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Analysis by liquid chromatography–electrospray ionization tandem mass spectrometry and acute toxicity evaluation for β-blockers and lipid-regulating agents in wastewater samples☆

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Abstract

This paper describes a multiresidue method for the extraction and determination of two therapeutic groups of pharmaceuticals, lipid-regulating agents (clofibric acid, bezafibrate, gemfibrocil, fenofibrate) and β-blockers (atenolol, sotalol, metoprolol, betaxolol) in waters by solid-phase extraction followed by liquid chromatography-electrospray ionization tandem mass spectrometry (LC–ESI-MS–MS). Recoveries obtained from spiked HPLC water, as well as, from spiked real samples (sewage treatment plants influent and effluents, river and tap water) were all above 60%, with the exception of betaxolol with a 52% recovery. The quantitative MS analysis was performed using a multiple reaction monitoring. The LC–MS–MS method gave detection limits ranging from 0.017 to 1.25 μg/l in spiked effluent. Precision of the method, calculated as relative standard deviation, ranged from 3.7 to 18.5%. Individual and combined effects on *Daphnia magna* were evaluated for both therapeutic groups. Individual effects in culture medium showed these compounds as not harmful and not toxic, an exception is fenofibrate that was found to be harmful, but at high, in the environment unrealistic concentrations (EC_{50} of 50 mg/l). Combined effect in wastewater showed synergistic toxic effects at low concentration level (2 μg/l).

Keywords: Water analysis; Lipid-regulating agents; Environmental analysis; Toxicity; *Daphnia magna*; β-Blockers

1. Introduction

During the last years, the occurrence of pharmaceuticals is an emerging issue in environmental research. In recent investigations carried out in Europe and the USA, more than 80 compounds including pharmaceuticals and drug metabolites have been detected in the aquatic environment [1]. Several of these compounds are among the most frequently prescribed drugs. Lipid-regulating agents, which are used for the treatment of hypercholesterolemia, are the leading therapeutic group with worldwide sales of $26.1 billion in 2003 [2]. β-Blockers are other group extensively used to treat angina and hypertension and they are in the top 200 prescribed medications in the USA [3,4]. Pharmaceutical residues enter wastewater treatment facilities and the incomplete removal in sewage treatment plants (STPs) is pointed out by many authors, as the major source of discharge of these compounds to the environment [5–7]. For example, Stumpf et al. found that the removal of lipid-regulating agents (clofibric acid, gemfibrocil or bezafibrate) from a conventional STP was between 34 and 50% [8]. Trace concentrations of lipid-regulating agents (up to low μg/l level) have been reported in sewage effluents, groundwater, surface waters [5,8–11], whereas in drinking water clofibric acid has been detected in concentrations of

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up to 270 ng/l [12,13]. Also, several β-blockers (metoprolol, propanolol, betaxolol, bisoprolol or nadolol) have been detected in municipal sewage effluents in low μg/l level [5,14,15].

Although these compounds can be degraded in the environment, it is assumed that they could act as persistent compounds simply because of their continual infusion into aquatic media via STP effluents [16,17]. It has been hypothesized that pharmaceuticals released into the environment could have subtle effects on aquatic wildlife and humans [18,19]. Few scientific indications are available on ecotoxicity or the potential adverse effects on humans of indirect exposure to pharmaceuticals via drinking water [20,21]. The available toxicity data indicate that high, environmentally unrealistic concentrations will be needed to cause negative effects on aquatic species [22,23]. The range of reported acute ecotoxicity endpoints vary from >100 mg/l for metoprolol to >200 mg/l for clofibric acid [24,25]. Generally, available acute toxicity data reveal no observed negative effects for these pharmaceuticals at concentrations below 25 μg/l, however, the lack of data clearly indicates that the ecotoxicity of pharmaceuticals at environmentally relevant concentrations is a major unaddressed area.

