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Simultaneous determination of acidic, neutral and basic pharmaceuticals in urban wastewater by ultra high-pressure liquid chromatography-tandem mass spectrometry

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ABSTRACT

In this work, an ultra high-pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method has been developed for the simultaneous quantification and confirmation of the 20 most consumed pharmaceuticals in Spain in urban wastewater and surface water samples. The scope of the method included acidic, neutral and basic compounds belonging to different therapeutic classes and allows their simultaneous determination in just a single injection, giving realistic information of the most widely consumed pharmaceuticals in only one analysis. An enrichment step based on solid-phase extraction using Oasis HLB cartridges was carried out, followed by UHPLC-MS/MS measurement with a fast-acquisition triple quadrupole mass analyzer. It allowed working with short dwell times and made possible to acquire three simultaneous SRM transitions per compound to assure a reliable identification. Several isotope-labelled internal standards were used as surrogates to correct SPE losses, as well as matrix effects that notably affect quantification of analytes. The method was validated in surface water and effluent and influent urban wastewater at different concentrations from 0.005 μg/L (surface water) to 1.25 μg/L (influent wastewater). The optimized method was applied to the analysis of 84 urban wastewater samples (influent and effluent), with the result that 17 out of 20 compounds monitored were detected in the samples. Analgesics and anti-inflamatories, cholesterol lowering statin drugs and lipid regulators were the major groups found, with diclofenac, ketoprofen, naproxen, 4-aminoantipyrine, bezafibrate, gemfibrozil and venlafaxine being the most frequently detected. The highest concentration level reached was 277 μg/L for salicylic acid in influent wastewater.

1. Introduction

Investigation of pharmaceuticals in the environment has become an important issue in the last years due to their large worldwide consumption and to their potential adverse effects on the animal and human health. Previous studies have demonstrated that pharmaceuticals are continuously being released in the environment, mainly through excreta, disposal of unused or expired products, or from pharmaceuticals discharges. Most of them are not completely removed from wastewater treatment plants (WWTPs), and can enter in ground and drinking water at low concentrations, from ng/L to μg/L [1,2]. Nowadays, not reliable data are available about long-term effects in the environment yet, and there is a need of performing ecotoxicological studies to know their concentration levels and to evaluate the possible toxic effects associated to their exposition. Therefore, the development of sensitive, selective and wide-scope methods is of major importance to have realistic data on their presence in both surface and wastewater.

As most pharmaceuticals are polar compounds, the technique of choice is, at the moment, HPLC coupled to mass spectrometry (MS), preferably to tandem MS. The development of faster and more sensitive methods is nowadays feasible using techniques like ultra high-pressure liquid chromatography (UHPLC), which has become one of the most suitable analytical tools for the determination of contaminants in environmental samples [3,4]. This technology provides greater resolution, increased sensitivity and high speed of analysis. The use of UHPLC in combination to tandem MS using fast analyzers makes possible working with short dwell times, thus increasing the number of selected reaction monitoring (SRM) transitions acquired simultaneously per compound. This increases confidence in the identification of analytes detected in samples.

UHPLC-MS/MS is increasingly being used for the determination of different organic contaminants in water. Typically, an off line preconcentration step is required to reach the sensitivity necessary to

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detect the low concentrations normally present in samples, in the range of ng/L [5–9].

Until now, most of attention has been paid to the presence of antibiotics in water [10–14]. In the last few years, methods developed for pharmaceuticals tend to simultaneously determine compounds belonging to different therapeutical groups, in contrast to previous methods that were focused on one specific group [15–17]. Thus, multi-class methods provide a more realistic knowledge about the presence of pharmaceuticals in water. As pointed out by some authors, in many published methods target analytes were selected because they could be included in a single method, due to their similar charge or ionization mode, or because reference standards or isotope-labelled internal standards were commercially available, or because these compounds had been previously detected [7]. However, to have a realistic view of the presence of pharmaceuticals in the environment, the most relevant compounds from the consumption point of view should be selected. In the present work, we have compiled information about the most consumed pharmaceuticals in Spain with medical prescription in the last years [18]. All these compounds were included in the method developed, together with some other pharmaceuticals that had been previously detected in surface water and urban wastewater by other authors [2,15–17,19].

A drawback when developing multi-residue multi-class methods comes from the quite different physico-chemical characteristics of the analytes, which makes difficult to find the most suitable chromatographic and MS conditions for all compounds; then a satisfactory compromise should be reached for the simultaneous analysis of all of them. However, regarding ionization mode, it is hard to find in the scientific literature applications analyzing positive and negative ionized pharmaceuticals in a single injection. Typically, positive and negative ionized analytes are determined in separate analysis, even using different column and mobile phases [7,20,21]. This is also problematic in the solid-phase extraction (SPE) step, because the extraction efficiency is compound dependent, and is affected by several variables such as the type of the sorbent used, sample pH, polarity of the solvent used for elution, or elution volume. Another key point is that selected chromatographic and MS conditions must be satisfactory for all type of water samples analyzed. This aspect is problematic when dealing with complex matrices, like urban wastewater samples, because co-eluting substances may lead to undesirable signal suppression/enhancement effects. In order to solve matrix effects, the use of isotope-labelled internal standards (ISs) seems to be the preferred strategy. This approach has been widely applied in the field of pharmaceuticals analysis [1,2,6,7,20,21].

The aim of this work is to develop rapid, selective and sensitive analytical methodology based on simultaneous sample enrichment by off-line SPE followed by UHPLC-MS/MS for the simultaneous determination of 20 acidic, neutral and basic pharmaceuticals widely consumed in Spain. All compounds, both measured under electrospray positive and negative ionization mode, are determined simultaneously in just one injection and acquiring three SRM transitions per compound. This allows their simultaneous detection, quantification and confirmation, making possible to reach more than 4 identification points (IPs) [22,23]. The most sensitive transition is used for quantification, while the other two allow the safe confirmation of the identity of the compounds detected in samples. The suitability of using several isotope-labelled ISs was evaluated to compensate matrix effects in surface water, but especially in wastewater where severe matrix effects were observed. The developed method was applied to the analysis of 84 wastewater samples from three WWTPs located at the Castellón province (Spain).

2. Experimental

2.1. Reagents and chemicals

The pharmaceuticals analyzed were selected accordingly to the following criteria: (i) the most consumed active principles with medical prescription in Spain [18] (ii) previous information reported in scientific literature about occurrences in surface and wastewater.

Acetaminophen (paracetamol), salicylic acid, ibuprofen, 4-aminoantipyrine, omeprazole, ketoprofen, naproxen, bezafibrate, diclofenac, gemfibrozil, pravastatin sodium and enalapril maleate salt were purchased from Sigma–Aldrich (Steinheim, Germany). Lorazepam, alprazolam, venlafaxine hydrochloride, risperidone and paroxetine hydrochloride were from LGC Promochem (London, UK). Atorvastatin and olanzapine were from Toronto Research Chemicals (Ontario, Canada). Pantoprazole was obtained by dissolving Anagastra® powder in HPLC-grade water. Isotopically labelled compounds were omeprazole-d_3, acetaminophen-d_4, diclofenac-d_4, salicylic acid-d_3 and ibuprofen-d_3 from CDN Isotopes (Quebec, Canada) and atorvastatin-d_5, paroxetine hydrochloride-d_4 and olanzapine-d_3 from Toronto Research Chemicals (Toronto, Canada). HPLC-grade methanol and HPLC-grade acetonitrile were purchased from Scharlau (Barcelona, Spain). HPLC-grade water was obtained by purifying demineralised water in a Milli-Q Gradient A10 (Millepore, Bedford, MA, USA). Formic acid (HCOOH, content >98%) and ammonium acetate (NH_4Ac, reagent grade) were supplied by Scharlau (Barcelona, Spain).

