Anal Bioanal Chem
DOI 10.1007/s00216-006-0781-y

REVIEW

LC-MS analysis and environmental risk of lipid regulators

M. D. Hernando · A. Agüera · A. R. Fernández-Alba

Received: 11 June 2006 / Revised: 1 August 2006 / Accepted: 15 August 2006
© Springer-Verlag 2006

Abstract This article presents a review of liquid chromatography-mass spectrometric (LC-MS) methods applied to the determination of lipid-regulating agents, "fibrate" and "statin" classes, and some of their metabolites (clofibric acid and fenofibric acid) in environmental samples. Concentration levels of this therapeutical group have been reported in the ppt to ppb range for different compartments (wastewater, surface water and sediments) in several monitoring studies. Part of this article is dedicated to hazard assessment of lipid-regulating agents according to the approaches of the European Union (EU) and Environmental Protection Agency (EPA) for identifying persistent, bioaccumulable and toxic (PBT) substances. The pharmacodynamic and pharmacokinetic activities of these pharmaceuticals are well known and, based on this information, the derivation of the potential long-term effects, which may be induced on eco-organisms at low concentration levels, is discussed. Studies of environmental risk assessment (ERA) for lipid regulators carried out through the framework of the European Agency for the Evaluation of Medicinal Products (EMEA) and Food and Drug Administration (FDA) are presented.

Keywords Lipid regulators · Metabolites · LC-MS · PBT · ERA

Introduction

Different therapeutical groups of pharmaceuticals present in the environment have been the focus of attention for some time due to the fact that they are designed to produce biological responses and because of their high production and consumption. Antibiotics, antineoplastics or hormones are of concern due to their effects on bacteria, cells or their high potential activity. Other examples include medicines of high consumption such as non-steroidal anti-inflammatory drugs (NSAID), beta-blockers and antidepressants. Several survey studies have shown the presence of pharmaceuticals and their metabolites in the environment. In the mid-1970s, clofibric acid (the bioactive metabolite of certain serum triglyceride-lowering pharmaceuticals) was detected in a groundwater reservoir that was refilled with treated sewage water [1]. Since then, several monitoring studies have been carried out showing the detection of pharmaceuticals in water and sediments, at concentration levels of ppt or low ppb [2–7].

Due to high persistence and widespread occurrence of lipid-regulating agents in aquatic environments, their presence in drinking water has also been reported in some studies [4, 8–12]. Examples related to the "fibrate" class of the lipid regulators are the detection of clofibric acid (low ng/L to 270 ng/L), bezafibrate (27 ng/L) or gemfibrozil (70 ng/L) [9, 13, 14]. In contrast to the extensive information related to the fibrate class in the environment, very few papers have been published reporting the presence of pharmaceuticals belonging to the "statin" class (cholesterol-reducing agents). Both classes are some of the most prescribed therapeutic groups (the first top-selling prescription group of pharmaceuticals in the USA during 2003) [15]. Available data for the statin class refers to the detection of atorvastatin (Lipitor) in wastewater (1–117 ng/L) and in rivers at low ng/L [6, 16]. Regarding the detection of metabolites of this group of pharmaceuticals in the environment, several publications have reported the detection of clofibric acid [2, 5, 17–21] but scarce data (i.e. fenofibric acid) [2, 7, 8] or no information is available for other metabolites.

M. D. Hernando (✉) · A. Agüera · A. R. Fernández-Alba
Department of Analytical Chemistry, University of Almería,
04120 Almería, Spain
e-mail: dhernan@ual.es

Chemical analyses for the fibrate and statin classes have been performed with liquid chromatography-mass spectrometry (LC-MS/MS) in most publications [16–30]. The application of this technique has enabled the detection of trace concentration levels of these pharmaceuticals in different aqueous matrices and soil samples. In particular, for the measurement of highly contaminated samples (wastewater or sewage effluents), several approaches for reliable quantitative analysis have been described including efficient clean-up steps, dilutions of extracts or the use of appropriate surrogate standards [25].

The "chemical-based" analysis and the water quality evaluation have been the main focus of interest; however, the "ecologically based" quality assessment is also receiving special attention. The available ecotoxicity data of pharmaceuticals in the environment is still limited. Acute toxicity data are typically reported in the mg/L range for most of the tested pharmaceuticals [31–35]. Given the levels of concentration detected in the environment (at least 3 orders of magnitude below), this would indicate which that acute toxicity should not be expected on organisms of the ecosystem. A major concern and difficulty rely on the assessment whether there could be significant environmental damage with regard to long-term effects, since chronic toxicity data is lacking in most cases.

This article presents a review of LC-MS analysis applied to the fibrate and statin classes as well as their metabolites, and their occurrence in the environment. The hazard assessment of the lipid regulator agents according the European Union (EU) and Environmental Protection Agency (EPA) approaches for identifying PBT (persistent, bioaccumulable and toxic) substances is also discussed. The pharmacodynamic and pharmacokinetic activities of these pharmaceuticals are well known, and based on this information, the derivation of the potential long-term effects which may be induced on organisms of the ecosystem at low concentration levels, is discussed. Environmental risk assessment (ERA) studies of lipid regulators carried out through regulatory schemes by EMEA (European Agency for the Evaluation of Medicinal Products) and Food and Drug Administration (FDA) are presented.

LC-MS analysis

Although some gas chromatography-mass spectrometric (GC-MS)-based methods have been developed for the determination of lipid regulators [19, 36–38] in environmental samples, the polar character of many of them demands the application of labourious and time-consuming derivatization procedures. In fact, most of the analytical methods published in the literature are based on the use of LC-MS systems. Although atmospheric pressure chemical ionization (APCI) is generally regarded as a robust ionization technique and less susceptible to signal suppression effects, electrospray ionization (ESI) has been preferred in most methods developed for the analysis of lipid regulators. The ESI interface has been regarded as more versatile than APCI. The ESI interface is able to ionize extremely polar/non-volatile molecules, sometimes difficult for APCI, but also offers a high sensitivity (up to 10-fold higher than the APCI interface), which allows trace determination of pharmaceuticals in environmental samples [39]. The selection of negative ionization (NI) mode has been the starting point for most of acidic pharmaceuticals and positive-ion (PI) mode for the lactone compounds (i.e. lovastatin, simvastatin and mevastatin) of the statin group [6]. However, in some cases positive-ion mode has yielded better results, such as for fenofibrate [23, 39], atorvastatin [6] or bezafibrate [39].

LC separation of lipid regulators using a narrow-bore C18 column (2-mm i.d.) and the use of methanol or acetonitrile as organic mobile phase have been the optimal conditions which have enabled a better resolution and shorter retention times. Mobile phase additives for adjusting the pH and/or to enhance ionization, such as ammonium acetate or methylammonium acetate, have been common. The use of ion-pair liquid chromatography IP-LC with tri-*n*-butylamine (TrBA) as ion-pairing agent has allowed improvements in the detection limits by a factor of 2 for acidic pharmaceuticals. The result of addition of alkylammonium cations for strongly acidic compounds (i.e. sulfonates and phosphonates), has been attributed as the cause of the increasing response observed [39].

The formation of adduct ions to improve method sensitivity in ESI-MS detection has also been assayed by Miao and Metcalfe [6, 16], who reported the use of methylammonium acetate as mobile phase additive to obtain the precursor ion $[M+CH_3NH_3]^+$ for lovastatin, pravastatin and simvastatin. Atorvastatin was also investigated in this study, but the methylammonium adduct for this compound showed very low signal intensity. The mass analyzers, triple quadrupole or ion trap, have been successfully applied to the analysis of environmental samples, allowing the discrimination against matrix interference and achieving the sufficient sensitivity. The developed methods published in the literature, enable the analysis of lipid regulators at concentration levels up to low-ng/L (see Table 1). The sensitivity is achieved with previous enrichment of the analytes by solid-phase extraction (SPE) of the samples, allowing the determination of lipid regulators in natural or dinking water where trace concentrations may be detected.

