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CAMACHO-MUNOZ, D., PETRIE, B., CASTRIGNANÒ, E. and KASPRZYK-HORDERN, B.

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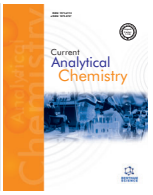

Dolores Camacho-Muñoz, Bruce Petrie, Erika Castrignanò and Barbara Kasprzyk-Hordern*

Department of Chemistry, University of Bath, Bath, United Kingdom

B. Kasprzyk-Hordern

Abstract: The issue of drug chirality is attracting increasing attention among the scientific community. The phenomenon of chirality has been overlooked in environmental research (environmental occurrence, fate and toxicity) despite the great impact that chiral pharmacologically active compounds (cPACs) can provoke on ecosystems. The aim of this paper is to introduce the topic of chirality and its implications in environmental contamination. Special attention has been paid to the most recent advances in chiral analysis based on liquid chromatography coupled with mass spectrometry and the most popular protein based chiral stationary phases. Several groups of cPACs of environmental relevance, such as illicit drugs, human and veterinary medicines were discussed. The increase in the number of papers published in the area of chiral environmental analysis indicates that researchers are actively pursuing new opportunities to provide better understanding of environmental impacts resulting from the enantiomerism of cPACs.

Keywords: Analysis, chiral chromatography, chiral drugs, environment, mass spectrometry, pharmaceuticals, wastewater.

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1. INTRODUCTION TO DRUG CHIRALITY AND ITS ENVIRONMENTAL RELEVANCE

The issue of drug chirality is now a major topic with an impact in various fields such as agriculture, pharmaceutical and chemical industries. Molecules consisting of the same number and types of atoms or groups, but differing only in their spatial arrangement, are called stereoisomers. If two stereoisomers are mirror images of each other then they are called enantiomers. Enantiomers have identical physical and chemical properties except for the fact that they rotate polarized light in opposite direction (phenomenon called optical activity) due to the presence of a chiral center originated by planes, axis or centers of asymmetry (e.g. asymmetric carbon). Enantiomers respond identically to an achiral environment. However, they can interact differently with other chiral molecules, such as receptors and enzymes which are at molecular level homochiral. As a result, enantiomers of the same chiral compound can differ in their biological properties such as distribution, metabolism and excretion.

Chiral pharmacologically active compounds (cPACs) (Fig. (**1**)) can undergo stereoselective disposition in the body, which can be affected by disease, ethnic difference, sex, age and lifestyle as well as co-administration of other drugs [1]. Thus, one enantiomer usually is favoured over the other. Additionally, during metabolism cPACs can show chiral inversion. Chiral inversion can be unidirectional (e.g. in 2- arylpropionic acids (profens) only an inactive R-enantiomer can undergo inversion into an active S-enantiomer) orbidirectional (racemization, e.g. in benzodiazepines) [1].

Enantiomers often exhibit different pharmacokinetics and pharmacodynamics that can result in stereoselective toxicity. Unfortunately, despite the fact that only one enantiomer is usually responsible for the desired activity whereas the other may be inactive or responsible for adverse effects; many drugs have been commercialized as 'racemates' or 'racemic mixtures'. To date, most studies do not explicitly account for individual enantiomers despite the growing evidence for enantiomer-selective toxicity of cPACs towards aquatic organisms as in the case of fluoxetine and propranolol [2, 3].

According to current legislation, a new veterinary or human medicine has to be subjected to an environmental risk assessment (ERA) to evaluate the potential risk of an active compound to the environment, based on protocols performed according to the current European Medicines Agency guidelines. They specify two phases: in Phase I an assessment of the potential extent of exposure to the environment takes place and if the so-called 'action limits' are exceeded then the drug will be subject to a risk assessment in Phase II. However in none of the phases chirality is considered. As a consequence, current approaches utilized in ERA might lead to erroneous and misleading results. It is therefore of the highest importance that more comprehensive ecotoxicity studies taking into account stereochemistry of cPACs, are be required for the approval of any new chiral drug by regulatory authorities.