Stock standard solutions were prepared dissolving 25 mg, accurately weighted, in 50 mL methanol, obtaining a final concentration of 500 mg/L. For LC-MS analysis, the individual stock solutions were mixed and diluted with methanol to give a final concentration of around 1 mg/L and subsequently diluted, when required, with HPLC-grade water to obtain working mixed solutions of pharmaceuticals. These working solutions were used for spiking samples in the validation study and also for preparation of calibration standards, which were prepared in methanol–water (10:90, v/v).

Individual stock solutions of isotope-labelled IS were also prepared in methanol. A mixed working solution at 100 μg/L (for IS ionizing in positive mode) and at 1 mg/L (for IS ionizing in negative mode), was prepared in water and used as surrogate.

Due to the low stability of some compounds, mainly omeprazole, working solutions of pharmaceuticals were renewed monthly.

SPE cartridges used were Oasis HLB (60 mg), Oasis HLB (200 mg) and Oasis MCX (150 mg) from Waters (Milford, MA, USA).

2.2. Liquid chromatography

UHPLC analysis was carried out using an Acquity UPLC system (Waters Corp., Milford, MA, USA), equipped with a binary solvent manager and a sample manager. Chromatographic separation was carried out with an Acquity UPLC BEH column, 1.7 μm, 50 mm × 2.1 mm (i.d.) (Waters) at a flow rate of 0.3 mL/min. The column was kept at 60 °C and the sample manager was maintained at 5 °C. Mobile phase consisted of water/methanol gradient both 0.1 mM NH_4Ac and 0.01% HCOOH. The methanol percentage changed linearly as follows: 0 min, 5%; 1.5 min, 5%; 2 min, 30%; 3 min; 50%; 5 min, 70%; 6 min, 90%; 7 min, 90%; 7.1 min, 5%. Analysis run time was 9 min. Mobile phases were filtered under vacuum through 0.22 nylon membrane filters.

2.3. Mass spectrometry

For UHPLC analysis, a TQD (quadrupole–hexapole–quadrupole) mass spectrometer with an orthogonal Z-spray-electrospray inter-

Table 1
MS/MS optimized conditions for selected compound.

Compound	Therapeutic group	LOD (pg)	MW	*Q* transition	Cone (V)	C.E. (eV)	q_1 transition	C.E. (eV)	q_2 transition	C.E. (eV)	Q/q_1	Q/q_2
Acetaminophen	Analgesic and anti-inflamatories	1.7	151.1	152.1 > 110.1	30	15	152.1 > 93.0	25	152.1 > 65.0	30	5.5	9.1
4-Aminoantipyrine (metabolite of metamizol)		0.5	203.3	204.2 > 56.0	30	20	204.2 > 83.0	15	204.2 > 94.0	20	4.9	8.1
Diclofenac		8.2	295.0	294.1 > 250.1	30	10	296.1 > 252.1	30	–	–	1.0	–
Ibuprofen		52.4	206.1	205.2 > 161.1	30	10	–	–	–	–	–	–
Ketoprofen		6.4	254.1	253.2 > 209.2	20	5	–	–	–	–	–	–
Naproxen		7.6	230.1	185.2 > 170.1	30	10	229.2 > 185.2[a]	5	229.2 > 170.1[a]	20	2.9	4.5
Salicylic acid		17.0	138.0	137.1 > 93.0	30	15	137.1 > 65.0	25	–	–	21.2	–
Atorvastatin	Cholesterol lowering statin drugs and lipid regulators	0.3	558.3	559.4 > 440.3	45	20	559.4 > 250.2	45	559.4 > 276.2	40	1.3	3.3
Pravastatin		12.4	424.2	423.4 > 321.2	40	15	423.4 > 101.1	30	423.4 > 303.2	20	1.0	2.1
Bezafibrate		1.6	361.1	360.2 > 274.1	30	15	362.2 > 276.2	20	360.2 > 154.0	30	4.1	1.8
Gemfibrozil		12.8	250.2	249.3 > 121.0	30	15	249.3 > 127.0	10	–	–	16.1	–
Paroxetine	Antidepressants	2.9	329.1	330.3 > 70.1	50	20	330.3 > 44.1	30	330.3 > 192.1	20	1.6	2.3
Venlafaxine		0.3	277.2	278.3 > 58.0	30	15	278.3 > 260.3	15	260.3 > 58.0[b]	15	2.1	3.3
Omeprazole	Anti-ulcer agents	1.3	345.1	346.3 > 198.1	30	10	346.3 > 136.1	35	346.3 > 151.1	20	1.3	1.9
Pantoprazole		4.4	383.1	384.2 > 200.1	25	35	384.2 > 138.1	10	384.2 > 153.1	15	1.0	1.6
Olanzapine	Psychiatric drugs	0.5	312.1	313.3 > 256.2	45	25	313.3 > 84.1	25	313.3 > 198.1	35	1.3	10.9
Risperidone		0.7	410.2	411.3 > 191.2	50	30	411.3 > 82.1	60	411.3 > 110.1	50	8.1	8.7
Alprazolam	Ansiolitics	0.3	308.1	309.2 > 281.2	60	25	309.2 > 205.2	40	309.2 > 274.2	25	0.9	3.6
Lorazepam		0.5	321.2	321.2 > 275.1	40	20	323.2 > 277.1	20	321.2 > 303.2	15	1.3	4.4
Enalapril	Cardiovasculars	0.2	376.2	377.4 > 234.2	35	20	377.4 > 91.1	55	377.4 > 160.2	30	1.5	3.1

Abbreviations: MW (monoisotopic molecular weigh), *Q* (quantification), *q* (confirmation), C.E. (collision energy).

[a] Cone voltage: 20 V.

[b] Cone voltage: 40 V.

face (Waters Corp., Milford, MA, USA) was used. Drying gas, as well as nebulising gas, was nitrogen generated from pressurized air in a N_2 LC-MS (Claind, Teknokroma, Barcelona, Spain). Cone gas and desolvation gas flows were set at 60 L/h flow and 1200 L/h, respectively. For operation in MS/MS mode, collision gas was Argon 99.995% (Praxair, Valencia, Spain) with a pressure of 2×10^{-3} mbar in the T-Wave cell. Capillary voltages of −3.0 and 3.5 kV were used in negative and positive ionization mode, respectively. Interface temperature and source temperature were optimized at 500 and 120 °C, respectively. Dwell times of 0.01 s/scan were selected.

Masslynx NT (Microsmass, Manchester, UK) software was used to process quantitative data.

2.4. Recommended procedure

All influent samples (IWW) as well as those effluent (EWW) and surface water samples (SW) with observable suspended particulate matter were centrifuged at 4500 rpm for 5 min before loading the SPE cartridges.

