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Water pollution

SIMULTANEOUS LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY DETERMINATION OF SOME PHARMACEUTICALS AND ANTIMICROBIAL DISINFECTANT AGENTS IN SURFACE WATER AND IN URBAN WASTEWATER

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Abstract. A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for the simultaneous quantification and confirmation of two diuretics (furosemide, hydrochlorothiazide), five nonsteroidal anti-inflammatory drugs (NSAIDs), (diclofenac, ketoprofen, piroxicam, ibuprofen, naproxen), one fibrate (gemfibrozil) and two antimicrobial agents (triclosan, triclocarban). The compounds were extracted from water samples using Oasis HLB cartridges. The analytes were then identified and quantified using LC-MS/MS with dual-polarity electrospray ionisation in the MRM mode. Good linearity was obtained for all tested compounds ($R^2 > 0.99$). The limits of detection (LODs) ranged between 0.15 and 24.0 ng l^{-1}. Spike recoveries ranged from 84 to 115% and from 81 to 117% for urban wastewater and surface water samples, respectively, with satisfactory precision (RSD < 20%). Finally, the method was used to analyse real effluent and surface water.

Keywords: pharmaceuticals, antimicrobial agents, LC-MS/MS, surface water, wastewater.

AIMS AND BACKGROUND

Public health concern over the occurrence of pharmaceuticals and disinfectants in the treated effluent and in the receiving surface waters is nowadays a subject of increasing interest. The presence of these substances, even in low concentrations, can cause adverse effects to several aquatic species and the proliferation of antibiotic resistant pathogens[1–3]. Triclosan (TCS) is a broad spectrum antimicrobial agent used in personal care products (toothpaste, cream, deodorants and soaps)[4]. This antimicrobial is considered to be an emerging substance due to its bioaccumulative characteristic, and producing in the last instance chronic toxicity in aquatic living (algae, fishes, etc.). TCS can also produce bacteria strains that are resistant to antimicrobials and antibiotics[5]. As a consequence, various analytical methods based on different techniques have been developed to detect and quantify pharmaceuticals residues in environmental samples[6–8]. Liquid chromatography-tandem

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mass spectrometry (LC-MS/MS) has become today the most important technique of a broad diversity applicable analytes in various environmental matrices[9–13].

The aim of this work was to develop and validate a sensitive and reliable method based on solid phase extraction (SPE) followed by analysis by LC-MS/MS for the simultaneous determination of two diuretics (furosemide, hydrochlorothiazide), five NSAIDs (diclofenac, ketoprofen, piroxicam, ibuprofen, naproxen), one fibrate (gemfibrozil) and two antimicrobial agents (triclosan, triclocarban) in surface water and in urban wastewater. The isotope dilution method, using an appropriate labelled internal standard (IS), is considerate to be one of the best approaches to compensate for matrix effects[14]. Moreover, by adding IS to the water sample before its treatment, the potential analytical errors associated to the sample manipulation can be also compensated. The applicability of the method was tested by determining target analytes in surface water and WWTP effluent wastewater.

EXPERIMENTAL

Chemicals and materials. Diclofenac (DCF), ketoprofen (KET), piroxicam (PIR), ibuprofen (IBU), naproxen (NPX), hydrochlorotiazide (HCT), furosemide (FUR), triclosan (TCS), triclocarban (TCC), and gemfibrozil (GEM) with purities higher than 98% were obtained from Sigma-Aldrich (Steinheim, Germany). The LC-MS grade formic acid (98%) and methanol, acetonitrile (ACN) used for the preparation of standards, mobile phases and pH adjustments were also provided by Sigma-Aldrich. HPLC-grade water was obtained by purifying demineralised water in a Milli-Q plus system from Millipore (Bedford, MA, USA). The isotopically labelled compounds: $^{2}H_{2}$-hydrochlorothiazide (D_{2}-HCT), $^{2}H_{3}$-^{13}C-naproxen (^{13}C-D_{3}-NPX), $^{2}H_{4}$-diclofenac (D_{4}-DCF), $^{13}C_{3}$-ibuprofen ($^{13}C_{3}$-IBU), $^{13}C_{6}$-Tticlocarban ($^{13}C_{6}$-TCC), $^{13}C_{12}$-triclosan ($13C_{12}$-TCS), $^{2}H_{6}$-gemfibrozil (D_{6}-GEM), $^{13}C_{6}$-trichlorofenoxyacetic acid ($^{13}C_{6}$-TCPAA) were used as surrogate or injection internal standard and were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Individual standard solutions of compounds (around 500 µg/ml) were prepared in methanol and stored at –20°C. Working standard mixtures were prepared by diluting the individual stock solution in methanol. All standard solutions were stored at 4°C in dark glass bottles to prevent photodegradation. SPE cartridges, built of a hydrophilic and a lipophilic monomer (Oasis HLB: 500 mg, 6 ml), were acquired from Waters (Milford, Massachusetts, USA).