Due to the importance of identifying such emerging risks there is an increased interest in the development of sensitive and selective analytical methods for the determination of pharmaceutical residues in the environment. To date, only few studies have published the detection of β-blockers in surface and ground waters at ng/l level [5,26]. Lipid-regulating agents or β-blockers are thermo labile and non-volatile, and previously have been analyzed by GC–MS (or MS–MS) after derivatization, which makes the sample preparation laborious and time consuming, increases the possibility of contaminations and errors and may lead to degradation of labile compounds [27,28]. LC–MS is more suitable to analyze polar and thermo labile compounds such as β-blockers (e.g. atenolol or sotalol) due to an incomplete derivatization of the functional groups [27]. For environmental analysis, LC–tandem MS is considered as one of the most powerful techniques for structural identification and quantitation because of its specificity and selectivity.

The objective of this work was to develop a LC–tandem MS method for the determination of β-blockers and lipid-regulating agents in environmental and wastewaters. The method includes the use of SPE that was used for preconcentration and cleanup of the sample in order to improve the detection of the pharmaceuticals. Also, this work offers acute toxicity data on aquatic organism *Daphnia magna*, for both therapeutic groups, in culture medium and wastewaters.

2. Experimental

2.1. Pharmaceutical standards and reagents

The lipid-regulating agents studied were: clofibric acid, bezafibrate, fenofibrate and gemfibrocil. The β-blockers analyzed were sotalol, atenolol, metoprolol and betaxolol. All pharmaceutical standards were of analytical grade (>90%) and purchased from Sigma (St. Louis, MO, USA). Stock solutions of the standards were prepared in methanol and stored at −20 °C. HPLC-grade acetonitrile (ACN), methanol and water (LiChrosolv) were supplied by Merck (Damstadt, Germany).

2.2. Sampling and sample preparation

Water samples were collected in amber glass bottles prerinsed with ultra-pure water. The different matrices (wastewater, river and tap water) used in this study were filtered with Rundfilter filter paper (Teknokroma, Barcelona, Spain) to eliminate the suspended matter and then filtered with the 0.45 μm nylon membrane filter (Teknokroma). The samples were stored at 4 °C until SPE extraction, which was performed within 24 h in order to avoid any degradation.

The SPE procedure was performed using Isolute SPE C_{18} (EC) cartridges (3 ml, 500 mg) from IST (Glamorgan, UK). The cartridges were preconditioned with 5 ml of MeOH and 3 ml of ultrapure water (HPLC grade). Two separate extractions were performed; one to extract acidic compounds (lipid-regulating agents) and another one for basic compounds (β-blockers). The pH of samples and ultrapure water for preconditioning and washing steps was adjusted to 2.8 (HCl) for the SPE of acidic pharmaceuticals and to 10.5 for the basic pharmaceuticals. A sample volume of 100 ml was applied to the cartridge and the flow was kept at no greater than 4 ml/min. A wash step with ultrapure water (1 ml) was applied after the sample loading. The cartridge was allowed to dry for about 30 min using vacuum to remove excess water. The analytes retained were eluted with 2 × 3 ml of MeOH. The extract was evaporated to dryness under stream of N_2 and redissolved with 1 ml of MeOH obtaining 100-fold preconcentration. After filtration with a 0.20 μm PTFE syringe filter (Millipore, Bedford, MA, USA), 20 μl of this solution was injected into the LC–ESI-MS–MS system.

2.3. LC–ESI-tandem MS analysis

The LC analysis were performed using a Waters 2690 HPLC separations module (Mildford, MA, USA) equipped with a Purospher Star RP-18 endcapped column (125 mm × 2.0 mm, particle size 5 μm) and a C_{18} guard cartridge supplied from Merck (Darmstadt, Germany). The mobile phase used in the chromatographic separation consisted of a binary mixture of solvents A (acetonitrile) and B (HPLC-grade water) at a flow rate of 0.2 ml/min. The gradient program began with a hold for 1 min at 30% of A followed by a linear ramp to 90% of A during 9 min, which was held constant at 90% for 1 min. The reequilibration time was 5 min.

The tandem MS analyses were carried out on a Micromass Quattro triple quadrupole mass spectrometer equipped with a Z-spray electrospray interface (Manchester, UK) in negative

mode (NI) for acidic pharmaceuticals and in positive mode (PI) for basic pharmaceuticals.

The parameters for the analysis of acidic pharmaceuticals were: ESI source block and desolvation temperature: 120 °C and 370 °C, respectively; capillary and cone voltages: 3.5 kV and 20 V, respectively; argon collision gas 2.5×10^{-3} mbar; cone nitrogen gas flow and desolvation gas: 109 and 508 l/h.