Oasis HLB (60 mg) cartridges were previously conditioned with 3 mL of methanol and 3 mL of HPLC-grade water. 100 mL of water sample were spiked with the mix IS working solution to give a final concentration of 0.1 μg/L for those isotope-labelled IS determined in positive mode (omeprazole-d_3, acetaminophen-d_4, atorvastatin-d_5, paroxetine-d_4, olanzapine-d_3) and of 1 μg/L for those determined in negative mode (salicylic acid-d_3, diclofenac-d_4, ibuprofen-d_3). Then, the sample was passed through the cartridge by gravity (flow rate around 3 mL/min). After drying under vacuum, analytes were eluted with 5 mL of methanol. The extract was evaporated to dryness under a gentle nitrogen stream (40 °C) and finally reconstituted with 1 mL methanol–water (10:90, v/v). Analyses were performed by injecting 20 μL of the final extract in the UHPLC-MS/MS system. Experimental MS conditions are given in Table 1. Quantification of samples was performed using calibration standards in solvent (methanol–water 10:90), which also contained the labelled IS. Thus, relative areas were used for quantification purposes.

2.5. Validation study

Method accuracy (expressed as recovery percentage) and precision (expressed as repeatability in terms of relative standard deviation (RSD)) were evaluated by recovery experiments of target compounds in surface water (SW), effluent wastewater (EWW) and influent wastewater (IWW), spiked at different concentration levels (0.005, 0.025 and 0.05 μg/L in SW; 0.1 and 0.5 μg/L in EWW; 0.25 and 1.25 μg/L in IWW). Experiments were performed by quintuplicate ($n = 5$) for each type of water sample tested and for each spiking level. Recoveries between 70 and 120% with RSD lower than 20% were considered as satisfactory.

The limit of quantification (LOQ) was estimated for a signal-to-noise (S/N) ratio of 10 from SRM chromatograms of samples spiked at the lowest validation level tested, from the quantification transition. In those particular cases where a sample blank was not feasible (several analytes were normally present in all effluent and influent wastewaters), the LOQ was estimated from the "blank" chromatograms without spiking the sample. In this case, the analyte concentration for the peak observed in the "blank" sample was quantified. Then, the LOQ was estimated for S/N = 10 taking into account the analyte concentration found in the "blank". The instrumental limit of detection (LOD) was estimated for S/N = 3 from the chromatograms of standards at the lowest concentration level tested in the calibration curve.

The linearity of the method was studied by analyzing standard solutions in triplicate at concentrations typically ranging from 0.25 to 500 μg/L, although final concentrations tested depended on the sensitivity reached for each analyte. Satisfactory linearity using weighed ($1/X$) least squares regression was assumed when the correlation coefficient (r) was higher than 0.99, based on analyte peak areas measurement, and when residuals were lower than 30% without significant trend.

2.6. Application to real samples

A total number of 84 urban wastewater samples (42 IWW and 42 EWW) were collected in polyethylene high-density bottles and stored at <−18 °C until analysis. Before analysis, samples were thawed at room temperature. Samples consisted on 24-h composite urban wastewater samples, and were collected from three WWTPs of the Castellón province (Benicàssim, Burriana and Castellón de la Plana). Samples were collected along one complete week in two different months (June 2008 and January 2009).

3. Results and discussion

3.1. MS and MS/MS optimization

Full-scan and MS/MS mass spectra were obtained from infusion of 1 mg/L methanol/water (50:50, v/v) individual standard solutions at a flow rate of 10 μL/min. The multi-class characteristics of selected pharmaceuticals made that 12 out of 20 compounds presented positive ionization meanwhile the rest were determined under negative mode. Acetaminophen and ibuprofen were ionized in both negative and positive modes. For the first compound, positive ionization was selected, while negative ionization mode was used for ibuprofen because of the better sensitivity reached under these modes.

All compounds showed an abundant $[M+H]^+$ or $[M-H]^-$ ion. These were selected as precursor ions, except for naproxen that showed better sensitivity when using an in-source fragment as precursor ion by increasing the cone voltage. For venlafaxine an additional sensitive transition was also obtained under in-source fragmentation (see Table 1).

For diclofenac, bezafibrate and lorazepam the presence of one chlorine atom in their structure allowed using two different precursor ions (corresponding to ^{35}Cl and ^{37}Cl isotopes, respectively).

In this work, a fast-acquisition triple quadrupole analyzer has been used. It allows reducing dwell times and increasing the number of SRM transitions acquired simultaneously. Dwell times as low as of 0.01 s could be used without resolution and/or sensitivity losses. This made feasible to acquire three simultaneous SRM transitions for each compound to assure a reliable identification. 4 out of 20 analytes showed poor fragmentation (ibuprofen, ketoprofen, salicylic acid and diclofenac). For these specific compounds, only one or two transitions could be monitored.

In order to acquire at least 10 points per peak and to ensure that enough time was spent on each transition to avoid data loss, SRM transitions where divided into seven overlapping elution-time windows (four elution windows for compounds determined under positive ionization mode and three windows for those under negative mode). It is worth to mention that the low positive-to-negative-switching time (0.02 s) of the tandem mass instrument used in our work allows this favourable overlapping between positive and negative time windows.

Mass spectrometry parameters selected, precursor and product ions, as well as instrumental LODs are shown in Table 1.

3.2. Chromatography optimization

In order to optimize the chromatographic separation, different mobile phases (methanol and acetonitrile) with different addi-

tives (HCOOH and NH_4Ac at various concentrations) were tested. A short UPLC BEH column (50 mm × 2.1 mm, 1.7 μm) was chosen. It allowed performing an efficient chromatographic separation for all the 20 analytes in only 7 min.

For those compounds determined under positive ionization, sensitivity improved when NH_4Ac was added, both in water and in methanol mobile-phase solvents. For those compounds determined in negative ionization mode, the use of mobile phases without any additive provided better ionization yield, but it resulted in a non-desirable peak shape. This problem was solved by adding NH_4Ac, which allowed improving the poor chromatographic behaviour of these compounds.

The addition of formic acid (0.01% HCOOH) improved the chromatographic separation (reduction of peak tailing and better resolution) of several compounds measured in positive mode. It also favoured the retention of the negatively charged compounds in the LC column. Therefore, methanol and water, both containing 0.1 mM NH_4Ac and 0.01% HCOOH, were finally chosen as mobile phases for the simultaneous chromatographic separation of both positive and negative ionized analytes.

Enalapril exhibited two poor resolved peaks because it is present as a mixture of cis- and trans-conformers around the amide bond [21,24]. To obtain a single peak for quantitative analysis of enalapril, the column temperature was increased from 40 to 60 °C without affecting the chromatographic separation of the other compounds.

3.3. Solid-phase extraction

A detailed study was carried out on the most relevant parameters – type of sorbent, pH of the sample and elution conditions – that affect the recovery of target compounds.

First of all, the extraction efficiency of two cartridges was tested using HPLC-grade water spiked with the analytes. Cartridges used were Oasis HLB (200 mg) and Oasis MCX (150 mg). Oasis HLB was tested at four pH values (8.5, 7, 4.5 and 2) while Oasis MCX, a mixed polymeric-cation exchange sorbent, was tested at pH 2, by acidifying the water sample with HCOOH. As methanol seems to be an efficient solvent for the elution of polar contaminants from different SPE cartridges, it was chosen for elution when evaluating the SPE process [25]. The vast majority of compounds determined in negative mode showed satisfactory recoveries using both cartridges, except for salicylic acid, which was partially lost during the SPE process. However, the best recoveries for pharmaceuticals determined in positive mode were obtained with Oasis HLB. Therefore, HLB cartridge was chosen for subsequent experiments. The performance of the sorbents tested at different pHs for all analytes is summarized in Fig. 1.