With a triple quadrupole mass analyzer, multiple reaction monitoring (MRM) has been carried out for the selective determination and quantification of lipid regulators in

Table 1 LC-MS methods for the determination of lipid regulating agents in environmental samples

Compounds	Matrix	Sample pretreatment	Extraction method	LC column	Mobile phase	MS	LOD/ LOQ (ng/L)	Ref.
Atorvastatin, lovastatin, pravastatin, simvastatin, mevastatin	Surface and WW	pH 4.5	SPE/HLB/ MeOH	C18	Aq.methylamine/ aq.HAc/ACN	LC-ESI (+)-MS/ MS	0.1–15.4	[16]
Gemfibrozil, clofibric acid	River sediments	–	UASE/SPE/ MCX	C18	Aq. HAc/ACN	LC-APCI (−)-MS/ MS	0.4*	[22]
Bezafibrate, gemfibrozil, clofibric acid, fenofibrate	Surface and WW	pH 2.8	SPE/C18/ MeOH	C18	ACN/water	LC-ESI (+/−)- MS/MS	50–1250	[23]
Bezafibrate, clofibric acid	Surface and WW	pH 2.0–2.5	SPE/HLB/ MeOH	Phenyl-hexyl	Aq. HAc, TrBA/MeOH HAc, TrBA	IP-LC- ESI(−)- MS/MS	0.3–2.1	[17]
Bezafibrate, clofibric acid	WW	pH 1.5–2.0	SPE/MCX/ MeOH/NH_3/ NaOH)	C8	Aq.TEA(pH 8)/ACN	LC-ESI (−)-MS/ MS	0.1–0.36	[18]
Bezafibrate, clofibric acid	WW	pH 2.0	LPME	Phenyl-hexyl	20% MeOH, HAc, TrBA/95% MeOH HAc, TrBA	IP-LC- ESI(−)- MS/MS	0.5–1.8	[41]
Fenofibrate, bezafibrate, clofibric acid	Surface and WW	pH 3.0	SPE/MCX/ MeOH:NH_3	C18	Aq. NH_4Ac/MeOH	LC-ESI (+/−)- MS/MS	5–10	[39]
Atorvastatin	Surface and WW	pH 4.0	SPE/HLB/ MeOH	C18	Aq. NH_4Ac/ACN	LC-ESI (+)-MS/ MS		[6]
Bezafibrate, clofibric acid	Sludge	–	USE/SPE/ MCX	C18	Aq. HAc/ACN	LC-APCI (−)-MS/ MS	20*	[24]
Bezafibrate, gemfibrozil, clofibric acid	Surface water	pH 2.0	SPE/LiChrolut EN/MeOH	C18	Aq. formic acid/ACN/ MeOH	LC-ESI (+/−)- MS/MS	6–18	[46]
Gemfibrozil	Surface and WW	pH 2.0	SPE/LiChrolut EN/MeOH: Acet.	C18	Aq. formic acid/ACN	LC-ESI (−)-MS	56	[27]
Bezafibrate, gemfibrozil	Surface and WW	pH 3.0	SSPE/C18 +HLB/ MeOH:Acet	C18	Aq. acetic acid/ACN	LC-ESI (−)-MS	2	[29]
Bezafibrate, gemfibrozil	WW	pH 2.0	SPE/C18/ MeOH	C18	Aq. NH_4Ac/MeOH/ ACN	LC-ESI (−)-MS/ MS	5–10	[42]
Bezafibrate, clofibric acid	Surface water	pH 2.0	SPE/C18/ MeOH	C18	Aq. NH_4Ac/MeOH	LC-ESI (+/−)- MS/MS	0.2–0.7	[26]

UASE ultrasound-assisted solvent extraction; *WW* wastewater
*ng g^{-1}

environmental samples. For statin compounds (simvastatin, lovastatin and pravastatin), the product ion mass spectra of the adduct $[M+CH_3NH_3]^+$, typically show an abundant fragmentation with common fragments at *m/z* 199, 267, 269 and 285. The dominant loss processes are the loss of CH_3NH_2 and the ester side-chain followed by dehydration and dissociation of the lactone moiety [40]. According to the characteristic fragment ions and the signal intensity, the selected reaction monitoring (SRM) transitions are 436→285 (lovastatin), 456→269 (pravastatin) and 450→199 (simvastatin). The most sensitive mass transition for atorvastatin is from *m/z* 559 to 440. A major cleavage occurs at both sides of carbonyl group, with benzyl ring A as neutral loss, generating the product ion at *m/z* 440 from

the precursor ion $[M+H]^+$. In the PI mode, the transition chanel 559→440 is approximately 10 times more sensitive than the NI mode with the transition channel 557→397. Statins can be unstable under conditions of high or low pH, leading to interconversion processes between acid and lactone forms and making the quantitation less accurate. However, within the typical period between sample storage and analysis (24 h), the observed variation is around 10%.

For fibrate compounds, MS/MS detection provides enough structural information for a reliable determination. The product ion spectrum for most of fibrate compounds shows at least two fragments ions. For instance, the metabolite clofibric acid shows a major and a minor fragment ion at *m/z* 127 and 85, respectively. The former ion is formed by the loss of the dimethyl-propionic acid group; the latter ion, $[C_4H_5O_2]^-$, also remains in the product ion spectrum of bezafibrate and results from the ether fission. In addition, the product ion mass spectrum of $[M-H]^-$ for bezafibrate show two fragment ions at *m/z* 274, $[M-H-C_4H_6O_2]^-$ and 154, $[M-H-C_{12}H_{14}O_3]^-$. The ion at *m/z* 274 corresponds to the ether cleavage and the formation of a phenolic anion [41]. Thus, the confirmation and quantitation of bezafibrate is possible by the monitoring of three transitions [42]. MS/MS fragmentation of gemfibrozil gives only one ion product at *m/z* 121, $[M-H-C_7H_{12}O_2]^-$, which is formed by the loss of the dimethyl-pentanoic acid group. In this way, the determination in water samples is performed using the MRM transition 249→121. Under ESI in PI mode, MS/MS fragmentations of $[M+H]^+$ ions for fenofibrate and bezafibrate have been assayed. For bezafibrate, ESI(+) and ESI(−) ionisation mode show similar sensitivity, whereas ESI(+) gave a higher sensitivity for MS detection of fenofibrate. The MS/MS ions used for confirmation and quantitation are *m/z* 316 and 276, respectively [39]. The MS/MS spectrum of fenofibrate shows a major fragment ion at *m/z* 233 $[C_{13}H_{10}ClO_2]^+$ and a second ion product at *m/z* 139, which is used for confirmation purposes [39].

Matrix effects

Matrix effects are a major drawback for quantitative trace determination of analytes in environmental samples by LC-MS systems, since reproducibility and accuracy of the method can be affected. Signal suppression or, less frequently, signal enhancement, can originate from co-eluting compounds arising from the matrix. Although LC-MS/MS systems are selective techniques, interferences may affect the ionization efficiency of the analytes when both enter into the ion source at the same time and can also induce to the loss of sensitivity. To reduce matrix effects in LC-MS analysis, different approaches have been presented e.g. optimization of chromatographic conditions, sample extraction and effective clean-up steps [6, 16, 23].

Matrix effect and its intensity are dependent on the sample complexity and the method of extraction applied. SPE has been commonly used for the extraction and clean up of liquid samples, but this procedure also concentrates the matrix components and signal suppression may be observed, in particular, with the ESI interface. The extraction efficiency of different sorbents and its relationship with matrix effects have been evaluated using various SPE cartridges (polymeric HLB, Bond Elute C8 and DSC-18). The comparison of four major statin drugs showed better extraction efficiency with C18 silica sorbents than with HLB sorbent (ca. 20%), but severe signal suppression was also observed in LC-MS analysis. In this way, HLB sorbent, which provided similar recoveries to silica-based sorbents, has been the choice for the analysis of complex matrices, such as raw wastewater in various studies [6, 16]. The SPE stationary phase, Oasis MCX (mixed mode sorbent) has also been applied by some authors as a selective extraction approach to reduce matrix effects. This sorbent contains sulfonate groups bonded to Oasis HLB co-polymer to provide both strong cation exchange (for basic compounds) and reversed-phase capacity (for acidic and neutral compounds) [6, 17]. As result, acidic compounds can be efficiently extracted at low pH values (e.g. clofibric acid and bezafibrate at 76–81%, respectively), while for other compounds, such as fenofibrate, low recovery has been obtained (36%) with Oasis MCX sorbent.

Alternative extraction strategies to SPE, such as hollow fibre liquid-phase microextraction (LPME), have also been applied as a one-step concentration/clean-up technique with the aim to not lead to signal suppression during LC-ESI-MS/MS analysis [41]. The three-phase LPME system has shown the capacity for providing high selectivity for the extraction of acidic compounds and clean extracts. However negligible, matrix effects can occur for complex matrices (raw water) due to alterations in the partitioning process of the analyte with the acceptor solution. The LPME technique is proposed as a good alternative to other extraction/clean-up technique, but still needs to be improved on precision for more reliable results.

The use of size-exclusion processes as a clean-up step to reduce matrix components in wastewater samples has been evaluated [43]. Restricted access materials (RAMs) are sorbents able to exclude high molecular weight materials with a nominal mass above 15 kDa. The application of RAMs has been effective for the analysis of groundwater and sediments, reducing matrix effects due to humic substances. The result obtained in the analysis of pharmaceuticals in wastewater samples is different, where matrix effects have been attributed to the presence of matrix interferences of low molecular weight (<1 kDa) [43]. The

same authors also investigated the reduction of the eluent flow that is delivered to the ESI interface. This approach is proposed on the basis that the amount of organic material that requires ionization at a given period of time can be minimized. In addition, the reduced droplet size which provides an increased droplet surface may minimize competitive processes of desolvation and ionization between analytes and matrix interferences. In this study, eluent flows from 20 to 100 µL/min were selected as the optimum for reducing matrix effect (45–60%), especially for bezafibrate. Furthermore, the authors also achieved improved sensitivity for some of the analytes tested (benzothiazoles) by reducing the eluent flow [43].