The parameters for the analysis of basic pharmaceuticals were: ESI source block and desolvation temperature: 80 °C and 350 °C, respectively; capillary and cone voltages: 2.5 kV and 55 V, respectively; argon collision gas 2.5×10^{-3} mbar; cone nitrogen gas flow and desolvation gas: 109 and 510 l/h.

Following the selection of the precursor ions, product ions were obtained at a series of collision energies and were selected the fragmentation that produced a useful abundance of fragment ions. The optimal collision energy and transitions chosen for the multiple reaction monitoring (MRM) experiment are listed in Table 1. The mass spectrometer was operated in MRM mode with unit mass resolution on both mass analyzer and a dwell time of 180 ms.

2.4. Acute toxicity analysis

The acute toxicity of all pharmaceuticals was tested in culture medium and in wastewaters to evaluate the single and combined toxicity of each pharmaceutical in both kinds of matrices respectively. For this study, individual stock solutions of the pharmaceuticals were prepared in culture medium and used to evaluate the single toxicity for each pharmaceutical by determining its effective concentration (EC_{50}). To evaluate the combined toxicity of each pharmaceutical in wastewater, the individual stock solutions prepared in culture medium were used to spike the wastewater to a final concentration of each pharmaceutical at 2 μg/l. With this purpose, preliminary toxicity studies were made on effluent samples and not toxic effluents were selected (0% of inhibition). The concentration of 2 μg/l was selected like arbitrary level of a possible concentration of the studied compounds in wastewaters.

The single and combined toxicity data of all pharmaceuticals were evaluated according to the toxicity categories established in the Directive 93/67/EEC using the EC_{50} toxicity endpoint which is the 50% of inhibition in the evaluated biological response. Wastewater samples or standard compounds are considered as "harmful to aquatic organisms" (10 mg/l EC_{50} 100 mg/l), "toxic" (1 mg/l EC_{50} 10 mg/l), "or very toxic" (EC_{50} 1 mg/l) [29]. In addition, for practical reasons, the toxicity category "not harmful to aquatic organisms" was added and used by us for the compounds with an EC_{50} above 100 mg/l.

Daphnia immobilisation test was conducted following the standard protocol described in the European Guideline [30]. The dormant eggs (ephipia) were incubated in standard freshwater at 21 ± 1 °C under continuous illumination of 6000 lx to induce hatching and the experiments were performed on less than 24-h-old daphnids. Between hatching and test steps, the daphnids were fed with the micro algae *Spirulina* in order to provide an "energetic reserve" and to preclude mortality by starvation during the subsequent 48 h test exposure. The EC_{50} was determined as the concentration of the sample required to immobilize 50% of the daphnids after 48 h of exposition. The neonates are considered immobilized, after 48 h of incubation, if they lie on the bottom of the multi-well test play and do not resume swimming within 15 s of observation. The test was carried out in the dark at a constant temperature of 20 ± 1 °C. The pH of samples was adjusted to be in the tolerance interval of the test organisms before testing by adding drop wise proanalysis HCl and NaOH (both 0.1 M).

3. Results and discussion

3.1. Analytical method

An analytical method was developed to determine the presence of lipid-regulating agents and β-blockers in environmental and wastewater samples. The ESI interface parameters were optimized by flow injection analysis (FIA) for all individual compounds in the PI and NI mode. Deprotonated molecules $[M - H]^-$ and $[M - 2H]^-$ at *m/z* 213 for clofibric acid, *m/z* 249 for gemfibrocil and *m/z* 360 for bezafibrate were selected in the first quadrupole as precursor ions. Protonated molecules $[M + H]^+$ were obtained for all β-blockers and fenofibrate, and were used as precursor ions in MS–MS experiments.