Regarding the effect of sample pH, satisfactory data were obtained using Oasis HLB cartridges at pH 7 for most compounds, although olanzapine, 4-aminoantipyrine and atorvastatin were poorly recovered. A slight improvement was observed for 4-aminoantipyrine and atorvastatin at pH 8.5, but this pH affected negatively to the recoveries of omeprazole and pantoprazole.

The difficulty for extraction of atorvastatin, the pharmaceutical most commonly used for the treatment of hypercholesterolemia, has been related to its instability, due to a possible interconversion of the lactone and acidic form [26,27]. Therefore, pH is one of the most important variables to minimize this interconversion. In this work, the potential problems associated to this analyte were solved by using its own isotope-labelled IS, obtaining satisfactory recoveries in all matrices tested.

As the objective of this work was to simultaneously extract the 20 selected acidic, neutral and basic pharmaceuticals, with quite different physico-chemical characteristics, SPE at pH 7 with Oasis HLB was selected as a compromise.

In order to determine if low recoveries for olanzapine, 4-aminoantipyrine and atorvastatin were consequence of exceeding the breakthrough volume, different volumes (10, 25, 50 and 100 mL) of spiked water were passed through the HLB cartridges at pH 7. Similar results were obtained in all cases; therefore, the volume of water samples was maintained at 100 mL.

Once the type of cartridge and sample pH was selected, three elution solvents (methanol, acetone and acetonitrile) were evaluated. Using acetone, enalapril and paroxetine were partially recovered. Methanol and acetonitrile did not show relevant differences although recoveries were slightly better when eluting with methanol; so, this solvent was selected for elution (5 mL).

A comparison between Oasis HLB 60 and 200 mg was carried out, eluting with 5 and 10 mL methanol, respectively. Similar results were obtained for all compounds, except for atorvastatin and risperidone, which recoveries were slightly improved using HLB 60 mg cartridge. A possible explanation might be that these analytes were less retained in 60 mg cartridges, and subsequently they might be more easily eluted. Finally, Oasis HLB 60 mg cartridges were selected. This allowed reducing solvent volume and the time necessary to evaporate the extract.

The slightly high recovery for paroxetine (around 130%) might be due to unknown compounds released from the SPE cartridges that would coelute with this analyte producing a slightly signal enhancement [28], as the blank performed with HPLC-grade water did not show any interferent peak. In any case, the use of paroxetine isotope labelled as surrogate IS allowed us to obtain satisfactory recoveries when the method was applied to spiked real-world samples (see next section).

3.4. Method validation

Analytical characteristics of the method were evaluated in three types of water samples (SW, EWW and IWW) that were spiked at different concentrations.

Linearity was studied in the range 0.25–500 μg/L for all selected compounds. Depending on the sensitivity reached for each analyte different linear responses were obtained: (1) alprazolam, lorazepam, enalapril, omeprazole, atorvastatin, venlafaxine, risperidone and 4-aminoantipyrine showed satisfactory linearity along this range; (2) acetaminophen, olanzapine, pantoprazole, diclofenac, bezafibrate and gemfibrozil showed linear response from 1 to 500 μg/L; (3) the rest of compounds showed good results in the range 5–500 μg/L. In all these cases, residuals were below 30% and correlation coefficients by linear or quadratic curves (risperidone) were greater than 0.99.

Accuracy and precision were estimated from recovery experiments of target analytes at different concentration levels. Recoveries were determined by comparing the concentrations obtained in spiked samples after applying the recommended procedure, using calibration curves with standards in solvent. Several isotope-labelled ISs were added as surrogates in order to compensate compounds' losses during the SPE process and/or matrix effects. In the case of EWW and IWW, it was not feasible to get a true blank, as all samples analyzed contained one or more analytes included in this work. So, EEW and IWW were previously analyzed and the concentrations found for target compounds present in the "blank" samples were subtracted from the spiked samples. It must be taken into account that subtracting the analyte amount present in the "blank" sample normally leads to higher errors in the recovery and RSD calculation.

The method was tested at three fortification levels in SW. As Table 2 shows, the 0.025 μg/L level could not be validated for four analytes determined in negative mode due to their lower sensitivity, but all of them were determined satisfactorily at 0.05 μg/L