To compensate matrix effects in the quantitative analysis, the use of appropriate internal standards, standard addition or matrix-matched samples for external calibration, have been frequently proposed [6, 16, 23]. The use of internal standard is limited in practice, since isotopically labelled standards are not always available. A mayor disadvantage of standard addition is the labourious and time-consuming procedure involving addition into each sample. The preparation of calibration curves obtained with spiked wastewater extracts and the dilution of the SPE extracts have been presented as reliable alternatives [6, 17, 23]. Concentration of interfering substances can provoke severe ion suppression effects. For that, dilution of the SPE extracts has been proposed as an effective approach to reduce matrix effects [23].

Some authors [17] have described a clear tendency of decreasing signal suppression with increasing LC retention time. Severe signal suppression in early-eluting compounds (i.e. acidic compounds) can be associated to moderately polar matrix compounds, whereas the late-eluting analytes can be affected by compounds with hydrophobic character. For instance, signal reduction of 38–54% has been described for early-eluting analytes such as gemfibrocil, clofibric acid and bezafibrate in sewage treatment plant (STP) effluents [23]. For more complex matrices, such as STP influent, signal suppression may be even pronounced for late-eluting compounds. An example is fenofibrate with a signal reduction of up to 60%. For those cases, the dilution of SPE extracts (i.e. 1:2, 1:4) has shown to be an effective procedure to reduce matrix effects, but also a decreasing of sensitivity can be observed when SPE extracts are prepared at a superior dilution factor [23]. The influence of electrolytes may also be a cause of analyte signal suppression, since these can inhibit ionization, to a large degree by limiting desolvation. For this reason, low concentrations of additives are recommended; furthermore, this it can reduce ESI cleaning times.

For the analysis of solid samples, USE (ultrasound solvent extraction) or PLE (pressurized liquid extraction) methods have been applied combined with an additional clean-up or purification steps, mainly with SPE procedures. Few studies have been reported related to the enhancement of analyte signal in LC-MS analysis. Löffler et al. [22] observed this effect in the determination of acid pharmaceuticals (ketoprofen, naproxen and bezafibrate) in river sediment extracts. To compensate the over-determination, they make use of surrogate labelled standard, but the method was not effective for all analytes [17, 22]. The quantitative analysis of bezafibrate using internal standard was not reliable. In this case, the enhancement of signal was observed using the APCI interface [22].

Occurrence

Presence of lipid regulators in natural waters is a consequence of the high human consumption and persistence against conventional biological treatments applied in STPs. Complete elimination by STPs is not usually achieved and removal rates through conventional treatments for gemfibrozil, bezafibrate and metabolites (clofibric acid and fenofibric acid) have been published in percentages lower than 50% [7]. Clofibric acid is eliminated at 15 to 51% [44]. For other compounds, although total elimination can be achieved, their metabolites or degradation products could be not removed. This is the case for fenofibrate, whose metabolite, fenofibric acid, is partially removed (64%) [2]. As a consequence of the incomplete elimination in STPs, receiving water may be potentially contaminated. Lipid regulators have been found in different environmental compartments. Table 2 summarizes the data of occurrence of these compounds detected in wastewater, surface water and sediments.

Several monitoring programs have been carried out at STPs in different European countries during the recent years for the screening of pharmaceuticals in effluents [2–7, 17, 18]. In these studies, the compounds of the fibrate class have been more frequently detected in STP effluents than other pharmaceuticals such as the statin class. For this class, data referring to the detection of atorvastatin, lovastatin and pravastatin in effluents are scarce and no data is available concerning their removal in STPs. The concentrations detected in a survey study in Canada were 1–59 ng/L in treated wastewater; in only one surface water sample was atorvastatin found at low concentration level (1 ng/L). Regarding the fibrate class, concentrations up to 4 µg/L of gemfibrozil and bezafibrate have been reported in monitoring programs located in Germany and Italy. The average concentration of bezafibrate in STP effluents, in Germany, was 2.2 µg/L [2, 7]. A pilot survey study carried out in other European countries (Belgium, Spain and Slovenia) for the determination of pharmaceuticals, published the detection of clofibric acid in natural and treated waters at trace

Table 2 Occurrence of lipid regulators in the environment

Lipid regulator agents	Wastewater (ng/L)		Natural water (ng/L)		Ref
	Range/max. conc	Average conc.	Range/max. conc.	Average conc.	
Atorvastatin	37		1 (river) n.d.		[6, 16]
		22.4		–	[6]
Lovastatin	14		n.d.		[6, 16]
Pravastatin	59		n.d.		[6, 16]
Simvastatin	1		n.d.		[6, 16]
Mevastatin					
Bezafibrate	565		847 (lake)		[17]
	0.3–117				[18]
	20–70				[42]
	68–72	70			[47]
			780 (river)		[46]
	<LOD–1,100	1,050			[7]
	<LOD–1,070	990			[5]
			41–76 (river)	51	[29]
	4,600		3,100 (river, streams)		[2]
			1.6–12.5 (river)		[26]
		2,200			[2, 7]
	4,600		3,100		[3]
			1.6–202.7		[4]
Gemfibrozil	35–55				[42]
	59–84	71			[47]
	319–1,200		<LOD–1,550 (river)		[27]
	0.84–4.76		1–170 (river)		[20]
	<LOD–2,450	600		250	[7]
	40–4,760	1,510			[5]
			<LOD–790	48	[28]
	80–2,090	410			[37]
	1,500		510		[3]
	80–478		3.9–35.3 (river)		[38]
		7–400			[2, 7]
Fenofibrate	n.d–160	120			[5]
	30		n.d		[2]
		380			[2, 7]
	30		n.d.		[3]
Clofibrate	n.d.–800	800			[5]
	n.d.		n.d.		[2]
Clofibric Acid	109		279 (lake)		[17]
	0.5–82				
		n.d.–60	5–25		[18]
	n.d.				[19]
	680–880	110		90	[20]
	n.d.–680	456			[7]
	44–338				[5]
	1,600		550 (river, streams)		[21]
					[2]
	1,600		550		[3]
	<LOD–740	310			[37]
	22–107		24–35		[45]
Fenofibric acid	220–400	70	<LOD–540	60	[7]
	1,200		280		[3]

n.d. not detected

concentration levels. Clofibric acid was found at maximum concentrations of approximately 100 ng/L in effluents and low ppt levels in rivers (24–35 ng/L) [45]. In France, higher concentration levels of bezafibrate were detected in rivers (780 ng/L) [46]. This study showed that in urbanized river basins, pharmaceuticals could heavily contribute to the whole water quality deterioration together with other organic wastewater contaminants (personal care products, disinfectants and surfactants). In Canada, bezafibrate and gemfibrozil were detected at average concentrations of 70 ng/L in effluents [47] .The concentrations of clofibric acid in STPs effluents were at a maximum of 76 ng/L and of 175 ng/L in surface water adjacent to discharges of STP effluents [6, 16]. These follow-up studies indicated that concentrations of acidic and neutral drugs in surface waters near the point of sewage discharge into the river were approximately equal or even higher than the concentrations in the final effluent from the STP included in this monitoring program [48].

The compiled data indicate that the fibrate compounds and metabolites more frequently detected in both wastewater and river are bezafibrate, fenofibrate and clofibric acid compared to the other compounds of this group. On the other hand, to date, information regarding their detection in sediments or in soils is limited [7]. Clofibric acid has been one of the most common pharmaceutical residues found in effluents of STPs. For example, clofibric acid was detected in earlier investigations of drug residues [1, 7] at the µg/L range. There are also multiple references to the presence of clofibric acid in groundwater and drinking water, since it is frequently included in pharmaceutical screening programs [4, 8–11]. Heberer and Stan found clofibric acid in the majority of drinking water samples collected from 14 waterworks in the Berlin area, with maximum concentrations of 270 ng/L. This fact was related to an artificial groundwater enrichment process [12]. However, positive findings of clofibric acid have decrease in the recent years, probably because of the increasing use of alternative parent drugs (bezafibrate and gemfibrozil) [49]. Besides clofibric acid, reports of the presence of other metabolites, e.g. fenofibric acid, in wastewater and surface water have been scarce [2, 7]; one occurrence in Rio de Janeiro (Brazil) has been reported but no other literature evidence has been found.

PBT characteristics and environmental risk assessment

EU and EPA approaches for identifying PBT substances

In addition to persistent organic pollutants (POPs), substances which show a PBT profile are also of particular concern. If a substance is persistent (or its rate of release is higher than its rate of disappearance) and bioaccumulable, the duration and level of exposure of living organisms increases, and could lead to irreversible effects, probably difficult to detect in the early stages. Another aspect is related to the exposure of organisms towards a substance at low levels of concentrations, since it could lead to chronic effects. To prioritize the hazard level of chemicals, various PBT criteria and cut-off values have been applied by organisations such as the United Nations Environment Programme (UNEP), Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR), US EPA, EU or the UK Chemicals Stakeholder Forum [50–54]. Using the PBT criteria of EU and EPA approaches, the hazard posed by the lipid-regulating agents has been evaluated. Table 3 shows the cut-off values for identifying PBT substances which have been applied by the EU Water FrameWork Directive [55] and by OSPAR, as well as those criteria applied by the EPA. For the hazard assessment of lipid regulators, available data concerning their acute and chronic toxicity, as well as persistence, have been compiled from scientific publications. It should be noted that experimental data of PBT properties for these compounds are scarce and this data has been completed using a predictive model. For that reason, PBT Profiler software developed by the EPA has been used. This tool is a predictive screening tool which facilitates the identification of substances which may need further evaluation due to their potential PBT characteristics [56]. The PBT Profiler uses a chronic (long-term) toxicity value called ChV to estimate a chemical's relative toxicity for fish following the quantitative structure–activity relationship (QSAR) model through ECOSAR software. The ChV value corresponds to the geometric mean of the lowest observed effect concentration (LOEC) and the non-observed effect concentration (NOEC). The half-life for degradation of a chemical in water, soil and sediment is determined using the biodegradation survey module of the BIOWIN estimation program [57]. Bioaccumulation is estimated through BCFWIN $\log K_{ow}$ -based estimation program [58]. BIOWIN, BCFWIN and ECOSAR subprograms are part of the EPA's EPIWIN estimation software package [59–62].