The product ion scan of clofibric acid (*m/z* 213) produced two fragment ions corresponding to $[C_6H_4ClO]^-$ (*m/z* 127) and $[C_4H_5O_2]^-$ (*m/z* 85). Precursor ion $[M - 2H]^-$ for bezafibrate, gave three fragment ions $[M - H - C_4H_6O_2]^-$ (*m/z* 274), $[M - H - C_{12}H_{14}O_3]^-$ (*m/z* 154) and $[C_4H_5O_2]^-$ (*m/z* 85). In contrast, gemfibrocil produced one fragment ion $[M - H - C_7H_{12}O_2]^-$ (*m/z* 121). The spectra of β-blockers presented fragment ions (*m/z* 133 and 159) common to metoprolol and betaxolol, corresponding to $[C_6H_{15}NO_2]^+$ and $[C_8H_{17}NO_2]^+$. Two fragment ions were produced from the protonated ion $[M + H]^+$ of atenolol and sotalol. Atenolol produced fragment ions *m/z* 190 and 145 corresponding to $[M - H_2O - NH_3 - \text{isopropyl} + 2H]^+$ and $[190 - CO - NH_3]^+$, respectively. From the protonated ion $[M + H]^+$ of sotalol, the *m/z* 255 and 213 attributed to $[M - H_2O + H]^+$ and $[M - C_3H_9N + H]^+$ were obtained. Fenofibrate produced one fragment ion corresponding to *m/z* 233 $[C_{13}H_{10}ClO_2]^+$. Characteristic precursor–product transition useful for confirmation and quantitation are listed in Table 1.

The most intensive fragment ion from each precursor ion was selected and was chosen as transition ion for detection and quantitative analysis. For this purpose, two criteria for positive identification were set, the correlation of the retention time with the standards (±2%) and the first selected precursor–product ion transition. A choice of a less intensive secondary transition was used as second criteria of confirmation purposes. An example of extracted MRM

Table 1
Detection, quantification and confirmation transitions for β-blockers and lipid-regulating agents

Pharmaceutical standards	MRM 1 (detection and quantification)		MRM 2 (confirmation)		*m*/*z*	Product ions
	Precursor (*m*/*z*) → product (*m*/*z*)	Collision (eV)	Precursor (*m*/*z*) → product (*m*/*z*)	Collision (eV)		
Bezafibrate	360 → 274	14	360 → 85	14	274	$[M - H - C_4H_6O_2]^-$
					154	$[M - H - C_{12}H_{14}O_3]^-$
					85	$[C_4H_5O_2]^-$
Clofibric acid	213 → 127	14	213 → 85	14	127	$[C_6H_4ClO]^-$
					85	$[C_4H_5O_2]^-$
Gemfibrocil	249 → 121	14	–	14	121	$[M - H - C_7H_{12}O_2]^-$
Atenolol	267 → 145	25	267 → 190	25	190	$[M - H_2O - NH_3 - \text{isopropyl} + 2H]^+$
					145	$[190 - CO - NH_3]^+$
Sotalol	273 → 255	15	273 → 213	15	255	$[M - H_2O + H]^+$
					213	$[M - C_3H_9N + H]^+$
Metoprolol	268 → 133	27	268 → 159	27	159	$[C_8H_{17}NO_2]^+$
					133	$[C_6H_{15}NO_2]^+$
Betaxolol	308 → 133	29	308 → 159	29	159	$[C_8H_{17}NO_2]^+$
					133	$[C_6H_{15}NO_2]^+$
Fenofibrate	361 → 233	26	–	26	233	$[C_{13}H_{10}ClO_2]^+$

chromatograms for target pharmaceuticals in spiked STP effluent is shown in Fig. 1. Analysis of different environmental and wastewaters revealed that the matrix affected the signal intensity and typically resulted in severe signal suppression. The susceptibility of ESI interface to co-extracted matrix component was especially pronounced in STP influent and effluent samples, as shown in Fig. 2. The extent of ion suppression was checked by comparing the signal intensity obtained for each analyte in spiked ultrapure water with signal obtained for spiked river, tap or wastewater, respectively.

Ion suppression is the common problem in LC–MS–MS analysis of organics in complex matrices. Several authors reported on the influence of mobile phase composition and mobile phase additives, including methanol or acetonitrile as organic mobile phase and ammonium acetate or formic acid as additives [31–34]. Limitations associated with ion suppression in the LC–MS analysis were observed in all cases studied.