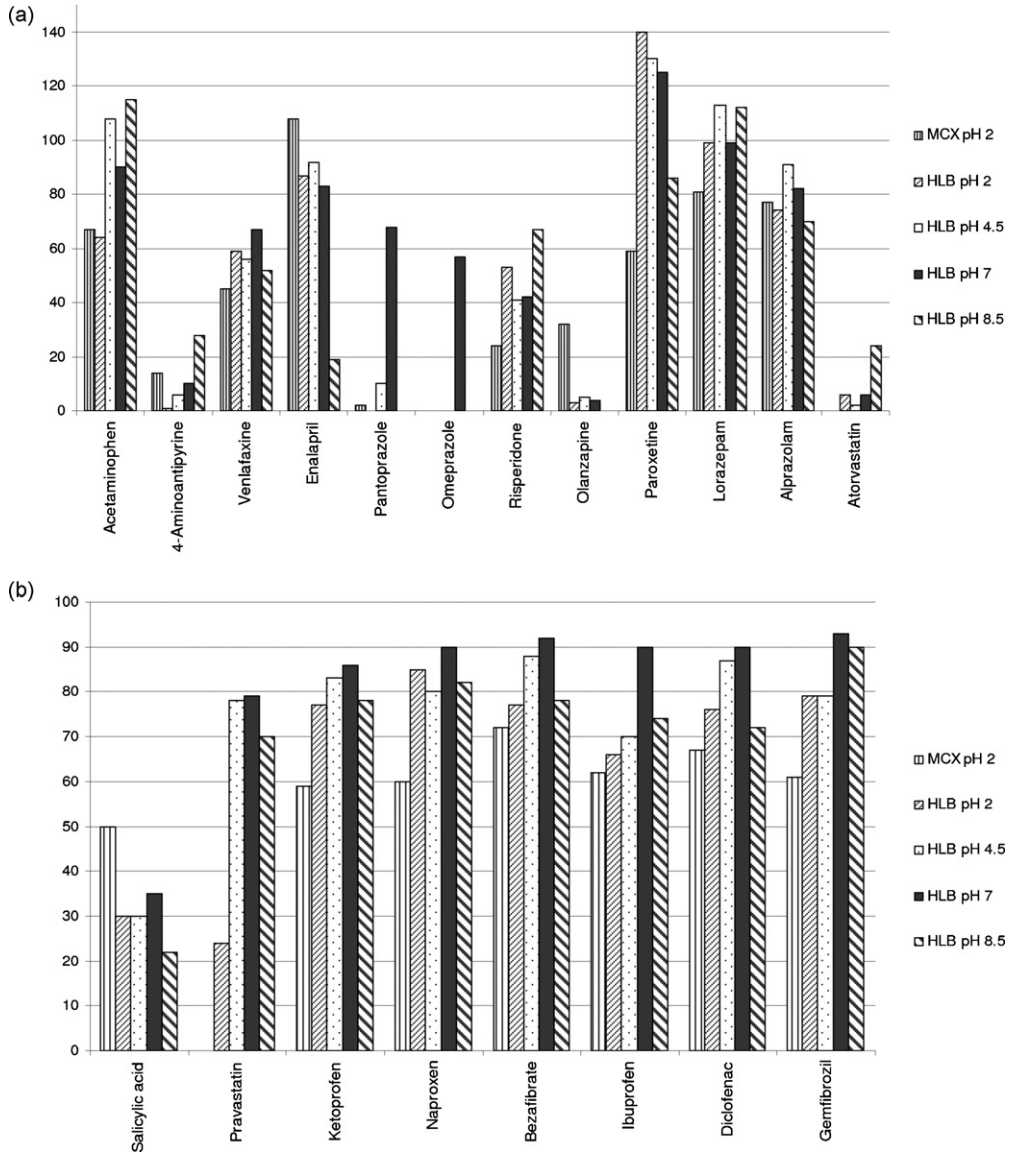

Fig. 1. Recoveries obtained after extraction of selected analytes with Oasis HLB (200 mg) and Oasis MCX (150 mg) cartridges at different sample pH values. (a) Compounds analyzed in positive mode and (b) compounds analyzed in negative mode.

(recovery from 70 to 120%). The high sensitivity typically observed for analytes determined in positive mode allowed us validating satisfactorily the method at a level as low as 0.005 μg/L. All compounds, which isotope-labelled IS was available, were quantified using its corresponding analyte-labelled IS. The rest of selected compounds, as matrix effects in surface water tested were not much relevant, could be quantified without using IS. Only 4-aminoantipyrine presented low recoveries at all fortification levels due to SPE pre-concentration losses that could not be properly corrected.

As can be seen in Table 3, recoveries and precision in effluent wastewater were satisfactory at 0.1 μg/L for 16 compounds out of 20 tested. 4-Aminoantipyrine and gemfibrozil were not validated at this level due to high concentration found in the "blank" sample (around 8–10 times higher than the spiking level). Salicylic acid and ibuprofen could not be validated at the lowest level assayed due to the lower sensitivity observed for these compounds.

As expected, method validation in influent wastewater was the most complicated case, especially at the lowest fortification level. Because of the impossibility to obtain true blanks, several samples were previously analyzed and that sample containing the lowest pharmaceutical concentration levels was selected for validation. Due to their high complexity and elevated organic matter content, it was necessary to dilute five times the IWW samples before validation. Spiking levels tested were 0.25 and 1.25 μg/L in the non-diluted raw sample (i.e. 0.05 and 0.25 μg/L in the 5-fold diluted sample). In general, recoveries and precision were satisfactory for most compounds at both fortification levels. 4-Aminoantipyrine, naproxen, ibuprofen and atorvastatin could not be validated at the low level due to high concentrations found in the "blank". As can be seen in Table 4, at 1.25 μg/L concentration level, a few compounds (salicylic acid, risperidone, ibuprofen, lorazepam and atorvastatin) showed recoveries around 120%, but precision was satisfactory in all cases (RSD < 10%).

The high complexity of sample matrix in wastewater samples (both EWW and IWW), affected considerably the recovery values of many compounds. Thus, in this type of samples, the use of IS to correct matrix effects was compulsory. This led to recoveries mostly within the desired range of 70–120%. Each compound

Table 2
Method validation for surface water (SW). Recovery (%) and relative standard deviation (RSD%) for five replicates.

Compound	Polarity (ES)	t_R (min)	0.005 μg/L		0.025 μg/L		0.05 μg/L		LOQ (ng/L)	I.S. used
			Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)		
Acetaminophen	+	1.60	[a]	–	115	3	107	3	9.3	Acetaminophen-d_4
Olanzapine	+	2.66	[a]	–	63	11	68	11	5.7	Olanzapine-d_3
4-Aminoantipyrine	+	2.76	55	5	48	1	27	1	0.8	
Salicylic acid	–	3.13	[a]	–	[a]		99	15	44	Salicylic acid-d_4
Risperidone	+	3.18	82	9	76	11	88	6	2.0	
Venlafaxine	+	3.30	70	8	70	4	110	2	0.2	
Enalapril	+	3.76	84	5	90	3	97	6	3.3	
Omeprazole	+	3.80	83	8	77	9	85	7	3.2	Omeprazole-d_3
Pantoprazole	+	3.83	[a]	–	87	15	88	13	20	
Paroxetine	+	3.76	[a]	–	94	9	97	9	19	Paroxetine-d_4
Lorazepam	+	4.39	93	19	86	2	96	2	4.3	
Alprazolam	+	4.42	82	8	78	4	83	4	2.9	
Pravastatin	–	4.40	[a]	–	[a]	–	90	22	25	
Ketoprofen	–	4.57	[a]	–	[a]	–	83	5	23	
Naproxen	–	4.69	[a]	–	76	12	103	6	21	
Bezafibrate	–	4.81	[a]	–	95	10	99	10	7.6	
Atorvastatin	+	5.52	101	13	100	7	106	4	0.8	Atorvastatin-d_5
Diclofenac	–	5.58	[a]	–	117	12	90	7	11	Diclofenac-d_4
Ibuprofen	–	5.75	[a]	–	[a]		106	12	39	Ibuprofen-d_3
Gemfibrozil	–	6.16	[a]	–	80	14	93	5	12	

Abbreviations: ES (electrospray ionization). t_R (retention time).
[a] Not estimated due to the low sensitivity at the fortification level tested.

was corrected with its labelled IS, when available, and the others were corrected with ISs eluting at closer retention times. For example, gemfibrozil and pravastatin were corrected using an analogue IS (diclofenac-d_4), obtaining satisfactory recoveries. Alprazolam, lorazepam, risperidone and enalapril could be quantified without using IS correction with acceptable recoveries and precision (see Table 3).

The method presented satisfactory precision for all type of water samples with most RSD values below 15%. LOQs were estimated for the three water samples tested. LOQs ranged from 0.2 to 25 ng/L for SW, from 3.6 to 85 ng/L for EWW and from 8.6 to 200 ng/L for IWW. The two exceptions were salicylic acid and ibuprofen, as the sensitivity was lower for these compounds, with the result of higher LOQs (see Tables 2–4). Concerning instrumental LODs, they ranged from 0.2 to 13 pg.

3.5. Application to environmental water samples

The method developed in this paper was applied to the analysis of 84 urban wastewater samples (42 influents and 42 effluents) (see Table 5).

In every sequence of analysis, the calibration curve was injected twice, at the beginning and at the end. Two quality control samples (QCs), i.e. a "blank" water sample (previously analyzed) fortified at the two validated levels, were also analyzed for quality control. QC recoveries were considered satisfactory if they were in the range 70–120% for every analyte.

Confirmation of positive findings was carried out by calculating the peak area ratios between the quantification (Q) and the two confirmation (q_1 and q_2) transitions, and comparing them with ion-ratios from a reference standard. The finding was con-

Table 3
Method validation for effluent wastewater (EWW). Recovery (%) and relative standard deviation (RSD%) for five replicates.