The need to develop enantioselective methods has arisen for the quantitative assessment of the contribution of each enantiomer and to determine the enantiomeric excess

*Address correspondence to this author at the Department of Chemistry, University of Bath, P.O. Box: BA2 7AY, Bath, United Kingdom; Tel: +44 (0) 1225 385013; E-mail: B.Kasprzyk-Hordern@bath.ac.uk

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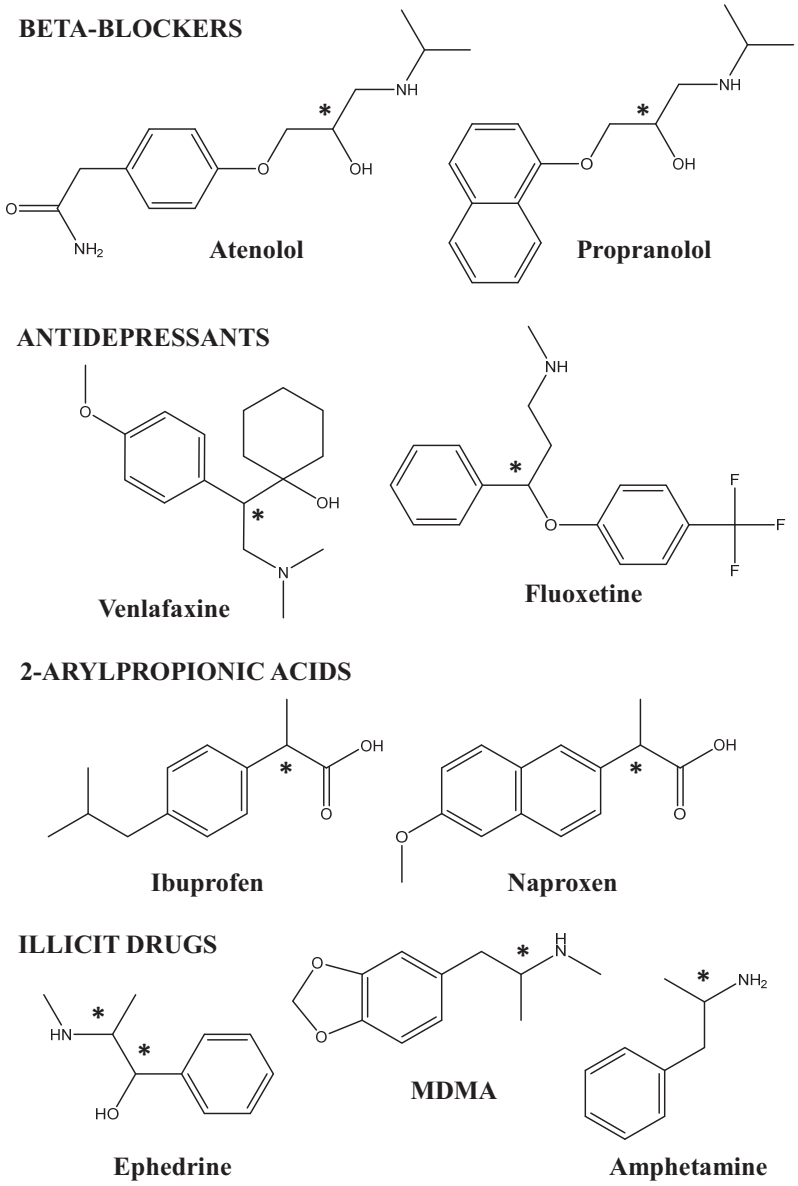

Fig. (1). Structures of chiral drugs (* denotes chiral centers; MDMA: 3,4-methylenedioxymethamphetamine).

[4]. In order to discriminate chiral compounds, the analyte of interest should be added into a chiral environment (e.g., a chiral derivatizing agent or a chiral stationary phase (CSP)) which enables differentiation of enantiomers through the formation of distinguishable diastereomers with distinct physico-chemical properties via multipoint interactions. The chiral recognition mechanism is usually explained by the "three point interaction model". Here a minimum of three simultaneous interactions being stereochemically dependent, are required [5]. In chiral analysis two approaches can be used: so called direct and indirect methods. Direct enantioseparation methods are based on interactions with a chiral selector (either bound to the stationary phase of the column or as chiral additives in the mobile phase) resulting in the temporary formation of diastereomers. In the indirect enantioseparations, analytes are derivatized with enantiomerically pure chiral derivatizing agents resulting in the formation of a pair of diastereomers, which can be afterwards separated in an achiral environment. As an example, enantioseparation of 2-arylpropionic acids has been achieved with direct methods using cyclodextrins bound to the stationary phase [6, 7] or as mobile phase additives [8, 9], and with indirect methods by the derivatization of the R- and S-enantiomers to amide diastereomers using (R)-1-phenylethylamine [10].

The relative proportion of a pair of enantiomers is commonly expressed in terms of the enantiomeric fraction (EF), which equals 0.5in the case of a racemic compound and 1.0 or 0.0 in the case of an enantiomerically pure compound. The accurate determination of EF can provide insights of the compound's history, as well as pointing to the nature and sources of environmental pollution [10].

