration level of 58 µgL^{-1}. Ion ratio is also shown.

Method validation

The linearity of the MS analyser response was investigated by performing duplicate injections standard solutions. The range tested was from 1 to 500 µgL^{-1}. A linear response was observed and correlation coefficients (R^2) ranged from 98.77% to 99.86% for all analytes. Linearity range varied for each analyte investigated. For cephalexin and penicillin V the lower limit of linearity was 1 µg/L, and for ampicillin, amoxicillin and oxacillin was 10 µgL^{-1}. Penicillin G was found to be less sensitive in terms of the method giving a lower limit of linearity of 60 µgL^{-1}. Figures 2 shows the calibration curve obtained for penicillin V and figure 3 the LC-MS/MS spectra and MRM chromatograms of $[M+H]^+$ for this compound at a concentration level of 58 µgL^{-1}. As it can be observed in these figures, a series of values (retention times, peak areas and calculated concentrations) are obtained as well as information on ion ratios and calibration curve data.

Recoveries achieved for all target compounds ranged from 75% to 88% for spiked effluent wastewater samples. Only ampicillin as well as penicillin G showed lower recoveries rates (54 and 61 %, respectively). Detection limits obtained are reported in Table 3. LODs ranged from 20 to 150 ngL^{-1} and from 8 to 60 ngL^{-1} for influent and effluent, respectively. Precision of the method was studied by analysing five replicates of 200 ngL^{-1} standard. Intra-day precision was calculated as the percent relative standard deviation (RSD) from the five measurements and ranged between 2.4 and 6.1%. Inter day precision was performed by analysing spiked water extracts in five consecutive days. Results are indicated in Table 3 for each compound, showing a precision from 4.7 to 11.2%. As can be deduced from Table 3, the developped method allows the quantification of six β-lactam antibiotics in wastewater using calibration with standards in solvent and a labelled internal standard.

Table 3. Results obtained in the validation study (n=5) for the determination of 6 β-lactams in spiked wastewater samples

Compound	200 ngL^{-1}				LODs (ngL^{-1})	
	Influent		Effluent		Influent	Effluent
	Rec[a] (%)	RSD[b] (%)	Rec (%)	RSD (%)		
Ampicillin	54	11.2	56	9.5	80	48
Cephalexin	79	8.1	75	4.7	20	8
Penicillin V	86	6.7	89	6.4	47	18,8
Amoxicillin	75	5.3	83	6.8	80	48
Penicillin G	61	10.4	70	11.0	150	60
Oxacillin	88	7.3	91	7.9	70	36

[a] Rec.: recovery.
[b] RSD: relative standard deviation.

Analysis of real wastewater samples

Table 4. Measured concentration in Magurele influents/effluents WWTP and removal rates

Compound	Effluent (ngL^{-1})	Influent (ngL^{-1})	Removal rates (%)
Ampicillin	25	65	61,5
Cephalexin	36	270	86,7
Penicillin V	<18,8	57	100
Amoxicillin	170	251	32,3
Penicillin G	<60	<150	-
Oxacillin	254	449	43,4

To demonstrate the applicability of the developed method, samples from a WWTP were analyzed. Another aim was to have a first estimation on the quantities discharged by WWTP effluents in environmental waters, to know if the treatment was able to totally remove β-lactam antibiotics from water. Results obtained are summarized in Table 4 and show that five of the six target analytes were detected.

Conclusions

An analytical procedure that allows quantification of six β-lactam antibiotics in influents and effluents from wastewater treatment plants has been developed. The sensitive and reliable analytical method is based on solid-phase extraction coupled to liquid chromatography/electrospray tandem mass spectrometry in Multiple Reaction Monitoring mode with two transitions. Recoveries obtained for all compounds using Oasis HLB cartridges were higher than 75%, excepting ampicillin (54%) and penicillin G (61 %). The overall precision of the method was found to be less than 15% for these compounds.This method was applied on environmental samples and it permited the detection of β-lactams in WWTP influents and effluents. This demonstrates that these compounds are a matter of concern in an aquatic environmental context and should be part of monitoring studies.

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