Compound	Polarity (ES)	t_R (min)	0.1 μg/L		0.5 μg/L		LOQ (ng/L)	I.S. used
			Recovery (%)	RSD (%)	Recovery (%)	RSD (%)		
Acetaminophen	+	1.60	114	4	103	2	85	Acetaminophen-d_4
Olanzapine	+	2.66	66	9	70	5	11	Olanzapine-d_3
4-Aminoantipyrine	+	2.76	[a]	–	79	2	44[b]	Acetaminophen-d_4
Salicylic acid	–	3.13	[c]	–	104	9	427	Salicylic acid-d_4
Risperidone	+	3.18	114	9	91	4	5.9	
Venlafaxine	+	3.30	88	12	91	4	3.6	Omeprazole-d_3
Enalapril	+	3.76	80	1	87	1	6.6	
Omeprazole	+	3.80	88	13	79	6	11	Omeprazole-d_3
Pantoprazole	+	3.83	103	18	89	3	33[b]	Omeprazole-d_3
Paroxetine	+	3.76	92	3	91	5	43	Paroxetine-d_4
Lorazepam	+	4.39	85	2	87	2	30[b]	
Alprazolam	+	4.42	75	4	78	3	11	
Pravastatin	–	4.40	96	13	70	3	22	Diclofenac-d_4
Ketoprofen	–	4.57	87	12	84	15	72[b]	Diclofenac-d_4
Naproxen	–	4.69	91	10	84	8	30[b]	Diclofenac-d_4
Bezafibrate	–	4.81	86	13	86	4	9.5[b]	Diclofenac-d_4
Atorvastatin	+	5.52	106	4	99	2	7.4	Atorvastatin-d_5
Diclofenac	–	5.58	83	8	84	2	53[b]	Diclofenac-d_4
Ibuprofen	–	5.75	[c]	–	120	15	247	Ibuprofen-d_3
Gemfibrozil	–	6.16	[a]	–	102	17	18[b]	Diclofenac-d_4

Abbreviations: ES (electrospray ionization). t_R (retention time).
[a] Not estimated due to the high analyte levels found in the "blank" sample (around 0.8 μg/L for 4-aminoantipyrine and 0.9 μg/L for gemfibrozil).
[b] LOQ determined from the "blank" sample chromatogram (non-spiked).
[c] Not estimated due to the low sensitivity at the fortification level tested.

Table 4
Method validation for influent wastewater (IWW). Recovery (%) and relative standard deviation (RSD%) for five replicates.

Compound	Polarity (ES)	t_R (min)	0.25 μg/L		1.25 μg/L		LOQ (ng/L)	I.S. used
			Recovery (%)	RSD (%)	Recovery (%)	RSD (%)		
Acetaminophen	+	1.60	127	8	105	3	112[a]	Acetaminophen-d_4
Olanzapine	+	2.66	66	7	68	8	13	Olanzapine-d_3
4-Aminoantipyrine	+	2.76	[b]	–	113	5	31[a]	Acetaminophen-d_4
Salicylic acid	–	3.13	[c]	–	123	5	974	Salicylic acid-d_4
Risperidone	+	3.18	86	6	120	5	8.6	
Venlafaxine	+	3.30	78	7	93	5	10	Omeprazole-d_3
Enalapril	+	3.76	112	14	95	6	23	
Omeprazole	+	3.80	102	7	102	2	29	Omeprazole-d_3
Pantoprazole	+	3.83	121	24	100	8	66	Omeprazole-d_3
Paroxetine	+	3.76	68	17	89	6	196	Paroxetine-d_4
Lorazepam	+	4.39	120	8	117	2	54	
Alprazolam	+	4.42	97	5	91	3	33	
Pravastatin	–	4.40	[c]	–	99	8	118	Diclofenac-d_4
Ketoprofen	–	4.57	55	8	71	4	109[a]	Diclofenac-d_4
Naproxen	–	4.69	[b]	–	100	6	49[a]	Diclofenac-d_4
Bezafibrate	–	4.81	113	8	98	7	20[a]	Diclofenac-d_4
Atorvastatin	+	5.52	[b]	–	116	1	31[a]	Atorvastatin-d_5
Diclofenac	–	5.58	112	13	98	9	137[a]	Diclofenac-d_4
Ibuprofen	–	5.75	[b]	–	124	6	642[a]	Ibuprofen-d_3
Gemfibrozil	–	6.16	118	6	96	6	48[a]	Diclofenac-d_4

Abbreviations: ES (electrospray ionization). t_R (retention time).
[a] LOQ determined from the "blank" sample chromatogram (non-spiked).
[b] Not estimated due to the high analyte levels found in the "blank" sample (around 1.7 μg/L for 4-aminoantipyrine. 1.0 μg/L for naproxen, 0.5 μg/L for atorvastatin and 6.9 μg/L for ibuprofen).
[c] Not estimated due to the low sensitivity at the fortification level tested.

sidered positive when experimental ion-ratios were within the tolerance range [22,23]. In spite that two SRM transitions per compound are normally considered sufficient for a reliable confirmation of the compound identity, in this work we acquired three transitions in order to increase the confidence of the confirmation process. This is in the line of our previous work, where we have described some drawbacks when using two transitions [29].

It is interesting to mention the advantages of acquiring three SRM transitions per analyte. Fig. 2 shows the UHPLC-MS/MS chromatograms for bezafibrate reference standard (Fig. 2a) and for an effluent wastewater sample that might had been reported as negative for bezafibrate if only the q_1 transition had been acquired (Fig. 2b). The reason for doubting about this positive (or negative) sample was that the ion-ratio was out of the tolerance range. However, the second confirmation transition (q_2) was in agreement with the reference standard indicating that the sample was positive to bezafibrate actually. It seemed that q_1 transition was interfered by a co-eluting isobaric compound sharing one product ion (m/z 276) with the analyte. Under these circumstances, the acquisition of the third transition allowed us to confirm the presence of bezafibrate in the sample.

Table 5
Summary of the results obtained in the monitoring of pharmaceuticals in influent and effluent wastewater from three urban WWTP of the Castellón province (total number of samples analyzed 84).

Compound	Therapeutic group	Influent wastewater (n = 42)		Effluent wastewater (n = 42)	
		% positive findings	Maximum level (μg/L)	% positive findings	Maximum level (μg/L)
Acetaminophen	Analgesic and anti-inflamatories	100	201.3[a]	0	n.d.
4-Aminoantipyrine (metabolite of metamizol)		100	6.45	100	1.68
Diclofenac		100	1.49	100	0.74
Ibuprofen		98	39.8[a]	33	<LOQ
Ketoprofen		100	1.17	100	0.62
Naproxen		100	3.58	100	0.72
Salicylic acid		76	276.7[a]	26	236.1[a]
Atorvastatin	Cholesterol lowering statin drugs and lipid regulators	100	0.45	76	0.16
Pravastatin		26	0.24	30	0.17
Bezafibrate		100	0.46	100	0.39
Gemfibrozil		100	2.12	100	1.24
Paroxetine	Antidepressants	0	n.d.	0	n.d.
Venlafaxine		100	0.52	100	0.30
Omeprazole	Anti-ulcer agents	0	n.d.	43	0.10
Pantoprazole		0	n.d.	65	0.18
Olanzapine	Psychiatric drugs	0	n.d.	0	n.d.
Risperidone		0	n.d.	0	n.d.
Alprazolam	Ansiolitics	0	n.d.	38	<LOQ
Lorazepam		0	n.d.	55	0.06
Enalapril	Cardiovasculars	96	0.29	0	n.d.