The importance of enantiomeric analysis of cPACs in the environment is mainly linked to four research are as that include:

(i) *Fate of cPACs during wastewater treatment*. After the administration of cPACs their enantiomeric composition can be altered owing to human or animal metabolism. Once they reach wastewater treatment plants (WWTPs) they are subjected to biotic processes that lead to further changes in their enantiomeric composition. Accordingly, it may be expected that their EF in untreated sewage would differ from the one observed in treated effluents. It is necessary to distinguish between untreated and treated sewage because the effluent might be enriched with one of the enantiomers and current risk assessment does not consider it. Only a few studies have reported these changes in EF in wastewater samples [1, 11-21];

(ii) *Wastewater-based epidemiology (WBE)*. This is an innovative approach that enables retrieving epidemiological information from wastewater via the analysis of human metabolic excretion products called biomarkers. It is for example employed to estimate illegal drug use within a community [22]. Enantiomeric profiling can supplement WBE data with valuable information on abuse trends and potency of chiral drugs and can also help with distinguishing between legal and illicit use of drugs as well as providing an indication of actual consumption as opposed to direct disposal of unused drugs [23];

(iii) *cPACs as chemical markers of water contamination with wastewater*. Several studies reported change in EF of chiral contaminants during biological wastewater treatment, which provides information about their history and makes possible to distinguish between untreated effluents and treated effluents discharged from WWTPs. Therefore some cPACs that undergo consistent and significant measurable EF changes during wastewater treatment could serve as effective indicators of human sewage contamination in water courses (e.g. propranolol) [11];

(iv) *ERA and fate in the environment.* Current ERA is often inaccurate because it evaluates environmental impacts based on a whole drug and does not account for individual enantiomers [24-29]. This might lead to inaccurate estimation of environmental fate and toxicity and might result in misleading conclusions regarding cPAC in question. As already mentioned, biologically mediated processes are oftenenantioselective and may change the EF of the chiral drug, whereas abiotic processes (e.g. sorption, photochemical transformation, air-water, and soil-water exchange) are generally notenantioselective. This phenomenon makes cPACs (those undergoing enantioselective metabolism) useful biotransformation markers.

2. CHIRAL LIQUID CHROMATOGRAPHY COUPLED WITH TANDEM MASS SPECTROMETRY FOR ENANTIOMERIC PROFILING OF CHIRAL HUMAN PHARMACOLOGICALLY ACTIVE COMPOUNDS AND ITS RECENT ADVANCES

To date, the majority of work undertaken in this field of research has focused on whole PAC concentration. This typically involves reverse phased chromatography which can analyse a large number of PACs (>50) simultaneously [30-32]. However, there is increased knowledge of enantiomer specific toxicity towards some aquatic species [2, 3]. This has driven research of PACs in environmental waters at the enantiomeric level. Traditionally though, methods capable of enantiomeric separation have been limited to use in quality control purposes for the pharmaceutical industry. These typically use ultra-violet detection with mobile phases not compatible with mass spectrometry (MS) [33]. However, compatibility with MS is essential as the high sensitivity and selectivity of tandem mass spectrometry is needed for environmental analysis. Several liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods have now been developed and applied to the chiral analysis of several human PAC sub-groups including beta-blockers, antidepressants and profens (2-arylpropionic acids) in environmental matrices (Table **1**).

Table 1. Validated chiral liquid chromatography methods coupled with tandem mass spectrometry for enantiomeric profiling of chiral human pharmaceutical active compounds in environmental matrices.

cPAC senantioseparated	Environmental matrix	Sampling	Sample preparation	Chromatography	Mass spectrometry	MQLs ($ng\ L^{-1}$)	Ref.
Amphetamine, methamphetamine, mephedrone, MDMA, MDA, MDEA, HMMA, HMA, PMA, fluoxetine, norfluoxetine, venlafaxine, desmethylvenlafaxine, tramadol, ephedrine/pseudoephedrine, norephedrine	Influent wastewater (100 mL)	Grab	Filtration (0.7 µm), Oasis HLB SPE	Chiral-CBH column (100 x 2 mm, 5 µm particle size). 85: 15 H_2O: MeOH with 1 mM NH_4OAc (pH 6.4)	Triple quadrupole		[38]
Ibuprofen, naproxen, ketoprofen, chloramphenicol, ifosfamide, 10,11 dihydrocarbamazepine, fexofenadine, 3-n-dichloroethylifosfamide, dihydroketoprofen, tetramisole, aminorex, praziquantel	Effluent wastewater (250 mL), river water (500 mL)	Grab	Filtration (0.7 µm), Oasis HLB SPE and Oasis MCX	AGP (100 x 2 mm, 5 µm particle size). 99: 1 H_2O with 10 mMNH_4OAc: ACN pH 6.7	Triple quadrupole		[43]