It is well known that the effect of co-eluting residual matrix components may results in the suppression or less frequently in the enhancement of the analyte response [35,36]. In our case, the suppression of the signal was below 13% in spiked tap and river extracts, for all compounds, except for clofibric acid and bezafibrate, which showed 17 and 28% signal reduction, respectively. In spiked STP effluents ion suppression was limited to 15% for five compounds eluting after 4 min (atenolol, sotalol, metoprolol, betaxolol and fenofibrate), whereas for early eluting compounds (gemfibrocil, clofibric acid and bezafibrate) reduction was 38%, 47% and 54%, respectively, indicating that compounds eluting in an area at the beginning of the LC gradient are more affected by the matrix effect. For the most complex matrix analyzed (STP influent) the phenomenon was also pronounce at the end of the chromatographic separation and the suppression signal varied from 49 to 60% for betaxolol and fenofibrate, respectively. Similar results were reported by Quintana and Reemtsma [36], they observed severe signal suppression in raw and treated municipal wastewater samples. Signal suppression measured for early eluting compounds was almost 80% and the response of the target compounds (acidic drugs) showed a clear tendency of decreasing signal suppression with increasing retention time. Such behaviour is indicative of non-specific matrix effects associated with the sample nature and the ionization techniques. The suppression in the beginning of the chromatogram can be attributed to moderately polar matrix components while hydrophobic interferences can affect the late eluting compounds. Additionally, both the hydrophilic or hydrophobic matrix components could interfere at different times showing located areas of suppression in the LC analysis of the influent samples.

The signal irreproducibility that leads to erroneous results can be compensated, over a limited retention time window, by the use of an appropriate internal standard or by the use of time-consuming and laborious standard addition method.

Another approach to cope with matrix is aimed at the reduction of matrix components prior to the LC–MS–MS analysis applying a selective extraction and improved sample clean-up. SPE preconcentration and clean-up, which is necessary to obtain adequate sensitivity for trace level determination, is often proposed as a solution for matrix effect. However, it is observed that the pre-concentration step increases the concentration of interfering substances and may magnify the matrix effect [37,38]. A simple solution to this problem is dilution of the extracts obtained by the exhaustive extraction of complex samples. Dilution of samples proved to be an effective approach in cases when the preconcentration of matrix components during sample preparation magnified matrix effect and is often used in the analysis of bio-fluids