[a] Samples were previously diluted to fit to the linearity range of the method.

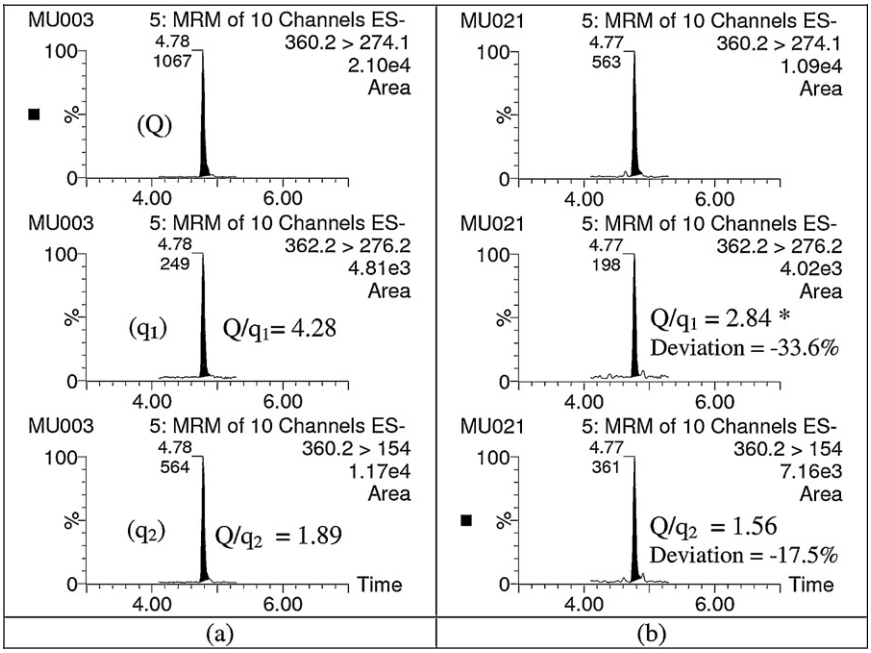

Fig. 2. Selected UHPLC-MS/MS chromatograms for (a) bezafibrate reference standard (5 μg/L) and (b) effluent wastewater sample (0.05 μg/L of bezafibrate). Quantification transition (*Q*), confirmation transitions (q_1 and q_2). *Ion-ratio deviation out of tolerance.

Analytes were quantified as described in the previous sections. However, in only a few EWW and IWW samples, QCs recoveries for venlafaxine were not satisfactory using omeprazole-d_3 as IS (>150%). This fact is accordance to other studies published about the use of analogues IS [8,30,31]. Only using the own analyte-labelled IS assures a satisfactory correction in all types of samples, because the use of analogues IS does not always assures an efficient matrix effects correction.

Analgesics and anti-inflamatories, cholesterol lowering statin drugs and lipid regulators were the major groups detected in urban wastewater. The highest concentrations corresponded to acetaminophen, salicylic acid and ibuprofen in IWW. In relation to analgesics and anti-inflamatories, acetaminophen was found in all IWW analyzed, and 84% of IWW samples had to be diluted and re-analyzed to fit the linearity range of the method. However, this compound was not detected in effluent wastewater. Diclofenac, naproxen, ketoprofen and 4-aminoantipyrine were present in all IWW and EWW samples analyzed at concentration levels normally in the range of high ng/L or low μg/L. Among them, the highest concentrations corresponded to 4-aminoantipyrine with average concentrations of 2.78 μg/L in IWW and 0.89 μg/L in EWW.

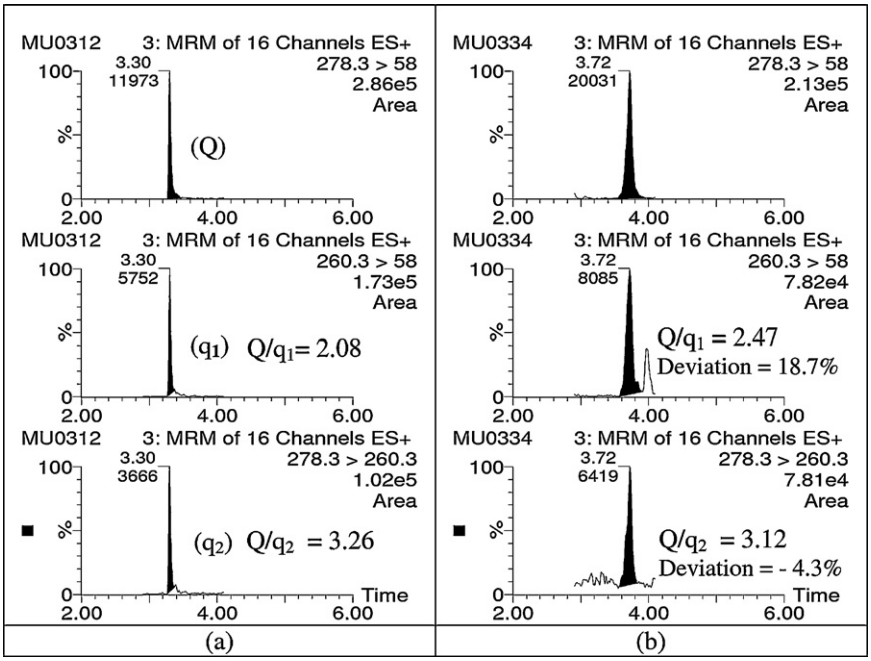

Fig. 3. Selected UHPLC-MS/MS chromatograms for (a) venlafaxine reference standard (5 μg/L) and (b) influent wastewater sample (0.3 μg/L of venlafaxine). Quantification transition (*Q*), confirmation transitions (q_1 and q_2).

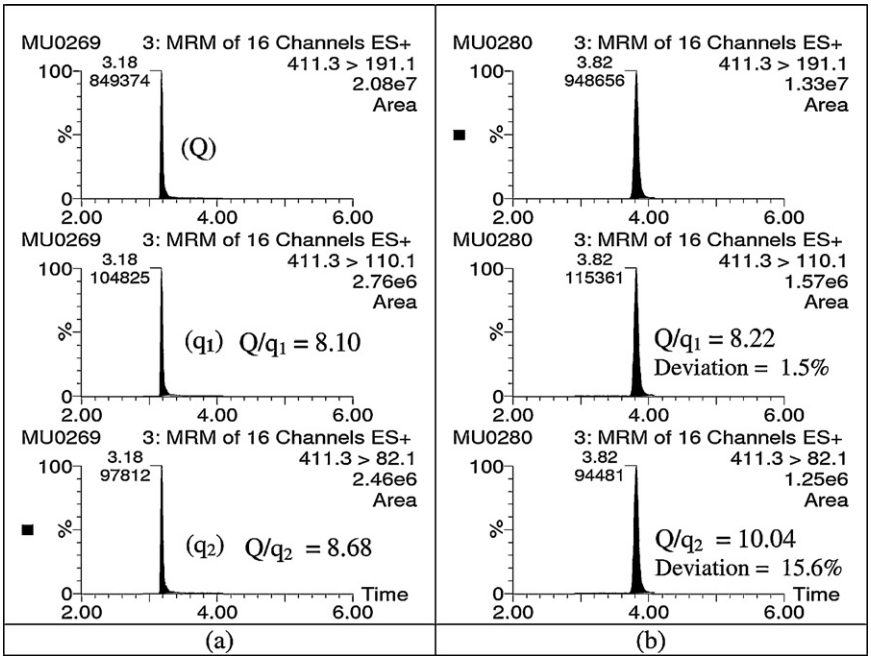

Fig. 4. Selected UHPLC-MS/MS chromatograms for (a) risperidone reference standard and (b) influent wastewater spiked at the highest level validated. Quantification transition (Q), confirmation transitions (q_1 and q_2).