Persistence

At present, little data is available on the persistence of pharmaceuticals in the aquatic environment, and in particular in sediments [63]. This information could be obtained using a combination of experimental investigations (OECD, Organisation for Economic Co-operation and Development, guidance 301 and 308) [64, 65]. For instance, studies based on laboratory degradation tests in water/sediment systems have shown which some pharmaceuticals can persist a long time in water and sediments (>100 days) [66], but these

Table 3 Cut-off values for identify PBT substances according EU and EPA guidelines

PBT criteria	Persistence	Bioaccumulation	Toxicity
EU PBT criteria	Half-life >60 days in marine water >40 days in freshwater >180 days in marine sediments >120 days in freshwater sediments	BCF >2,000	Chronic NOEC<0.01 mg/L or CMR or endocrine-disrupting effects
EU vPvB criteria	Half-life >60 days in marine or freshwater >180 days in marine or freshwater sediments	BCF >5,000	Not applicable
EPA criteria	≥60 days (persistent)	BCF ≥1,000 (bioaccumulative)	>10 mg/L, low concern
	>180 days (very persistent)	BCF >5,000 (very bioaccumulative)	0.1–10 mg/L, moderate concern
			<0.1 mg/L, high concern

BCF bioconcentration factor
NOEC no observed effect concentration:
vPvB very persistent and very bioaccumulable
CMR carcinogenic, mutagenic or toxic to reproduction

experiments can be costly and time consuming. An alternative approach is to use predictive models developed using factors related to chemical structure or environmental conditions [67]. The vast majority of publications deal with quantitative structure–biodegradation relationships (QSBRs), which rely on octanol/water partition coefficients (K_{ow}), van der Wall's radii, alkaline (abiotic) hydrolysis rate constants and various molecular connectivity indices. The application of these models could be limited to a very specific class, and could be inappropriate for predicting the biodegradation rates for chemicals outside a determined class. The PBT Profiler estimates the half-life for the degradation of a chemical in water, soil and sediments by using the biodegradation survey module of the BIOWIN estimation program [57]. With PBT Profiler, for most of the target lipid regulators, the average half-life in soil (75–120 days) and sediments (340–540 days) exceed the EPA criteria of ≥2 months and ≤6 months, respectively. Similar DT_{50} values were also obtained for the metabolites, clofibric acid and fenofibric acid, in soil and sediments. By applying EU criteria for sediments, all target compounds could be classified as "very persistent" (vP, DT_{50} >180 days) with the exception of pravastatin and lovastatin. On the other hand, given the rate of adsorption (K_{oc}) of simvastatin, estimated as 8,400, should also indicate that simvastatin is expected to be immobile in soil [68]. Therefore, most of fibrate and statin compounds are expected to be persistent in sediments. The half-life (DT_{50}) values of lipid-regulating agents in water, soil and sediments are shown in Table 4.

In water, the half-lives of fenofibrate, bezafibrate and atorvastatin exceed the EPA criteria of ≥2 months and subsequently they could be identified as persistents. The rest of the compounds did not exceed the cut-off values to be classified as persistents. Using EU criteria, atorvastatin, bezafibrate, fenofibrate, clofibric acid and fenofibric acid are expected to be persistent, since they exceed the cut-off value of DT_{50} water >60 days.

On the other hand, due to the continuous input of pharmaceuticals into the environment, they could to be considered as "pseudo-persistents", even if the environmental half-life of pharmaceuticals is not relatively great. In addition, the metabolites of pharmaceuticals and early degradation products can also be of environmental concern, such as in the case of lipid regulators, clofibrate, etofibrate and fenofibrate, in which their polar metabolites, clofibric acid and fenofibric acid, are detected. In the case of the statin class, the major metabolites are hydroxylated and hydroxyacid derivatives, but to our knowledge, there is no data reporting the detection of their metabolites in the environment. For the pharmaceuticals which are highly metabolized (e.g. atorvastatin) and therefore poorly excreted, it should be noted that a negative correlation may occur, i.e. having a low degradability in the environment, such as in the case of clofibrate and clofibric acid.

Bioaccumulation

Wastewater and sewage sludge are the major vectors for the entry of these compounds into the environment, and this will depend on their physico-chemical properties. The most widely accepted measure of bioaccumulation is the bioconcentration factor (BCF), but in the absence of BCF values, the bioaccumulation potential may be indicated from $\log K_{ow}$ values. Another alternative to the measured values of BCF, is the use of predictive models such as quantitative structure–property relationship (QSPR)-based models [69] or by the use of molecular connectivity indices and polarity

Table 4 PBT profile of lipid-regulating agents. Reported ecotoxicity and bioaccumulation data

Lipid-regulating agents	Acute toxicity			Cronic toxicity			Persist. DT_{50} (days)	Ref.	Bioaccum. BCF/logK_{ow}	Ref.
	Test organism/endpoint toxicity	EC_{50}	Ref.	Test organism/endpoint toxicity	NOEC	Ref.				
"Fibrate" class										
Clofibrate	Gambusia holbrooki, effects on AChE, LDH and CAT	7.7 mg/L (96 h)	[31]	Gambusia holbrooki, effects on AChE, LDH and CAT	–	[31]	DT_{50} water=38	[56]	BCF=120	[56]
							DT_{50} soil=75 DT_{50} sed=340		LogK_{ow}=3.6	[37]
	Rainbow trout, fatty acyl-CoA oxidase (FOA)	46–152 mg/kg/day	[2]	*Daphnia magna.*/reproduction	0.01 mg/L	[34]				
	Cytotoxicity in fish cell	0.46 mg/L (24 h)	[5]	Fish ChV(estimated)	0.64 mg/L	[56]				
	Algae/growth inhibition	12 mg/L	[34]							
	Algae (estimated)	0.50 mg/L	[47]							
	Fish (estimated)	5 mg/L	[47]							
	Daphnia magna Immobilisation	28.2 mg/L	[34]							
	Daphnid (estimated)	6.50 mg/L	[47]							
	EROD	96 mg/L	[5]							
Fenofibrate	Cytotoxicity in fish cell	3.25 mg/L (24 h)	[5]	Fish ChV(estimated)	0.048 mg/L	[56]	DT_{50} water=60 DT_{50} soil=120 DT_{50} sed=540	[56]	BCF=290 LogK_{ow}=5.19	[56] [37]
	Daphnia magna Immobilisation	50 mg/L	[23]							
	Daphnid (estimated)	0.35 mg/L	[47]							
	Fish (estimated)	0.8 mg/L	[47]							
	Algae (estimated)	0.10 mg/L	[47]							
	EROD	25 mg/L	[5]							
Bezafibrate	*Daphnia magna*	30 mg/L	[35]	Fish ChV(estimated)	3.8 mg/L	[35]	DT_{50} water=60 DT_{50} soil=120 DT_{50} sed=540	[56]	BCF=3.2 LogK_{ow}=4.25	[56] [73]
	Immobilisation	>200 mg/L	[23]							
	Daphnid (estimated)	25 mg/L	[47]							
	Fish (estimated)	5.30 mg/L	[47]							
	Algae (estimated)	18 mg/L	[47]	*Daphnia magna* Reproduction 21 days	ND	[35]				
Gemfibrozil	*Daphnia magna*	10.4 mg/L	[35]	Fish ChV(estimated)	0.93 mg/L	[35]	DT_{50} water=38	[56]	113 times in blood (goldfish)	[4]
	Immobilisation	100 mg/L	[23]				DT_{50} soil=75		BCF=3.2	[56]
	Daphnid (estimated)	6 mg/L	[47]				DT_{50} sed=340		LogK_{ow}=4.77	[74]
	Algae (estimated)	4 mg/L	[47]							
	Fish (estimated)	0.9 mg/L	[47]							
				Daphnia magna Reproduction 21 days	ND	[35]				

Table 4 (continued)