Sample extraction. The analytical procedure was based on a previously reported, validated method with some modifications[15]. Prior to analysis, all samples were vacuum-filtered through 1.6 µm glass fibre membrane (Whatman, Maidstone, UK) to remove suspended particles, and then through 0.45 µm disk filters. The filtered samples were adjusted to pH 2 ± 0.2 with 12N HCl. In the filtered aliquots of wastewater (100 ml for influent and 250 ml for effluent) and surface water

(500 ml) 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 (500 mg, 6 ml; Waters, Milford, MA, USA) using the protocol described in EPA Method 1694 for extraction of acid fractions of aqueous samples. Prior to extraction isotopically labelled compounds were added to water samples at a concentration of 50 ng/ml for each labelled compound. After successively preconditioning the cartridges with 20 ml of methanol and 10 ml of pH 2 water, samples were percolated through the cartridge at a flow rate of 5 ml/min. Then, the cartridge was washed with 6 ml water and dried under vacuum for 5 min. Analytes were eluted from the cartridge using 12 ml methanol and the extract was evaporated to near dryness under a gentle N_2 stream in a water bath held at 50 ± 5°C. The acid extract was spiked with the labeled injection internal standard and diluted to 1 ml with 0.1% formic acid solution (500 times concentration factor) and transferred to a HPLC autosampler vial.

LC-ESI-tandem MS analysis. Experiments were performed using an Agilent 1260 series LC system (Agilent, Waldbronn, Germany), which consisted of an autosampler and a quaternary pump coupled with an Agilent 6410B triple-quadrupole mass analyser fitted with an electrospray ionisation source. Data acquisition and analysis were performed using Mass Hunter software, revision B.04.01. All chromatographic runs were carried out on a Zorbax Eclipse Plus C_8 column (100 × 2.1 mm, 3.5 µm) from Agilent Technologies. The initial mobile phase composition was a mixture of aq. 0.1% formic acid and ACN in the ratio 90/10 (v/v). The elution program consisted of a 20%/min ballistic gradient applied in 2 min up to 50% ACN followed by a slower 12.5%/min gradient up to 100% ACN and an isocratic plateau at 100% ACN of 3 min. A low mobile phase flow-rate of 0.3 ml/min was chosen to enhance ESI ionisation and sensitivity. Column temperature and injection volume were 20°C and 10 µl. For increased sensitivity and selectivity, data acquisition was performed working in Multiple Reaction Monitoring (MRM) mode with dual polarity (±). Full scan MS mode coupled with different fragmentor voltage values was used to acquire mass spectra, precursor ions and product ions from standard solutions. Nitrogen gas was used as collision and nebulising gas. Analytes ionisation in the ESI source was done at 300°C drying gas temperature, 9 l/min drying gas flow, 40 psi nebuliser pressure and a variable capillary voltage between 2–4 kV. Analyte instrumental detection limits were lowered also after a rigorous MS optimisation procedure in which all MS parameters were optimised for each of the 10 target analytes. Collision energies set against precursor ions, fragmentor voltage and sampling capillary voltage, quadrupole resolution, collision cell accelerator voltage, MRM dwell time, drying gas flow and temperature were optimised to obtain highest S/N ratio.

Validation study. Prior to its application, the method was satisfactorily validated for surface water and effluent wastewater matrices considering the following parameters: linearity, limits of detection, intra-day and inter-day precision, accuracies and recoveries. The calibration curve was obtained by analysing standard solutions in duplicate at six concentrations between 1 and 200 mg/l. A mixed solution of labelled internal standards was added to all the calibration points at a fixed concentration of 50 µg/l. Linearity was assumed if the r^2 value was higher than 0.97. The limit of detection was estimated from the sample chromatograms at the lowest analyte concentration determined, as the analyte concentration giving peaks for which the signal-to-noise (S/N) ratio was 3. For intra-day experiments, three replicates were spiked, extracted and analysed in the same day, whereas for inter-day assays, the extraction and analysis were performed for one sample, in three days. Individual recoveries for overall analytical procedure were determined by spiking surface water and effluent wastewater with standard mixture at approximately 40 ng/l. Surface water and effluent wastewater samples were previously analysed to confirm the absence of any significant peak at the selected transitions and positive findings were substracted from spiked samples. The method was considered accurate if recoveries were in the 70–120% range, and precision was satisfactory if the relative standard deviation RSD was lower than 15%.