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cPAC senantioseparated	Environmental matrix	Sampling	Sample preparation	Chromatography	Mass spectrometry	MQLs (ng L^{-1})	Ref.
Amphetamine, methamphetamine, ephedrine/pseudoephedrine, MDA, MDMA, norephedrine, venlafaxine, atenolol,metoprolol, propranolol, mirtazapine, tramadol, desmethylvenlafaxine, desmethylcitalopram, fluoxetine, salbutamol, sotalol, citalopram	Influent wastewater (50 mL), effluent wastewater (50 mL) and digested sludge (1 g)	Grab	Liquid: filtration (0.7 µm), Oasis HLB SPE Solid: Microwave assisted extraction, Oasis HLB	Chirobiotic V (250 x 4.6 mm, 5 µm particle size). MeOH with 4 mM NH_4OAc, 0.005 % formic acid. Chiral-CBH column (100 x 2 mm, 5 µm particle size). 90: 10 H_2O: 2-propanol with 1 mM NH_4OAc (pH 5)	Triple quadrupole	0.1-29ng L^{-1} influent wastewater, 0.1-11 ng L^{-1} effluent wastewater, 0.1-113 ng g^{-1} digested sludge	[40]
Ibuprofen, ketoprofen, naproxen	Influent wastewater (72.2 mL), effluent wastewater (72.2 mL)	Grab	Microextraction with supramolecular solvent	Sumichiral OA-2500 (250 x 4.6 mm, 5 µm particle size). 90:10 tetrahydrofuran: 50 mM NH_4OAc in MeOH	Triple quadrupole/linear ion trap	0.5-1.2 (MDLs)	[20]
Fluoxetine, norfluoxetine, venlafaxine, alprenolol, bisoprolol, metoprolol, propranolol, salbutamol	Effluent wastewater (250 mL)	-	Filtration (0.45 µm), Oasis MCX SPE	Chirobiotic V (150 x 2.1 mm, 5 µm particle size). 92.5: 7.5 H_2O with 10 mM NH_4OAc: ethanol pH 6.8	Triple quadrupole	2.0-20	[37]
Betaxolol, propranolol, ibuprofen, pindolol, fluoxetine, salbutamol, sotalol, timolol, carazolol, clenbuterol, metoprolol, atenolol	Influent wastewater (100 mL), effluent wastewater (100 mL), river water (500 mL)	Composite (wastewater), grab (river)	Filtration (0.7 µm), Oasis HLB SPE	Chirobiotic V (250 x 4.6 mm, 5 µm particle size). MeOH with 4 mM NH_4OAc, 0.005 % formic acid.	Triple quadrupole	0.2-28 ng L^{-1} influent wastewater, 0.2-28 ng L^{-1} effluent wastewater, 0.2-9 ng L^{-1} river water	[39]
Amphetamine, methamphetamine, MDMA, propranolol, atenolol, metoprolol, venlafaxine, fluoxetine	Effluent wastewater (100 mL), river water (250 mL)	Grab	Filtration (0.7 µm), Oasis HLB SPE	Chirobiotic V (250 x 4.6 mm, 5 µm particle size). MeOH with 4 mM NH_4OAc, 0.005 % formic acid. Chiral-CBH column (100 x 2 mm, 5 µm particle size). 90: 10 H2O: 2-propanol with 1 mM NH_4OAc (pH 5)	Quadrupole time-of-flight	1.3-86 ng L^{-1} effluent wastewater, 0.3-39 ng L^{-1} river water	[16]
Fluoxetine, norfluoxetine	Influent wastewater (200 mL), effluent wastewater (500 mL)	Grab	pH adjusted to 4, Evolute CX-50 SPE	AGP (100 x 2 mm, 5 µm particle size). 97: 3 H_2O with 10 mM HN_4OAc: ACN	Triple quadrupole	0.9-4.3	[36]
Norephedrine, ephedrine/pseudoephedrine, amphetamine, methamphetamine, venlafaxine, MDEA, MDMA, MDA,	Influent wastewater (100 mL), effluent wastewater (100 mL)	Grab	Filtration (0.7 µm), Oasis HLB SPE	Chiral-CBH column (100 x 2 mm, 5 µm particle size). 90: 10 H_2O: 2-propanol with 1 mM NH_4OAc (pH 5)	Triple quadrupole	2.2-12 ng L^{-1} influent wastewater, 2.8-10 ng L^{-1} effluent wastewater	[68]
Atenolol, metoprolol, pindolol, propranolol, sotalol, citalopram, salbutamol	Influent wastewater (100 mL), effluent wastewater (500 mL)	Grab	Filtration (0.7 µm), Oasis HLB SPE	Chirobiotic V (250 x 4.6 mm, 5 µm particle size). 90: 10 MeOH: H_2O with 20 mM NH_4OAc, 0.1 % formic acid (pH 5)	Triple quadrupole	1-25 ng L^{-1} influent wastewater, 0.2-2.5 ng L^{-1} effluent wastewater	[18]
Atenolol, metoprolol, propranolol	Influent wastewater (100 mL), effluent wastewater (500 mL)	Grab	Filtration (0.7 µm), Oasis HLB SPE	Chirobiotic V (250 x 4.6 mm, 5 µm particle size). 90: 10MeOH: H_2O with 0.1 % triethyl ammonium acetate (pH 4)	Triple quadrupole	17-110 ng L^{-1} influent wastewater, 4.4-17 ng L^{-1} effluent wastewater	[92]