Lipid-regulating agents	Acute toxicity			Cronic toxicity			Persist. DT_{50} (days)	Ref.	Bioaccum. BCF/logK_{ow}	Ref.
	Test organism/endpoint toxicity	EC_{50}	Ref.	Test organism/endpoint toxicity	NOEC	Ref.				
"Statin" class										
Atorvastatin				Fish ChV(estimated)	0.086 mg/L	[56]	DT_{50} water=60 DT_{50} soil=120 DT_{50} sed=540	[56]	BCF=56 LogK_{ow}=4.46	[56] [72]
Simvastatim				Fish ChV(estimated)	0.056 mg/L	[56]	DT_{50} water=38 DT_{50} soil=75 DT_{50} sed=340	[56]	BCF=800 LogK_{ow}=4.68 Koc=8400	[56] [33] [33]
Fluvastatin				Fish ChV(estimated)	0.0014 mg/L	[56]	DT_{50} water=38 DT_{50} soil=75 DT_{50} sed=340	[56]	BCF=2000 LogK_{ow}=4.85	[56] [37]
Lovastatin				Fish ChV(estimated)	0.13 mg/L	[56]	DT_{50} water=38 DT_{50} soil=75 DT_{50} sed=340	[56]	BCF=380 LogK_{ow}=4.26	[56] [33]
Pravastatin				Fish ChV(estimated)	0.18 mg/L	[56]	DT_{50} water=15 DT_{50} soil=30 DT_{50} sed=140	[56]	BCF=3.2 LogK_{ow}=n.a.	[56]
Mevastatin				Fish ChV(estimated)	0.27 mg/L	[56]	DT_{50} water=15 DT_{50} soil=30 DT_{50} sed=140		BCF=220 LogK_{ow}=3.95	[56] [37]
Metabolites										
Clofibric acid	*Gambusia holbrooki*, effects on AChE, LDH and CAT	–	[31]	*C. dubia*	640 µg/L (7 days)	[79]	DT50 water=100	[56]	BCF=3.2	[56]
							DT_{50} water=38 DT_{50} soil=75 DT_{50} sed=340		LogK_{ow}=2.84–2.5 2.57	[6] [75]
	Scenedesmus subspicatus/ growth inhibition	89 mg/L (96 h)	[10]	*B. calyciflorus*	246 µg/L (2 days)	[79]				
	Photobacterium phosphoreum	NOEC5-40 µg/L	[78]	*D. rerio*	700 µg/L (10 days)	[79]				
	P. Pugio	No effect <1,000 µg/L	[77]	Fish ChV(estimated)	38	[56]				
	F. Heteroclitus	No effect <1,000 µg/L	[77]	*Daphnia magna* Reproduction 21 days	40 mg/L	[35]				
	D. tertiolecta Cell density or growth rate	No effect <1,000 µg/L	[77]	*P. subcapitata* 96 h	75 µg/L					
	Microtox 30 min	91 mg/L	[79]							
	Daphnia magna	106 µg/L	[3]	*Gambusia holbrooki*, effects on AChE, LDH and CAT		[31]				
	Immobilization	141 mg/L	[35]							
	Daphnid (estimated)	200 mg/L	[79]							
		150 mg/L	[23]							

		293 mg/L	[47]							
	Fish (estimated)	53 mg/L	[47]							
	Algae (estimated)	192 mg/L	[47]							
Fenofibric acid	Daphnid (estimated)	38 mg/L	[47]	Fish ChV(estimated)	5.5 mg/L	[56]	DT_{50} water=100	[56]	BCF=3.2	[56]
							DT_{50} water=38		LogK_{ow}=4.0	[47]
							DT_{50} soil=75			
							DT_{50} sed=340			
	Fish (estimated)	7.7 mg/L	[47]							
	Algae (estimated)	26 mg/L	[47]							

correction factors [70] The PBT Profiler estimates a BCF based on a chemical's physical and chemical properties using the SRC's BCFWIN estimation program [58]. In the PBT/vPvB (very persistent and very bioaccumulable) criteria defined in the Technical Guidance Document (TGD) [71], the B and vB triggers are BCF=2,000 and 5,000, respectively, which while related to K_{ow} , indicate that a substance may be bioaccumulated when logK_{ow} >3. For this therapeutic group, data of logK_{ow} were available for most of the target compounds [72–75] of this study with the exception of pravastatin. The logK_{ow} values exceed the EU and EPA criteria (logK_{ow} values >3 and >4, respectively), indicating that most of them show high lipo-affinity and therefore, the process of transference into cells and bioaccumulation would be favoured [52, 54]. However, using the PBT Profiler, the estimated BCF values obtained [56], were of 3.2 for bezafibrate, gemfibrozil, clofibric acid and fenofibric acid and >100 for clofibrate (BCF=120) and fenofibrate (BCF=290). In the statin class, BCF values of 800 (using a logK_{ow} value of 4.68) and 2,000 were calculated for simvastatin and fluvastatin, respectively [67]. Thus, fluvastatin is the only pharmaceutical of this group which meets the EU and EPA criteria and would be considered as bioaccumulable. The BCF value for lovastatin and mevastatin was of 380 and 220, respectively, and atorvastatin is not expected to be bioaccumulable (BCF=56).

The only compound with lower a logK_{ow} value (2.5) is clofibric acid, and could be considered as a compound with low lipophilic character and low sorption potential in soil or sediments. This characteristic would be associated with diffusion processes of clofibric acid from soil, or activated sludge toward water compartments, since it is absorbed in soil particulates in a very small proportion [76].

Toxicity

Most of the studies dedicated to assess the hazard posed by pharmaceuticals in the environment have been based on acute toxicity data. Pharmaceuticals occur in the environment at very low concentrations and for short exposure times which might not show acute toxicity effects. However, these compounds could induce disturbances which should achieve higher relevance and biological responses being detectable as adverse effects after a long time period of exposure. In this sense, further data on chronic toxicity analysis should contribute to the assessment of their potential impact in the environment. Extensive knowledge concerning adverse effects at the genetic level, related to biological signalling cascade effects, or effects on the ecosystem is still needed.

Regarding acute toxicity, most studies have been carried out with aquatic organisms, determining the effective concentration

(EC_{50}) through standardized methods. Different in vivo (algae, crustaceans and bacteria) and in vitro tests (cytotoxicity, enzyme-based assays) have been applied to evaluate acute toxicity, in particular for compounds of the fibrate class [23, 34, 35, 77, 78]. Clofibrate and clofibric acid have been studied more than their homologues. However, in spite of that, clofibric acid is a compound frequently detected in the environment; till now, the ecotoxicity studies developed for this and other lipid-regulating agents has been limited. Table 4 shows acute toxicity data for lipid regulator agents. For clofibric acid, the crustacean *Daphnia magna* was the most sensitive species (EC_{50}=100 μg/L) in comparison with algae and fish [3]. No acute toxic effects were observed for the species *P. pugio*, *F. heteroclitus*, *D. tertiolecta* and *Gambusia holbrooki* at the concentrations tested (<1,000 μg/L) [31, 77]. With the bacteria *Photobacterium phosphoreum*, the NOEC was determined as 5–40 μg/L. Regarding to the ecotoxicity of clofibrate, the EC_{50} for *Daphnia magna* is 28 mg/L; therefore, its metabolite, clofibric acid, is more toxic for this aquatic organism. With these EC_{50} values and according to the Directive 93/67/EEC (which establishes the following toxicity categories: very toxic to aquatic organisms (0.1–1 mg/L), toxic (1–10 mg/L), harmful (EC_{50} >100 mg/L), and non-toxic), the fibrate class has only two compounds that would be classified as toxic (clofibrate and clofibric acid), whereas the rest of compounds have no acute toxic effects on the tested organisms. It should be noted that different EC_{50} values have been reported for clofibric acid on the crustacean *Daphnia magna*. According to Ferrari et al. and Han et al. [35, 79] this species is relatively insensitive to clofibric acid; in another publication [77], clofibric acid could be classified as “very toxic”. Clofibrate causes very toxic effects in the cytotoxicity test performed with fish cells and with this classification it would also be toxic towards *Gambusia holbrooki*.

To our knowledge, chronic toxicity studies on aquatic organisms have mainly been performed for clofibrate and clofibric acid. The available data is related with experiments carried out with crustaceans and fish. With these organisms, the results obtained also indicate the toxicity of both compounds. The most sensitive species is *Brachyonius calyciflora* (NOEC=246 μg/L) and *C. dubia* (NOEC=640 μg/L) for clofibric acid and *Daphnia magna* through the test of reproduction with a NOEC of 0.01 mg/L for clofibrate [79].

The development of experiments with chronic toxicity tests can be unachievable in most cases due to the characteristics of this kind of test limiting the knowledge of their potential effects. Supplementary information can be achieved through predictive models. For instance, TGD provides recommendations for the use of QSARs to predict long-term toxicity to fish (NOEC, 28 days) and to *Daphnia* (NOEC, 21 days). In particular QSARs are recommended for chemicals acting by nonpolar narcosis and polar narcosis mechanisms of action [71]. Using PBT Profiler approach [56], the ChV values calculated for lipid regulators exceed the EU and EPA criteria and would show toxicity with NOEC at the ppb level for some of them. The lowest NOEC values were estimated for fluvastatin (1.4 μg/L), fenofibrate (48 μg/L), simvastatin (56 μg/L) and atorvastatin (86 μg/L). The cut-off value for toxic substances (<1 mg/L) was also exceeded for the rest of compounds with the exception of bezafibrate (NOEC of 3.8 mg/L) and the metabolites, clofibric acid (38 mg/L) and fenofibric acid (5.5 mg/L). Thus, with the estimated ChV values and under EPA criteria, clofibric acid would a substance of low concern. However, the available information obtained with experimental data indicates that clofibric acid could be classified as a toxic substance given the NOEC values at the ppb level (see Table 4).