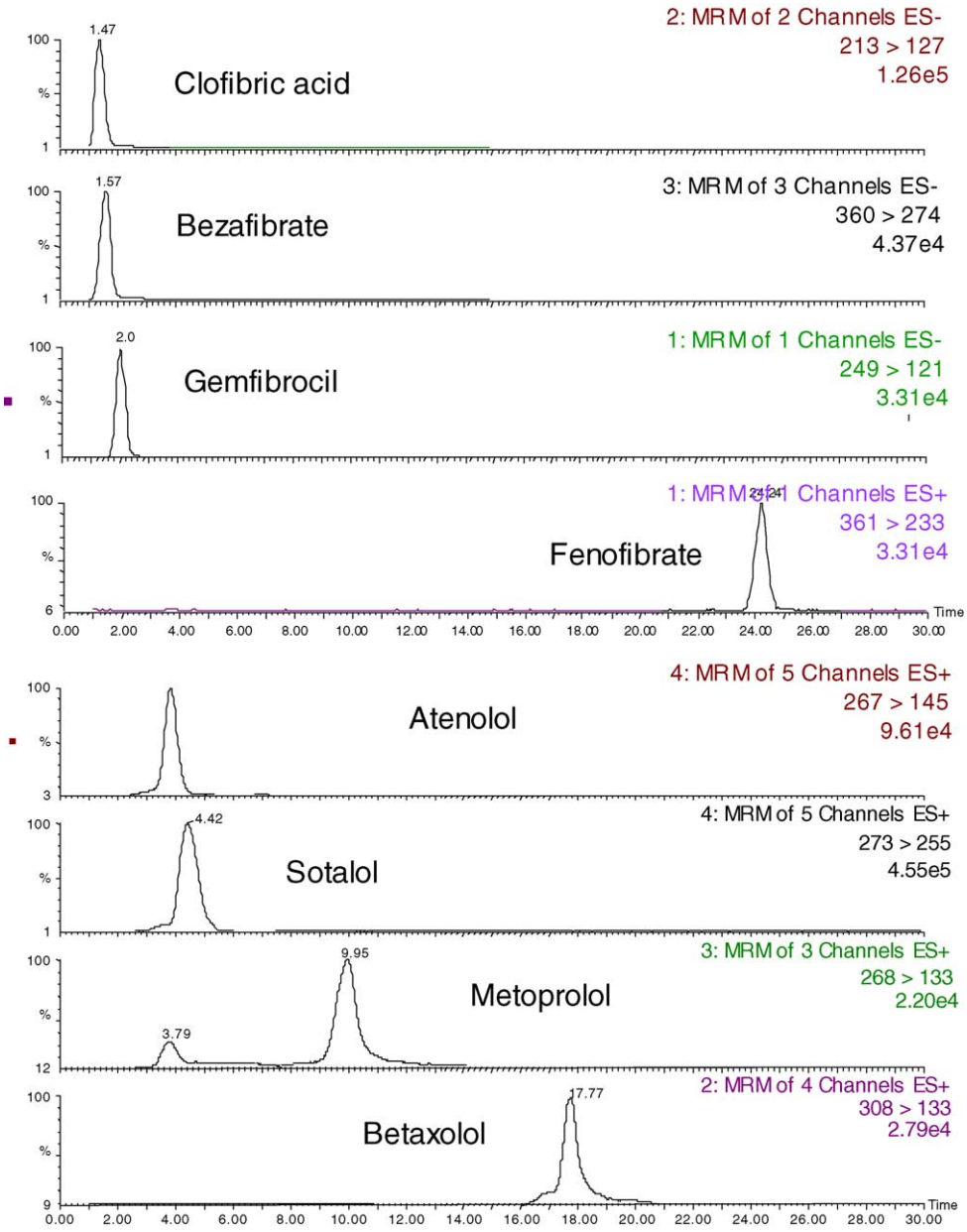

Fig. 1. Extracted MRM chromatograms for target pharmaceuticals in spiked STP effluents at 100 μg/l. Peak identification is as follows: (1) clofibric acid, (2) bezafibrate, (3) gemfibrocil, (4) atenolol, (5) sotalol, (6) metoprolol, (7) betaxolol and (8) fenofibrate. Time scale in minutes.

Table 2
Recoveries of β-blockers and lipid-regulating agents in spiked ultrapure water (HPLC-grade) and in spiked real samples (15 μg/l)

Pharmaceutical standards	LOD (μg/l)	Recoveries (%)				
		Ultrapure water	Tap water	River	Influent	Effluent
Bezafibrate	0.050	78.5	59.5	70.3	59.9	60.2
Clofibric acid	0.060	81.2	100.3	89.0	62.0	79.2
Gemfibrocil	0.090	92.3	45.2	69.2	29.6	30.5
Atenolol	0.017	80.3	58.7	48.7	27.5	50.1
Sotalol	0.018	89.7	76.2	63.2	18.0	52.3
Metoprolol	0.55	76.2	55.2	43.1	34.2	42.5
Betaxolol	0.75	52.0	40.3	42.5	27.5	45.1
Fenofibrate	1.25	60.0	49.2	45.1	28.0	39.5

Limits of detection (LODs) (μg/l) in spiked effluent.