Samples collection and storage. Wastewater effluent samples were collected from a WWTP located in Braila County and from the natural receiver, Danube River, immediately downstream of the WWTP near the urban area. Treatments in this WWTP include primary settling and biological processing (activated sludge). All samples were grab samples and were collected in between the 16th and 20th of November 2015. The water samples were collected in polypropylene bottles (1000 ml), stored at 4°C and pretreated within 3 days.

RESULTS AND DISCUSSION

Optimisation of LC-MS^2 parameters. The optimisation of MS parameters was performed by flow injection analysis (FIA) for each compounds. For each analyte, mass spectra from m/z 50 to 1000 were recorded in both ionisation modes. The NSAIDs (DEF, KET, PIR, IBU, NPX) were more sensitive in PI mode, whereas the analysis of the rest of compounds was carried out in NI mode. In order to select the precursor ions for each compound, chromatograms were recorded in full scan mode. After selecting the precursor ions, $[M–H]^-$ for NI mode and $[M+H]^+$ for PI mode, product ions were obtained with a combination of collision energies and fragmentor voltages. The optimised values of some of these parameters are given in Table 1. The table also includes the optimised values for deuterated analogs.

Table 1. MS/MS parameters for the analysis of target analytes by MRM negative and positive ionisation mode

Compound	Retention time (min)	MRM transition	Fragm. volt. (V)	CE (V)	Polarity	Cap. volt. (V)	Time segment (min)
D_2-HCT	4.053	298 → 269	150	15	(–)	2000	2.0–5.5
HCT	4.070	296 → 269	150	15			
PIR	6.371	332 → 164	135	15	(+)	4000	5.5–6.6
FUR	6.315	329 → 285	125	15	(–)	2500	
KET	6.855	255.1 → 209.1	140	10	(+)	4000	6.6–7.4
^{13}C-D_3-NPX	6.869	235 → 170.1	110	30			
NPX	6.873	231 → 170.1	110	30			
$^{13}C_6$-TCPAA	7.140	259 → 201	110	5	(–)	2500	
D_4-DCF	7.531	300 → 219	75	20	(+)	3700	7.4–7.9
DCF	7.542	296 → 215	75	20			
$^{13}C_3$-IBU	7.676	210.1 → 163	110	5			
IBU	7.678	207.1 → 161	110	5			
$^{13}C_6$-TCC	8.210	319 → 160	140	10	(–)	2500	7.9–9.0
TCC	8.211	313 → 160	140	10			
$^{13}C_{12}$-TCS	8.305	299 → 35.1	100	5			
TCS	8.306	287 → 35.1	100	5			
D_6-GEM	8.007	255.2 → 121.1	130	10			
GEM	8.016	249.2 → 121.1	130	10			

Collision energies were chosen to give the maximum intensity of the fragment ions obtained, whereas fragmentor voltages were selected according to the sensitivity of precursor ions. Finally, data acquisition was performed under time-segmented conditions based on the chromatographic separation of the analytes to maximise the sensitivity of detection. Figure 1 shows the chromatogram obtained for studied compounds in concentrations between 45 and 100 ng/l. Extracted ion chromatograms are overlaid for each of target analyte according to appropriate protonated/deprotonated molecule and product ions from MRM transitions.

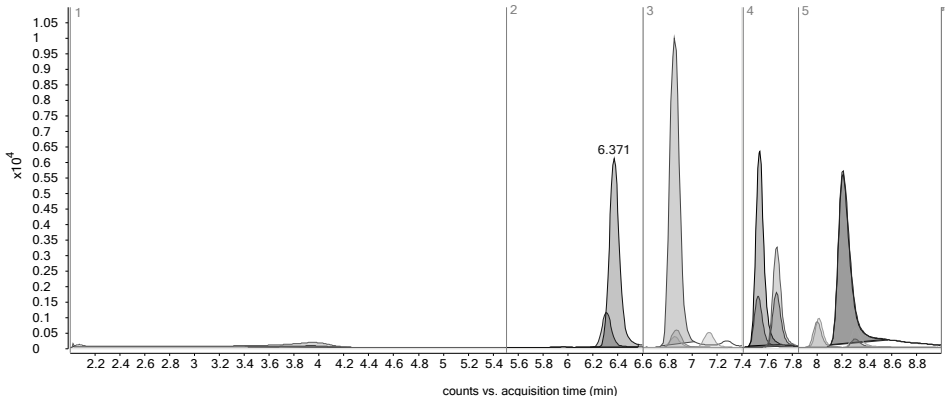

Fig. 1. Liquid chromatogram showing separation of the 10 analytes and corresponding isotopically labelled standards