On the other hand, the type of activity and mode of action of these pharmaceuticals, which are designed for the treatment of diseases in humans, could serve as important basis and source of information for deriving risks in the environment, since the pharmacodynamic activity (on receptors, tissues and organs) and pharmacokinetic properties of pharmaceuticals are well known. In this sense, this information can help in the selection of the appropriate test organisms to assess chronic toxicity. Thus, from the mammalian toxicology database of the pharmaceuticals, the ratio of acute to chronic toxicity, target organs, genotoxicity, reproduction or immunotoxicity could be useful for deriving environmental risk. For example, vertebrates (fish) have many physiological functions in common with mammals and it is possible to observe similar endpoints in both organisms [80]. Other relevant data are obtained from pharmacokinetics studies (i.e. the process of absorption, distribution, metabolism and excretion (ADME)) which provides information about the formation of metabolites, which can be relevant at an environmental level (i.e clofibric acid) as well as the comparison about the effective levels of a pharmaceutical in plasma (mammals) and environmental concentration levels. This could be a reference of the exposure which would be necessary for evaluating in eco-chronic toxicity tests.

The information regarding similar or identical physiological targets in organisms and a more broadened biological/phylogenetic understanding, should also contribute to the ERA of pharmaceuticals [81]. It should be noted that pharmacological and pharmacokinetic information from mammalian investigations could support ecotoxicity evaluations in vertebrates (fish); however, for invertebrates, approaches tailored in the pharmacological function and activity may be assumed by taking into consideration several aspects such as those related to the presence of enzymes, receptors, etc. for detecting the pharmacological activity or toxicological effects (secondary effects) in the test organisms. Thus, for the generation of effects in

organisms, these must possess the structure or function which is targeted in the therapeutic indication. In particular, some of compounds of the statin class have a mechanism of action by inhibiting the target enzyme hydroxymethyl-glutaryl-CoA reductase (HMG-CoA reductase) in a relatively non-specific way related to its localization in different tissues which could be responsible for no target effects. This is the case with cerivastatin which was withdrawn from the market because of its relation with rhabdomyolysis. On the other hand, other compounds of the statin class which act through an specific mechanism reducing the possibilities of adverse effects, could not show a direct correlation with damage in ecological species, but the hazard of these pharmaceuticals could be assessed by taking into consideration the implications of the adverse effects. Secondary effects of statins in humans have been described related to cholesterol biosynthesis, effects on gene expression and effects on immunologic parameters, which may not be relevant for the characterization of environmental hazards. With the available information regarding toxicity in mammals, there is no evidence concerning carcinogenic, mutagenic and toxic to reproduction (CMR) or endocrine effects and the statin class should therefore not be identified as a substance with potential chronic toxicity according to the EU criteria [82]. Toxicity studies performed for fluvastatin, simvastatin, atorvastatin, lovastatin and pravastatin have shown that in vitro (bacterial and mammal cells) and in vitro (rodents), they are not genotoxic [83]. In fertility studies with rats, no adverse effects of atorvastatin were observed on fertility and reproduction [82].

The fibrate class is known to enhance β-oxidation of lipids, increasing the amount of reactive oxidative species (ROS) in cells. This process could be responsible for the acute cytoxicity effects which are more active in the case of fenofibrate and clofibrate [28]. Studies performed with primary cultures of rainbow trout hepatocytes (PRTHs) and PLHC-1 fish cell line have shown the higher toxic effect of fenofibrate and clofibrate to PLHC-1 with respect to PRTH. The lipid-regulating agents act by enhancing or reducing the nuclear peroxisome proliferator-activated receptors (PPAR-α, -β or -γ) which play a key role in the catabolism and storage of fatty acids and therefore in the regulation of blood lipids. This mechanism of action has also been observed in aquatic organisms. Compounds such as clofibrate, bezafibrate and fenofibrate affect the PPARs in amphibians at the micromolar level and fish [84]. PPAR-γ has an important role in cellular differentiation, and inhibition of its activity could have consequences for the embryonal development of sensitive species. On the other hand, cellular processes related to transcriptional control of cell cycling, apoptosis and carcinogenesis are mediated by PPARs and the lipid-regulating agents could act at these levels [85, 86]. For the fibrate class, there is no consistent evidence in humans for the carcinogenicity of clofibrate; however, in studies performed with mammals, their link with carcinogenesis and mutagenesis has been described. The fibrate compounds such clofibrate, gemfibrozil and atrovastatin cause cancer in rodents at the equivalent doses used by man [87]. The effects of the main metabolite of clofibrate, etofibrate and etofyllin-clofibrate detected in the environment, clofibric acid, could hold endocrine disruption activity through interference with steroid genesis and cholesterol synthesis [88]. The induction of endocrine responses related to the bioconcentration and reduction of testosterone has been observed in fish after 14 days of exposure to gemfibrozil at environmental concentration levels [89]. Other effects could also be related to other enzymological markers of peroxisome proliferation such as acyl-CoA oxidase [88]. Regarding reproduction studies in both dogs and monkeys using clofibrate, doses approximately 4–6 times the usual human dos, have demonstrated arrest of spermatogenesis. However, the data obtained in experiments with mammals is limited and the extrapolation from rodents to human as well as the relationship with their potential CMR (carcinogenic, mutagenic or toxic to reproduction) or endocrine-disrupting activities cannot be considered as consistent [82].

Environmental risk assessment

ERA schemes in accordance with regulatory guidelines are generally based on a tiered procedure. In the European Union, legislation has introduced the requirement of ERA studies for veterinary products and today the draft Note for Guidance (CPMP/SWP/4447/00 draft) by the EMEA and the TGD [71, 90] are proposed as a framework for human pharmaceuticals. In the USA, the general concepts of the FDA's "Guidance for industry: environment assessment of human drug and biologics applications" [91] are established in a similar context-based framework. In these approaches, the risk of a compound is characterized by comparing the predicted environmental concentration (PEC) with the predicted no effect concentration (PNEC).

The ERA guidelines by EMEA and FDA have included noticeable improvements; nevertheless, some aspects have to be questioned in order to develop a more effective and reliable system of risk assessment [92]. These are related to the overestimation of the PEC and with some deficiencies in the toxicity screening (chronic and mechanism-specific toxicity screening) [93]. Exposure assessment is estimated in the context of a precautionary principle, and the PEC is first calculated as the worst-case scenario and is then refined in a second step taking into account environmental factors or local conditions, providing a more realistic environmental concentration. However, several reports have indicated the need to develop monitoring studies which offer information

about the occurrence of pharmaceuticals in the environment and to allow the corroboration of the PEC values with the measured environmental concentrations (MECs). The MEC values compiled in the "Occurrence" section of this article, show a high variability in the concentration levels reported in publications for each scenario. The lipid regulator agents occur in average concentrations of medium–high ng/L in wastewater, which are high compared to the PECs reported in some publications for influents (180 ng/L) and effluents (170 ng/L) [49]. In surface water, the PEC is close to mean MEC values reported at low ng/L levels. The differences between PEC and mean MEC in wastewater could be associated to several factors, but removal efficiency of sewage treatment plants, seasonal variations or consumption by country could be the main causes. On the other hand, taking into account the cut-off values of both EU and ERA approaches, for exposure assessment, PEC in surface water should exceed the action limit of 0.01 μg/L by EMEA as well as the expected introduction concentration (EIC) by FDA established as 1 μg/L. Therefore, following both EU and ERA procedures, environmental fate and effect analysis should be performed.

In phase II of the ERA schemes, the initial prediction of risk is based on data provided through a set of short-term ecotoxicological studies using three to four different species (*Daphnia* sp., algae and fish according to EMEA, 2001) [90], as well as in the environmental behaviour of the target compounds. However, for certain groups of pharmaceuticals, such as antibiotics, a different approach may be necessary that considers other species (i.e. blue-green algae rather than green algae for anti-microbials), endpoints, or exposure time of the test. On the other hand, the trigger concentration approach (PEC) for phase II may be under-protective or over-protective depending on the ecotoxicity of the pharmaceuticals. The screening studies for initial risk assessment of lipid regulators have shown different results, since the PEC values applied for determining the risk quotient, RQ (PEC/PNEC), have been estimated for different scenarios. For example, Tauxe et. al. determined a PEC/PNEC ratio of 0.1 for clofibric acid in effluent wastewater [49] with mean PEC in effluent of 170 ng/L, whereas other authors have reported RQs close to 1, for a mean PEC value of 3.85 μg/L [79], indicating that this metabolite does not constitute a cause of risk for aquatic ecosystem with the EMEA approach. For gemfibrocil, environmental risk should be expected considering mean MEC values reported in articles and with respect to its acute effects [94]. According to a study performed by Sanderson et al. [95] using the QSAR approach, with probabilistic hazard assessment, acute risk is not expected for more than 99.9% of a total of 67 pharmaceuticals and metabolites. However, they pointed out that all of them fulfilled the EU criteria of 0.01 μg/L and compounds such as bezafibrate and gemfibrozil also show potential bioaccumulation ($\log K_{ow}$ >3). As result, the authors of this study indicated that for bezafibrate and gemfibrozil, the implementation of the second-tier assessment of the ERA schemes should be developed [92]. In other study, this class was classified as the most hazardous among the therapeutic groups evaluated through a comprehensive QSAR screening [96].