2.1. Beta-blockers

The most well studied group of compounds at the enantiomeric level is beta-blockers such as atenolol, metoprolol and propranolol (Table **1**). Methods in the literature report the use of Chirobiotic V columns which contain an antibiotic based stationary phase. Chirobiotic V has an isoelectric point (pI) of 7.2 therefore under typical mobile phase conditions (pH 3.5-7.5) the ionisable groups of this CSP will be positively charged. This CSP is compatible with polar organic mobile phases (e.g., methanol) which is advantageous for environmental analysis due to ease of coupling to MS as well as the excellent sensitivity achieved. Ammonium acetate is often added as a mobile phase buffer as it improves ionisation as well as increasing the separation of enantiomers during chromatography [34]. Methods tend to operate at flow rates of ≤0.1 ml min^{-1} and run times of ~60 min to achieve adequate enantiomeric separation. Application of these methods have successfully shown that several beta-blockers (atenolol, metoprolol and propranolol) undergo stereoselective biodegradation during wastewater treatment [11, 15, 18, 34, 35]. This leads to the enrichment of one enantiomer in final effluent discharges. This information is missed by commonly used achiral methods, yet it is essential for establishing accurate ERA. Chiral analysis has also successfully indicated direct disposal of atenolol. A high estimated population usage coincided with a racemic EF suggesting metabolism by the human body had not occurred [35].

2.2. Anti-depressants

Several analytical methods reported in the literature (Table **1**) successfully separate enantiomers of the antidepressant fluoxetine and its active metabolite norfluoxetine [36-38]. This is underpinned by the enantiomer dependent toxicity of fluoxetine to various aquatic indicator species [3]. Again, the Chirobiotic V column is suitable for enantiomer separation here [37]. Successful separation of fluoxetine is also achievable by macrocyclic glycoprotein based columns such as CBH (cellobiohydrolase I) and AGP (α_1-acid glycoprotein) [36] columns. Similarly, venlafaxine has been separated by both Chirobiotic V (Fig. **2**) and CBH [13, 38-40] columns [16, 41]. A direct comparison of two different methods which can separate venlafaxine enantiomers at similar retention times (Rt) showed the Chirobiotic V method to be ~15 times more sensitive than the CBH method [16]. To demonstrate, venlafaxine method quantitation limits (MQLs) in river water were 8.1 and 7.9 ngL^{-1} for the *S-(+)-* and *R-(-)-*enantiomers using the Chirobiotic V method. For the CBH method, MQLs were 51.7 and 47.9 ngL^{-1}, respectively. This is likely to be caused by a lower MS signal from an aqueous based mobile phase (CBH method) in comparison to an organic one (Chirobiotic V method) [16]. Nevertheless, CBH has shown to be beneficial for achieving separation of a larger number of chemical groups including several illicit drugs and their metabolites [38].