Method of validation. Six-points calibration curves showed good linearity in the range from 1 to 100 µg/l, for most of target analytes, except for FUR, HCT and TCS for which the range was from 2 to 200 µg/l. The obtained calibration curves provided high correlation coefficients ($r^2 > 0.99$) and residuals lower than 30% for all 10 compounds. Table 2 details the linearity, LOD, the intra and inter-day precision of the method for each compounds and sample type tested. Instrumental detection limits (S/N = 3) ranged between 0.07–1.02 µg/l, except for HCT for which a higher instrumental LOD was obtained (6 µg/l) due to elution on the isocratic step and poor ESI ionisation.

Table 2. Method of validation parameters calculated in surface water and treated wastewater

Parameter	R^2 (%)	Surface water				Treated wastewater			
		LOD (ng/l)	recovery (%)	intra-day (%RSD)	inter-day (%RSD)	LOD (ng/l)	recovery (%)	intra-day (%RSD)	inter-day (%RSD)
HCT	99.27	12.0	81.5	13	15	24.0	115.0	7	14
PIR	99.85	0.17	106.0	8	10	0.34	99.4	10	13
FUR	99.85	2.03	86.0	9	12	4.05	85.8	9	12
KET	99.81	0.84	81.3	8	11	1.68	116.0	6	10
NPX	99.72	1.58	98.1	12	14	3.16	108.0	12	15
DCF	99.33	0.65	85.2	8	13	1.31	84.2	7	14
IBU	99.67	1.15	93.1	10	11	2.31	107.0	10	16
TCC	99.79	0.15	101.0	9	14	0.30	101.0	14	13
TCS	99.74	1.47	93.4	11	15	2.95	96.4	8	12
GEM	99.74	0.95	85.3	4	8	1.90	117.0	8	8

The method limits of detection in surface water varied between 0.15 and 12.0 ng/l, while in treated wastewater increased up to the range 0.30–24.0 ng/l. Intra-day precision of the developed method, calculated as relative standard deviation (%RSD) ranged from 4 to 13% in surface water and from 6 to 14% in wastewater. Similar values were obtained for the relative inter-day standard deviation, ranging from 8 to 15%. As regard to recovery, values higher than 81 and 84% were obtained in surface water and in treated wastewater, respectively.

Application of the method. The method was applied for the determination of some frequently used pharmaceuticals residues in wastewaters from a WWTP located in Braila County and in the natural receiver, Danube River. Results obtained are summarised in Table 3. The study has shown that all investigated WWTP effluent wastewater samples contained pharmaceuticals. Maximum concentrations were detected for piroxicam, with average concentration of 103 ng/l in WWTP effluent and 14.6 ng/l in surface water. Ibuprofen was not detected in any of the analysed samples.

Table 3. Average concentrations detected for target pharmaceuticals in effluent wastewaters from an urban WWTP and in the receiving surface water

Compound	Effluent wastewaters (ng/l)	Surface waters (ng/l)
Hydrochlorothiazide	73.7	9.2
Piroxicam	103.3	14.6
Furosemide	33.6	<LOD
Ketoprofen	20.8	5.3
Naproxen	80.5	3.5
Diclofenac	75.8	5.8
Ibuprofen	<LOD	<LOD
Triclocarban	15.6	7.1
Triclosan	14.1	6.5
Gemfibrozil	16.4	<LOD

CONCLUSIONS

A sensitive method for the analysis of two diuretics (furosemide, hydrochlorothiazide), five nonsteroidal anti-inflammatory drugs, one fibrate (gemfibrozil) and two antimicrobial agents (triclosan, triclocarban) in surface water and urban treated wastewater was developed. The method consists on SPE extraction and further MS/MS detection with electrospray ionisation. Recoveries obtained for all target compounds exceeded 80% in both matrices. The method yielded detection limits in the low ng/l range for both surface and wastewater thus providing a reliable tool for routine analysis of pharmaceutical residues in water samples.

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