Pharmaceuticals may yield chronic effects at low concentrations which may follow different toxicodynamic mechanisms than extrapolated from short-term studies and do not need to be correlated to the potential for an acute effect on the environment. The scarce or, in most cases, lack of chronic ecotoxicity data, hinders an adequate assessment of the risks of pharmaceuticals. For lipid-regulating agents, scarce ERA studies for long-term effects have been carried out through the application of standardized methods using algae, rotifers, crustaceans and fish [35, 79]. Moreover, the available data is mainly related to clofibric acid and clofibrate under European regulatory procedure. For instance, Schowanek and Webb [97] estimated the PNEC for clofibrate as NOEC/AF 10/50=0.2 μg/L, using published data of chronic toxicity for algae and crustaceans [98]. With an estimated PEC of 0.06 μg/L, the PEC/PNEC ratio was 0.3, indicating that clofibrate would not be a cause of risk. Ferrari et al. reported a PNEC value of 4.2 for clofibric, assuming a log-normal distribution of chronic NOEC values. The PNEC was calculated by using the HC5 values with a default safety factor of 5, as suggested in the TGD, 1996 [99], which mean that 95% of the species in the environment are protected. In this study, the PEC/PNEC ratio was close to 1 (0.92) and the risk could be conditioned to a higher value of PEC in the target scenario [79]. Different results were obtained in another ERA study, using a PEC value of 1,75 μg/L and chronic toxicity data with *C. Dubia* (NOEC=640 μg/L), where the hazard quotient was of 175 and clofibric acid was ranked as a compound of high risk [100]. For the rest of compounds of this therapeutical group, to our knowledge, there are no ERA studies published based on long-term effects. There is a need of further ERA information, but also for further considerations regarding the application of ERA studies under the framework of the regulatory schemes which provide a more reliable assessment. For instance, there are many more chronic sublethal endpoints than the recommended number in regulatory guidelines that could have significant effects in the ecosystem.

Conclusions

This article has attempted to provide an updated review of analytical methods developed for the determination of lipid

regulators in environmental samples, focussing on the major drawbacks for quantitative trace determination. Matrix effects can have a profound influence on sensitivity and assay reproducibility. Operational approaches to reduce matrix effects, including the optimization of chromatographic conditions, improvements in sample extraction and effective clean-up steps, have been presented. To compensate matrix effects in the quantitative analysis, the use of appropriate internal standards, standard addition, matrix-matched samples for external calibration, and dilution of SPE extracts have been frequently proposed in several publications.

In the last few years, a major issue is also the assessment of the hazard or risk of lipid regulators in the environment. However, there is still a general lack of data concerning their analysis or evaluation, particularly their ecotoxicity due to long-term effects towards representative organisms of the ecosystem; in spite of this, the pharmacodynamic and pharmacokinetic activities of pharmaceuticals are well known. To identify PBT substances, new challenges for harmonising criteria among different regulatory organisations have been proposed. These consider test representative species, test methods, end-points, the agreement in the assessment procedure of test results and its weight of evidence for providing more a reliable system of PBT and risk assessment. With the predictive model, PBT Profiler, most of the target lipid regulators exceeded the EPA criteria of $\geq$2 months (and $\leq$6 months) in soils and sediments. Using EU criteria, atorvastatin, bezafibrate, fenofibrate, clofibric acid and fenofibric acid are expected to be persistent, since they exceed the cut-off value of DT_{50} water >60 days. The $\log K_{ow}$ values exceeded the EU and EPA criteria ($\log K_{ow}$ values >3 and >4, respectively), indicating that most of them show high lipo-affinity and therefore the process of transference into cells and bioaccumulation would be favoured. To date, limited toxicological studies have evaluated risks posed by chronic exposure to trace concentrations of drugs.

Acknowledgements This study has been financially supported by the Spanish Ministerio de Educación y Ciencia Projects (CTM2004-06265-CO3-01, PPQ2002-04573-C04-03 and CE-CSD2006-004). M. D. Hernando acknowledges the research contract (contrato de retorno de investigadores) from Consejería de Educación y Ciencia de la Junta de Andalucía, Spain.

References

1. Garrison AW, Pope JD, Allen FR (1976) In: Keith CH (ed) GC/MS analysis of organic compounds in domestic wastewaters. Identification and analysis of organic pollutants in water. Ann Arbor Science, Ann Arbor, MI, pp 517–556
2. Ternes TA (1998) Wat Res 32:3245–3260
3. Daughton CG, Ternes TA (1999) Environ Health Perspect 107:907–938
4. Zuccato E, Calamari D, Natangelo M, Fanelli R (2000) Lancet 355:1789–1790
5. Andreozzi R, Raffaele M, Nicklas P (2003) Chemosphere 50:1319–1330
6. Miao XS, Metcalfe CD (2003) J Chromatogr A 998:133–141
7. Stumpf M, Ternes TA, Wilken RD, Rodrigues SV, Baumann W (1999) Sci Total Environ 225:135–141
8. Stumpf M (1996) Vom Wasser 86:291–303
9. Stan HJ (1994) Vom Wasser 83:57–68
10. Heberer T (1997) Fresenius Environ Bulll 6:438–443
11. Tauber R (2003) Quantitative analysis of pharmaceuticals in drinking water from ten Canadian cities. Enviro-Test Laboratories, Xenos Division, Ontario, Canada
12. Heberer T, Stan HJ (1996) Vom Wasser 86:19–31
13. Jones OA, Lester JN, Voulvoulis N (2005) Trends Biotechnol 23:163–167
14. Heberer T, Stan HJ (1997) Int J Environ Anal Chem 67:113–124
15. International Marketing Services (IMS) World Review 2004
16. Miao X-S, Metcalfe CD (2003) J Mass Spectrom 38:27–34
17. Quintana JB, Reemtsma T (2004) Rapid Commun Mass Spectrom 18:765–774
18. Castiglioni S, Bagnati R, Calamari D, Fanelli R, Zuccato E (2005) J Chromatogr A 1092:206–215
19. Ollers S, Singer HP, Fässler P, Müller SR (2001) J Chromatogr A 911:225–234
20. Bendz D, Paxeus NA, Ginn TR, Loge FJ (2005) J Hazard Mater 122:195–204
21. Roberts PH, Thomas KV (2006) Sci Total Environ 356:143–153
22. Löffler D, Ternes TA (2003) J Chromatogr A 1021:133–144
23. Hernando MD, Petrovic M, Fernández-Alba AR, Barceló D (2004) J Chromatogr A 1046:133–140
24. Ternes TA, Bonerz M, Herrmann N, Löffler D, Keller E, Bago B, Alder AC (2005) J Chromatogr A 1067:213–223
25. Petrović M, Hernando MD, Díaz-Cruz MS, Barceló D (2005) J Chromatogr A 1067:1–14
26. Ahrer W, Scherwenk E, Buchberger W (2001) J Chromatogr A 910:69–78
27. Farre M, Ferrer I, Ginebreda A, Figueras M, Olivella L, Tirapu L, Vilanova M, Barcelo D (2001) J Chromatogr A 938:187–197
28. Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT (2002) Environ Sci Technol 36:1202–1211
29. Loos R, Hanke G, Eisenreich SJ (2003) J Environ Monit 5:384–394
30. Schwab BW, Hayes EP, Fiori JM, Mastrocco FJ, Roden NM, Cragin D, Meyerhoff RD, D'Aco VJ, Anderson PD (2005) Regul Toxicol Pharmacol 42:296–312
31. Nunes B, Carvallo F, Guillermino L (2004) Chemosphere 57:1581–1589
32. Scarano LJ, Calabrese EJ, Kostecki PT, Baldwin LA, Leonard DA (1994) Ecotoxicol Environ Safety 29:13–19
33. Laville N, Ait-Aissa S, Gómez E, Casellas C, Porcher JM (2004) Toxicology 196:41–55
34. Halling-Sørensen B, Nielsen N, Lanzky PF, Ingerslev F, Lotten-Lützhoft HC, Jorgensen SE (1998) Chemosphere 36:357–393
35. Han GH, Hur HG, Kim SD (2006) Environ Toxicol Chem 25:265–271
36. Sacher F, Lange FT, Brauch HJ, Blankenhorn I (2001) J Chromatogr A 938:199–210
37. Lee H-B, Peart TE, Svoboda ML (2005) J Chromatogr A 1094:122–129
38. Verenitch SS, Lowe CJ, Mazumder A (2006) J Chromatogr A 1116:193–203