2.3. Profens

cPACs are often prepared and dispensed as racemic mixtures. Interesting though, some cPACs, such as naproxen, are prescribed as a single enantiomer because only the *S* enantiomer exerts a beneficial therapeutic response in the human body (the *R* enantiomer is suspected to be a liver toxin) [42]. Chiral inversion has been observed for ibuprofen and naproxen during wastewater treatment [10, 21]. This phenomenon can be used as a diagnostic tool to help distinguish between treated and untreated sources of contamination in the environment [21]. Due to chiral inversion, it is also necessary to develop chiral methods for cPACs which are dispensed as single enantiomers. At present, there is a lack of LC methods suitable for the enantiomeric determination of profens (ibuprofen, ketoprofen, naproxen). Caballo *et al*. [20] successfully achieved separation using (R)-1-naphthylglycine 3,5-dinitrobenzoic acid as CSP and a mobile phase consisting of 90% tetrahydrofuran and 10% ammonium acetate (50mM) in methanol (Table **1**). MQLs in influent and effluent wastewaters were ~1 ngL^{-1}. Also, other study used an AGP column for their separation with a mobile phase mostly aqueous (10mM ammonium acetate/acetonitrile, 99:1, v/v). This achieved MQLs on the range of low ng L^{-1} [43]. Gas chromatography-

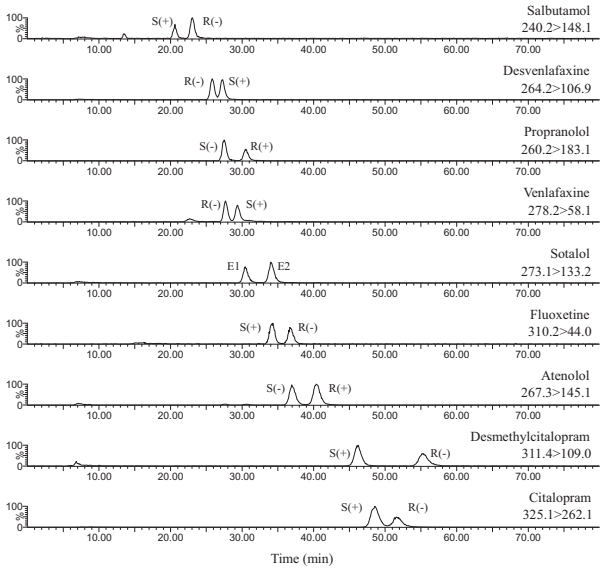

Fig. (2). Mass chromatograms of chiral human pharmaceutical sextracted from final effluent and analysed using Chirobiotic V.

tandem mass spectrometry (GC-MS/MS) methods also exist but derivatization is required prior to analysis [10].

2.4. Non-targeted Analysis

Chiral chromatography can also be applied (with suitable MS) for the identification of unknown compounds. Most methods reported in the literature use triple quadrupole tandem mass spectrometers due to their high sensitivity (Table **1**). Despite being excellent for quantitative determinations, they cannot perform non-targeted analysis. However, the increasing sensitivity of high resolution mass spectrometers enable quantitative (targeted screening) and qualitative (non-targeted screening) determinations simultaneously. To demonstrate, Bagnall *et al*. [16] successfully used quadrupole time-of-flight mass spectrometry (QTOF-MS) to identify and confirm several cPACs at the enantiomeric level in final effluent and river water extracts whilst using Chirobiotic V column. Coupling chiral chromatography with high resolution MS could therefore be used to help identify cPACs and estimate their enantiomeric distribution, as well as to identify transformation patterns. It is worth emphasizing here that transformation by-products can be also chiral. At present this is an area where little/no work has been conducted. This is mainly a result of poor understanding of the mechanisms of chiral separation using various stationary phases and the influence of mobile phase composition. For this concept to be successful, a greater understanding of chiral separations and modelling the likelihood of achieving separation of cPACs which possess a broad range of physico-chemical properties is needed. Furthermore, the development of chiral stationary materials capable of multi-dimensional chiral recognition of several structurally diverse chemical targets is required for successful application of high resolution MS in non-targeted analysis of cPACs.

3. CHIRAL LIQUID CHROMATOGRAPHY COUPLED WITH TANDEM MASS SPECTROMETRY FOR ENANTIOMERIC PROFILING OF CHIRAL VETERINARY MEDICINES

World production and consumption of PACs intended for veterinary applications has been steadily increasing at an alarming rate during the last decades. These compounds are widely used to protect animal health, prevent economic loss, and promote animal growth, ensuring a safe food supply [44]. Despite their benefits, the potential adverse impacts on biota and human health has become a matter of increasing concern due to their continuous release into the environment, either directly from aquaculture, by grazing animals, or indirectly during manure spreading [45]. The presence of significant amounts of residual PACs and their metabolites have been reported in different environmental compartments (wastewater, surface and ground waters, river sediments and soils) [46-55].