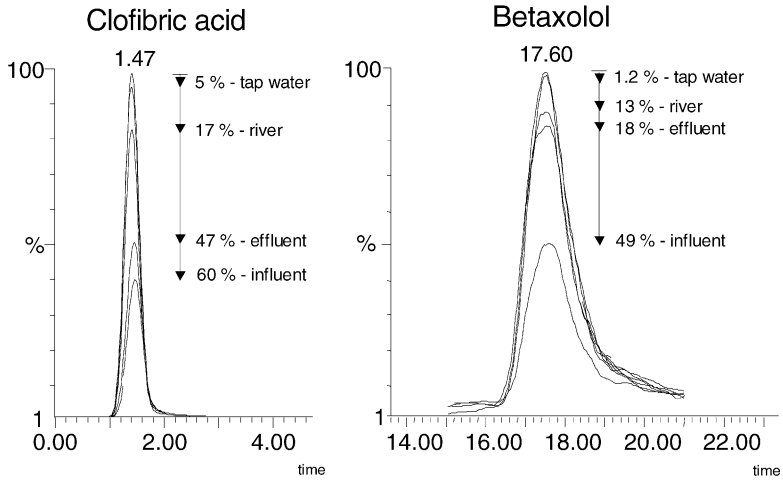

Fig. 2. Extracted MRM chromatograms for clofibric acid and betaxolol in spiked ultrapure water and in spiked real samples (sewage treatment plants influent and effluents, river and tap water). Signal suppression (%) for early (clofibric acid) and lately LC eluting compounds (betaxolol). Time scale in minutes.

[39]. In this work, the sequentially diluted (1:2, 1:4, 1:5 and 1:10) extracts of samples with severe ions suppression (STP influent, effluent and river water) were injected into LC–MS–MS and the signal intensity was compared to those obtained for spiked ultrapure water. A dilution of 1:4 was shown to be sufficient to minimize the signal suppression increasing the intensity of the signal of the analytes, thus making possible to correct the results of quantitative analysis. With the dilutions of 1:5 and 1:10, ion suppression was completely eliminated but the decrease of sensitivity (analyte signal) was also observed.

3.2. Validation of the method

Calibration curves were prepared for each compound from the spiked effluent wastewater by plotting the average total ion peak area versus the analyte concentration. Wastewater sample with no analyte peaks was used as a blank and for the calibration curves. Linearity was tested in the range 0.04–1000 μg/l depending of the pharmaceutical and all showed r^2 values >0.9967, indicating a good correlation.

Precision of the method was investigated by determining the short-term and long-term relative standard deviations (R.S.D.s) under identical conditions. R.S.D.s were obtained by analysing three replicates of spiked effluent samples at 15 μg/l. Intra- and inter-day precision were for all compounds lower than 18.5%. Limits of detection (LODs) calculated as signal-to-noise ratio (*S*/*N*) of 3, ranged from 0.017 μg/l to 1.25 μg/l depending of the compound in spiked effluent wastewater (Table 2).

The SPE procedure was evaluated using standards prepared in ultrapure water and not contaminated influent wastewater, effluent wastewater, river and tap water, with the studied compounds. Recoveries as evaluated in spiked ultrapure water as well as in spiked real samples are shown in Table 2. The recoveries of the analytes from the spiked ultrapure water at a concentration of 15 μg/l ranged from 52 to 92.3%, the highest recovery was for most of them above 76.2% and the lowest were for betaxolol and fenofibrate with a 52% and 60%, respectively. Low recoveries ranging from 18 to 62 were determined specially, in influent wastewater samples, probably due to the complexity of the matrix. This fact introduces high uncertainties in quantitation at very low concentrations but can allow the determination of their presence or absence. R.S.D.s for recoveries of standard in ultrapure water were determined by analysing three replicates and were lower than 7.8% for all standards except for gemfibrocil and fenofibrate that were 15% and 16.2%, respectively.

3.3. Acute toxicity data

The toxic effects of lipid-regulating agents and β-blockers were assessed on *D. magna*. This test was selected to evaluate the negative effects of these pharmaceuticals because its high sensitivity to detect toxic response from low concentration levels (ng/l or low μg/l) [40,41].

Usually the toxicological information is related to the individual risk of the chemical, however pollutants occur in real samples as mixtures and their potential toxicity is not easy to predict. In this sense, the single and combined toxic effects of the pharmaceuticals have been evaluated in culture medium and in wastewater respectively.