39. Stolker AAM, Niesing W, Hogendoorn EA, Versteegh JFM, Fuchs R, Brinkman UATh (2003) Anal Bioanal Chem 378:1754–1761
40. Wang H, Wu Y, Zhao Z (2001) J Mass Spectrom 36:58–70
41. Quintana JB, Rodil R, Reemtsma T (2004) J Chromatogr A 1061:19–26
42. Miao XS, Koenig BG, Metcalfe CD (2002) J Chromatogr A 952:139–147
43. Kloepfer A, Quintana JB, Reemstma T (2005) J Chromatogr A 1067:153–160
44. Ternes TA (2004) Assessment of technologies for the removal of pharmaceuticals and personal care products in sewage and drinking water facilities to improve the indirect potable water reuse. Poseidon EU Project Report. http://www.eu-poseidon.com
45. Hernando MD, Heath E, Petrovic M, Barceló D (2006) Anal BioAnal Chem 385:985–991
46. Comoretto L, Chiron S (2005) Sci Total Environ 349:201–210
47. Gagne F, Blaise C, André C (2006) Ecotoxicol Environ Safety (in press)
48. Metcalfe C (2003) Pharmaceuticals in the canadian environment. In: Kummerer K (ed) Pharmaceuticals in the environment: sources, fate, effects and risks. Springer-Verlag, Berlin Heidelberg New York, pp 67–90
49. Tauxe-Wuersch A, De Alencastro LF, Grandjean D, Tarradellas J (2005) Water Res 39:1761–1772
50. UNEP, Stockholm Convention in persistent organic pollutants (POPs) Stockholm, May 22, 2001
51. OSPAR Convention, cut-off values for the selection criteria used in the initial selection procedure of the OSPAR dynamic selection and priorisation mechanism, Annex 6, 4.12a. OSPAR Convention Meeting, Valencia, June 25–29, 2001
52. EC 2003, technical guidance document on risk assessment (TGD) in support of Commission Directive 93/67/EEC, Commission Regulation (EC) No 1488/94 and Directive 98/8/EC). European Chemicals Bureau, Ispra (It), 2003
53. Directive 2000/60/EC of the European Parliament and of the Council, of 23 October 2000 establishing a framework for Community action in the field of water policy
54. Substances of equivalent concern-a draft definition from the Advisory Committee on hazardous substances. Discussion paper presented to the UK Chemicals Stakeholder Forum (UK CSF), Meeting of the 25 January 2005
55. Directive 2000/60 of the European Parliament and of the Council establishing the framework for Community action in the field of water policy. Official J Eur Communities (OJ L 327, 22 December 2000)
56. PBT Profiler software developed by EPA. Available at http://www.pbtprofiler.net
57. Boethling RS, Howard PH, Meylan WM, Stiteler W, Beauman J, Tirado N (1994) Environ Sci Technol 28:459–465
58. Meylan WM, Howard PH, Boethling RS, Aronson D, Printup H, Gouchie S (1999) Environ Toxicol Chem 18:664–672
59. Exposure assessment tools and models. US EPA. Available at http://www.epa.gov/oppt/exposure/docs/episuitedl.htm
60. BIOWIN estimation program. Boethling RS, Howard PH, Meylan WM, Stiteler W, Beauman J, Tirado N (1994) Group contribution method for predicting probability and rate of aerobic biodegradation. Environ Sci Technol 28:459–65. Available at http://www.syrres.com/esc/est_soft.htm
61. BCFWIN estimation program. Meylan WM, Howard PH, Boethling RS, Aronson D, Printup H, Gouchie S (1999) Improved method for estimating bioconcentration factor (BCF) from octanol-water partition coefficient. Environ Toxicol Chem 18:664–72. Available at http://www.syrres.com/esc/est_soft.htm
62. ECOSAR: a computer program for estimating the ecotoxicity of industrial chemicals (EPA-748-R-93-002), and estimating toxicity of industrial chemicals to aquatic organisms using structure activity relationships (EPA-748-R-93-001). Available at http://www.epa.gov/oppt/newchems/tools/21ecosar.htm and http://www.syrres.com/esc/est_soft.htm
63. European Commission report on pollutants in urban waste water and sewage sludge. Chap 6: case studies. Available at http://europa.eu.int/comm./environment/waste/sludge/sludge_pollutants_6.pdf
64. OECD guideline for testsing chemicals No 308. Aerobic and anaerobic transformation in aquatic sediments systems, Paris, 2000
65. OECD Guidelines for testing of chemicals, Paris, 1993. OECD 301 A: DOC die-away-test; OECD 301 B: CO_2 evolution Test; OECD 301 C: modified MITI Test (I); OECD 301 D: closed bottle test; OECD 301 E: modified OECD screening test; OECD 301 F: manometric respirometry test
66. Loffler D, Rombke J, Meller M, Ternes TA (2005) Environ Sci Technol 39:5209–5218
67. Estimation programs interface (EPI) version 3.11 for Windows (2003) US EPA, Washington DC. Available at http://www.epa.gov/opptintr/exposure/docs/episuite.htm
68. Hansch C, Leo A, Hoekman D (1995) Amer Chem Soc 185
69. Lu X, Tao S, Hu H, Dawson RW (2000) Chemosphere 41:1675–1688
70. Liu H, Yao X, Zhang R, Liu M, Hu Z, Fan B (2006) Chemosphere 63:722–733
71. TGD, 1996, technical guidance document in support of Council Directive 93/67/EEC on risk assessment for new notified substances and Commission Regulation (EC) 1488/94 on risk assessment for existing substances. Office for Official Publications of the European Communities, Luxembourg
72. Jjemba PK (2006) Ecotoxicol Environ Safety 63:113–130
73. Beausse J (2004) Trends Anal Chem 23:10–11
74. Khan SJ, Ongerth JE (2002) Water Sci Technol 46:105–113
75. Alcock RE, Gemmill R, Jones KC (1999) Chemosphere 38:759–770
76. Drillia P, Stamatelatou K, Lyberatos G (2005) Chemosphere 60:1034–1044
77. Emblidge JP, DeLorenzo ME (2006) Environ Res 100:216–226
78. Kalbfus W, Kopf K (1997) Proceedings of the 51st meeting of the Bavarian Ministry of Water Economy, Munich
79. Ferrari B, Paxeus N, Lo Giudice R, Pollio A, Garric J (2003) Ecotoxicol Environ Safe 55:359–370
80. Bjerselius R, Liundstedt-Enkel K, Olsen H, Mayer I, Dimberg K (2001) Aquat Toxicol 53:139–152
81. Seiler JP (2002) Toxicol Lett 131:105–115
82. National Library of Medicine, ChemIDplus Advanced Record. Available at http://chem.sis.nlm.nih.gov/chemidplus
83. Manson JM, Freyssinges C, Ducrocq MB, Stephenson WP (1996) Reprod Toxicol 10:439–446
84. Kliever SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Wilson TM, Lenhard JM, Lehmann JM (1997) Proc Natl Acad Sci USA 94:4318–4323
85. Auwerx J (1999) Diabetologia 42:1033–1049
86. Vamecq J, Latruff N (1999) Lancet 354:141–148
87. Newman TB, Hulley SB (1996) JAMA 275:5–60
88. Pfluger P, Dietrich DR (2001) In: Kümmerer K (ed) Pharmaceuticals in the environment. Springer Verlag, Berlin Heidelberg New York, pp 11–17
89. Mimeault C, Woodhouse AJ, Miao X-S, Metcalfe CD, Moon TW, Trudeau VL (2005) Aquat Toxicol 73:44–54
90. European Agency for the Evaluation of Medicinal Products (EMEA, 2005) Note for guidance on environmental risk assessment of medicinal products for human use. EMEA CPMP/SWP/4447/00. Draft II. London, UK
91. US FDA, 1998. Guidance for industry. Environmental assessment of human drugs and biologic applications. Revision 1. US Department of Health and Human Services, Food and Drug Administration, CMC6
92. CSTEE. 2001. Opinion on: draft CPMP discussion paper on environmental risk assessment of medicinal products for human

use [non-genetically modified organism (non-GMO) containing]. 24th Scientific Committee on Toxicity, Ecotoxicity and The Environment (CSTEE) plenary meeting, 12 June 2001, Brussels, Belgium. Available at http://europa.eu.int/comm/food/fs/sc/sct/out111_en.pdf [accessed 10 May 2002]
93. Bound JP, Voulvoulis N (2004) Chemosphere 56:1143–1155
94. Hernando MD, Mezcua M, Fernández-Alba AR, Barceló D (2006) Talanta 69:334–342
95. Sanderson H, Johnson DJ, Wilson CJ, Brain RA, Solomon KR (2003) Toxicol Lett 144:383–395
96. Sanderson H, Johnson D, Reitsma T, Wilson C, Brain R, Solomon KR (2004) Regul Toxicol Pharm 39:158–183
97. Schowaneck D, Webb S (2002) Toxicol Lett 131:39–50
98. Köpf W (1995) Effects of endocrine substances in bioassays with aquatic organisms. Presentation at the 50th Seminar of the Bavarian Association for Water Supply
99. Technical guidance documents (1996) in support of Directive 93/67/EEC on risk assessment of new notified substances and Regulation (EC) No. 1488/94 on risk assessment of existing substances (Parts I, II, III and IV). EC catalogue numbers CR-48-96-001, 002, 003, 004-EN-C. Office for Official Publications of the European Community, 2 rue Mercier, 2965 Luxembourg
100. Kelli Berg (2005) Master of environmental toxicology. Available at http://www.ir.lib.sfu.ca/retrieve/2491/etd1839.pdf