Although there is a general move within the European Union towards reducing veterinary medicine use, the use of drugs to maintain animal health and welfare remains a necessity. Of the various PACs commonly used in veterinary medicine, special attention has been paid to antibiotics due to their high detection frequency in the environment and their potential health risk. Antibiotics may cause a direct toxic effect on microflora and microfauna [56], an emergence and spread of resistant bacteria [57] that would affect humans or other animals/organisms [58-60] or even multi-drug resistant pathogen strains, such as in the case of methicillin-resistant *Staphylococcus aureus*, and carbapenem-resistant *Enterobacteriuaceae* [61]. As evidence, the use of antibiotics as growth promoters in animal husbandry in the European Union has been banned since 2006 [62]. However, although monensin sodium or salinomycin sodium were banned for fattening in cattle and pigs, respectively, they are allowed for chicken and turkeys fattening in the United Kingdom [63].

To prevent the risks to human health and the environment the approval and use of veterinary medicines in the European Union are to a large extent regulated by the European Directive 2001/82/EC (amended by Directive 2004/28/EC) and by the European Regulation 726/2004/EC. However, none of the proposed guidelines consider key phenomena characteristic to veterinary medicines. These are: metabolism/excretion in target animals or the degradation processes that take place during manure storage or after the manure is applied onto the soils, as well as stereoselective environmental fate and ecotoxicological effects.

In order to achieve on above, there is a need for reliable multiresidue analytical methods, both for screening and confirmation purposes of veterinary medicines in the environment. Most papers published present procedures for active compounds in drug formulations, in various biological samples or in food of animal origin, but only a few have been focused in solid and aqueous environmental matrices. Most of the current available methods involve the use of LC-MS/MS [45, 53, 64-67]. Also, they have been focused primarily on therapeutic groups over which concern has been raised. Furthermore, there are no published methods, which allow for separation of chiral veterinary medicines at enantiomeric level.

Existing enantioselective multiclass analytical methods are focused on illicit drugs in environmental matrices, beta-blockers, anti-inflammatory drugs and antidepressants [16, 23, 34, 35, 37, 68]. Unfortunately, there are no published methods allowing for chiral analysis of veterinary medicines and their biotransformation by-products. Recently developed by Camacho-Muñoz and Kasprzyk-Hordern, [43] multiresidue cLC-MS/MS method enabled simultaneous analysis of several veterinary and human cPACs with successful enantioseparation of chloramphenicol, ifosfamide and its major metabolite (3-N-dechloroethylifosfamide), 10,11-dihydro-10-hydroxycarbamazepine (a chiral metabolite of carbamazepine), fexofenadine, ibuprofen, naproxen, tetramisole and its metabolite aminorex and partial resolution of praziquantel, ketoprofen and its metabolite dihydroketoprofen (Fig. **3**). Due to the variety of veterinary medicines used, appropriate methods that cover several therapeutic groups are required to evaluate the potential threat to aquatic and terrestrial environment.

4. ENANTIOMERIC PROFILING OF ILLICIT DRUGS IN THE ENVIRONMENT WITH CHIRAL CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY USING PROTEIN-BASED CHIRAL STATIONARY PHASES

Macromolecular stationary phases are the main group of chiral selectors used in chiral LC environmental analysis of drugs [33]. The interest in these protein based selectors

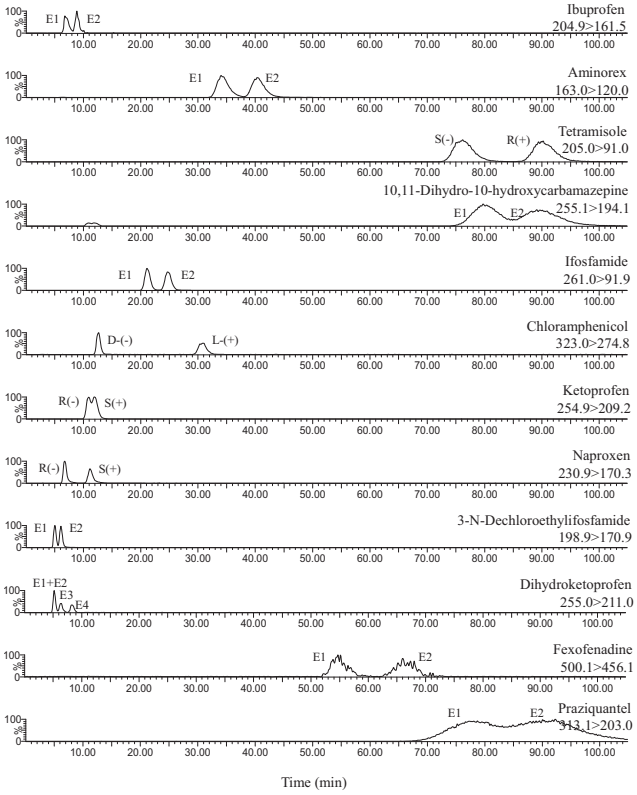

Fig. (3). LC-MS/MS chromatograms using an AGP column for chiral human pharmaceuticals, veterinary medicines and their metabolites in a standard solution of 600 μg L^{-1} of each racemic mixture.