The acute toxicity of the pharmaceuticals determined as EC_{50} in culture medium, that is the single toxic effect of each compound ranged from 50 mg/l to >200 mg/l for lipid-regulating agents and from 200 to >300 mg/l for β-blockers. Applying the toxicity categories established in the Directive 93/67/EEC and based on the EC_{50} values, fenofibrate and gemfibrocil can be considered as harmful to aquatic organisms because their EC_{50} is between 10 and 100 mg/l [29]. For the pharmaceuticals with an EC_{50} value above 100 mg/l, an additional category was added as a new toxicity category called “not harmful to aquatic organisms”. In this

Table 3
Single and combined acute toxicity effects of each pharmaceutical in culture medium and in spiked wastewater for *D. magna* test

Pharmaceutical	EC_{50} (mg/l)[a] obtained in culture medium (toxicity category[bc])	Percent inhibition[a] obtained in spiked wastewater[d] at 2 μg/l (toxicity category[be])
Fenofibrate	50 (harmful)	58 (very toxic)
Gemfibrocil	100 (harmful)	80 (very toxic)
Clofibric acid	150 (not harmful)	100 (very toxic)
Bezafibrate	>200 (not harmful)	100 (very toxic)
Betaxolol	>300 (not harmful)	80 (very toxic)
Metoprolol	200 (not harmful)	100 (very toxic)
Sotalol	>300 (not harmful)	80 (very toxic)
Atenolol	200 (not harmful)	81 (very toxic)

[a] EC_{50} = 50% of inhibition (EC_{50} toxicity endpoint is defined as the tested sample that immobilises a 50% of the daphnids after 48 h of exposure).
[b] Toxicity categories established according with the Directive 79/831/EEC: very toxic ($EC_{50} \leq 1$ mg/l); toxic (1 mg/l < $EC_{50} \leq 10$ mg/l); harmful (10 mg/l < $EC_{50} \leq 100$ mg/l). The toxicity category "not harmful to aquatic organisms" was added and used by us for the compounds with an EC_{50} above 100 mg/l.
[c] Toxicity categories corresponding to EC_{50} value determined in culture medium (single toxic effect).
[d] Wastewater is not toxic (% of inhibition).
[e] Toxicity categories corresponding to the inhibition effect determined in wastewater (combined toxic effect).

category were included most of the pharmaceuticals, clofibric acid, bezafibrate, betaxolol, metoprolol, sotalol and atenolol. Table 3 shows the EC_{50} value and the toxicity category for each pharmaceutical in culture medium. Considering this toxicity ranking, the single effect of most pharmaceuticals can be considered as not harmful to the aquatic environment, and on the other hand, even in the case of fenofibrate or gemfibrocil, high unrealistic environmental concentration will need to produce acute toxic effects.

To consider the risk of the pharmaceuticals in a real and complex exposure situation to *D. magna*, no toxic wastewater samples were spiked with individual pharmaceutical standards at realistic environmental concentrations (2 μg/l). In wastewater samples several pollutants can contribute to the total toxic effect on aquatic organisms. Thus, synergistic, additive or antagonistic effects can be produced. To evaluate the combined toxic effect of each pharmaceutical in wastewater, the percentage of inhibition was determined. The toxicity endpoint was established as the 50% of inhibition, which is the same concept of EC_{50}. Table 3 also shows the % of inhibition and the toxicity category for each pharmaceutical in wastewater. The combined effect of the pharmaceuticals was higher than 58% indicating in all cases toxic effects. Comparing the EC_{50} values in culture medium and the % of inhibition in wastewater, it is evident that there is a notable increment in the toxicity. This fact could be explained because the contribution of the pharmaceuticals even at very low concentration with others substances results in synergistic toxic effects. Therefore, the combined effects of the studied pharmaceuticals result in higher negative effects in wastewater than in culture medium. Applying the toxicity categories, the combined effect of each pharmaceutical in wastewater can be considered as very toxic.

4. Conclusions

The described method allowed the analysis of the two groups of pharmaceuticals at low concentration levels in waters of different origins. The determination of the pharmaceuticals was possible correlating the retention times, mass spectra and monitoring of the characteristic precursor–product transitions. The SPE procedures provide enrichment factor of 100-fold and acceptable recoveries, all above 60%, except for betaxolol with 52% recovery from spiked HPLC-grade water. LC–ESI-MS–MS gave detection limits ranging from 0.017 to 1.25 μg/l.

The acute toxicity data on *D. magna* indicates that both groups of pharmaceuticals are not harmful or not toxic, except fenofibrate, which can be considered as harmful when individual effects are evaluated in culture medium. However, the combined effects in wastewater samples showed a greater negative effects indicating synergistic effects when these compounds occurring in complex samples at low concentration levels of 2 μg/l.

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