Concerning lipid regulators, gemfibrozil and bezafibrate were present in all influent and effluent samples analyzed. The highest concentrations were found for gemfibrozil in IWW, with an average value of 1.38 μg/L, while in EWW the average level was 0.57 μg/L. Atorvastatin, the most consumed statin pharmaceutical, was also present in most of IWW and EWW, although its levels were lower than for lipid regulators.

A general overview to EWW pharmaceuticals data show that salicylic acid was by far the compound present at highest levels. Diclofenac, naproxen, ketoprofen and 4-aminoantipyrine and other compounds like venlafaxine, lorazepam and pantoprazole were frequently detected in EWW, although normally at concentrations below 0.5 μg/L.

Three of the compounds selected in this work were not detected in neither IWW nor EWW: the antidepressant paroxetine and the psychiatric drugs olanzapine and risperidone. It seems that searching for metabolites of these three compounds is necessary to follow their impact on aquatic environment.

When analyzing water samples with high matrix load, like urban influent wastewater, chromatographic retention time shifts may occur. Under this situation, the acquisition of additional confirmatory SRM transitions could help for analyte confirmation. As an example, Fig. 3 shows an influent wastewater sample positive for venlafaxine. The analyte retention time differed notably between the standard (3.30 min) and the sample (3.72 min). However, the ion-ratio when using the q_1 transition was within tolerance range.

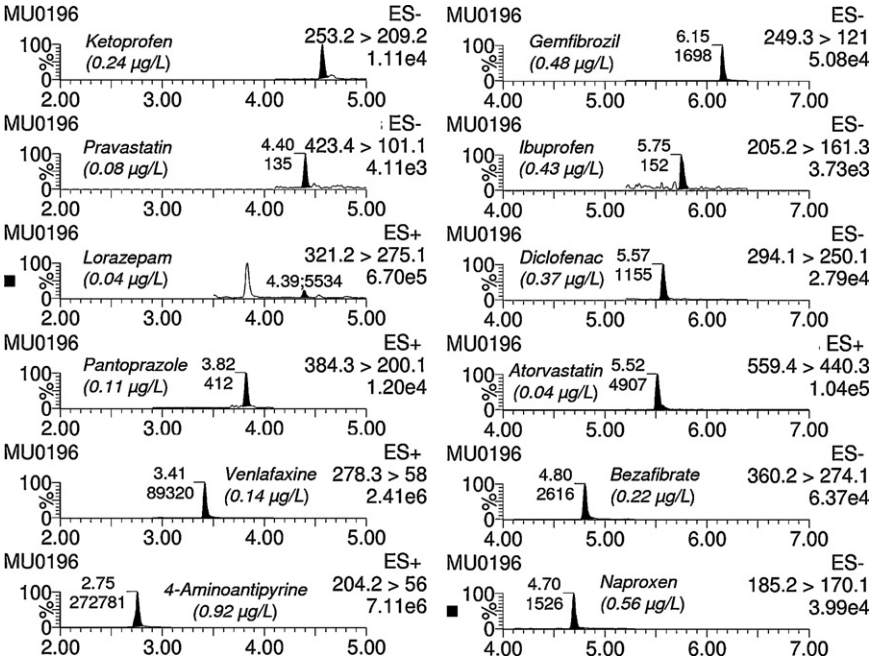

Fig. 5. UHPLC-MS/MS chromatograms (quantification transition) for an effluent wastewater sample (Burriana, January 2009).

This apparent contradiction was solved by acquiring a second confirmation transition (q_2) that allowed us to assure that the sample was positive for venlafaxine, as the second ion-ratio also was in agreement with the reference standard.

This fact was observed for other analytes as well, especially in IWW. Fig. 4 shows another illustrative example, where chromatograms of the reference standard (Fig. 4a) and the influent QC sample (Fig. 4b) presented different retention times for risperidone.

In all influent wastewater samples, strong signal suppression was observed for olanzapine and its IS (olanzapine-d_3). The IS signal even disappeared in some samples, notably decreasing sensitivity for this compound in IWW.

In the light of our preliminary data, WWTPs seemed to have good removal efficiency for some compounds, e.g. acetaminophen and enalapril. For the rest of pharmaceuticals, although their concentrations were lower than in IWW, positive samples were still found in EWW, and in some particular cases pharmaceuticals concentrations were slightly higher in the effluent.

As an illustrative example, Fig. 5 shows the UHPLC-MS/MS chromatograms for an effluent water sample (only quantitative transition is shown). As can be seen, the sample was positive for 12 out of 20 target compounds. However, none of the pharmaceuticals detected exceeded 1 μg/L.

4. Conclusions

Rapid, selective and sensitive analytical methodology, based on the use of UHPLC-MS/MS with triple quadrupole analyzer has been developed for the simultaneous multi-class determination of 20 acidic, neutral and basic pharmaceuticals in urban wastewater and surface water. Target analytes were selected among the most widely consumed in Spain and corresponded to the therapeutic groups of analgesic and anti-inflamatories, cholesterol lowering statin drugs and lipid regulators, antidepressants, antiulcer agents, psychiatric drugs, ansiolitics and cardiovasculars. The method allows the simultaneous extraction of all selected analytes, with quite different physico-chemical characteristics, in a single step using Oasis HLB cartridges at neutral pH. The use of fast-acquisition triple quadrupole analyzer makes feasible selecting short dwell times (0.01 s) and acquiring up to three simultaneous SRM transitions per compound to assure a reliable identification for all analytes. Thanks to the use of both, UHPLC and this MS analyzer, the safe quantification and identification of all analytes is feasible at very low concentration levels with a chromatographic run of only 9 min. Thus, it is not necessary to perform two analyses, for positive and negatively ionized compounds, as all of them can be determined in only one injection using an optimized mobile phase for positive and negative analytes. The method has been validated in three types of water at different concentrations depending on the sensitivity reached for every analyte/matrix combination. Illustrative of the excellent sensitivity of the method is that a level as low as 5 ng/L could be validated in surface water, still detecting the three SRM transitions acquired per analyte. Recoveries for most selected compounds were higher than 70% with very few exceptions. In urban wastewater the use of labelled internal standards has allowed a satisfactory correction of matrix effects that suffered most pharmaceuticals, mainly in influent wastewater.

The developed method has been applied to monitor pharmaceuticals in influent and effluent wastewater 24-h composite samples collected at two different seasons, showing a widespread occurrence of pharmaceuticals. The advantages of acquiring three SRM transitions per analyte for a reliable identification have been illustrated in several cases. The third transition helped us to confirm the presence of bezafibrate in EWW, where the ion-ratio of the other transition was out of tolerance limits. In influent wastewater a notable shift in chromatographic retention times was observed in positive samples. Again, the use of three SRM transitions reinforced reliability in the analyte confirmation process.

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