emerged because of the chiral distinction capability of enzymes and plasma proteins, natural chiral pool of selectors [69]. The most important chiral selectors belonging to this group are based on human and bovine serum albumin (HSA and BSA, respectively), glycoproteins such as AGP and crude ovomucoid (OVM)), enzymes (e.g., CBH) and amylose and cellulose based stationary materials. Amongst them, acidic chiral compounds are preferably resolved in HSA columns, basic chiral compounds are resolved in CBH columns while the broadest enantiomer separation capabilities are provided by AGP columns [69]. Since many illicit drugs are basic, CBH and AGP stationary materials are the most widely used for the environmental chiral analysis of drugs of abuse.

CBH column has a cellobiohydrolase enzyme immobilized on to spherical 5 μm silica particles as chiral selector with apIof 3.9. According to Henriksson *et al.* [70], three main active chiral-recognition areas are defined in the column: a catalytically active core, a connecting area and a cellobiohydrolase domain with 36 aminoacids forming two disulphide-bridged loops. The catalytically active core contains the dominating chiral binding site, whilst the cellulose one has the other enantioselective site [71]. Developed by Hermansson [72], AGP consists of a single peptide chain with 181 aminoacids and five heteropolysaccaride units, containing 14 residues of sialic acid. Due to the presence of this acidic component, AGP has a pI of 2.7. Sugar moieties are also present [72]. As the tertiary structure is missing, little information is available on the chiral recognition sites and mechanism of AGP, even if it is known that hydrophobic, electrostatic and hydrogen bonding interactions play a key role in retention and enantioselectivity [73]. Factors, such as temperature, pH and mobile phase composition, can influence chiral recognition on these CSPs. Also, they are particularly sensitive to these factors, with denaturing a very real possibility [74]. Hence, both pH and mobile phase composition are key parameters to achieve enantioselectivity of target compounds. Mobile phase pH affects the ionization of both solutes and CSPs. Both columns are positively charged at pH<pI and negatively charged when pH>pI, so raising the mobile phase pH within the recommended range (pH range: 3-7 and 4-7 for CBH and AGP, respectively) increases the negative charges of the CSP, thus determining ionic bonds between the CSP and the positively charged solute (i.e. amine). As a consequence, higher Rt and an increase of enantioselectivity are expected. Hydrophobic interactions and hydrogen bonding can be influenced by mobile phases containing different nature and percentage (<20%) of organic modifiers and ionic strength. The most frequently used uncharged organic modifiers are methanol, acetonitrile and isopropanol. Depending on their nature (i.e. methanol has lower elution strength than isopropranol) and their content (i.e., lower percentages lead to higher Rt in the case of amines) different Rt and enantioselectivities will be observed. Furthermore, the presence of buffers in mobile phases can ionise the analytes and alter their interactions with the CSP at molecular level. The most used buffers in MS methods are ammonium acetate/formate due to their compatibility with electrospray ionization (ESI) interfaces in MS [75].

So far, in environmental analysis, chiral methods have been used as complementary tools for the investigation of just a few specific chiral compounds (as CSPs were chosen

on the basis of specific enantiomeric resolution) alongside non-chiral multi-residue methods utilizing C18 stationary materials [15, 16]. This required an *ad hoc* sample preparation, which meant a higher quantity of sample, more time consuming and more effective cost analysis. A recently developed multi-residue method combined chiral recognition capability of the CBH-based stationary materials with multi-residue separation potential of the C18-based materials [38]. It enabled the detection and quantification of all targeted (both chiral and non-chiral) human biomarkers in wastewater along with satisfactory enantiomeric separations of 18analytesand a unique single sample preparation step.

In order to study the enantiomeric profiling of illicit drugs in the environment and in wastewater, some precautions are required during the sample collection and preparation. Indeed, an incorrect assessment of the relative concentration of enantiomers might occur at this stage due to the enantioselective microbial metabolic degradation of target analytes [76]. In order to reduce enantioselective degradation, a correct storage protocol is highly recommended. To minimize microbial activity samples must be kept at low temperatures (preferentially frozen) during transport. A controversial question is about the acidification of the sample and the addition of sodium azide to eliminatemicrobial activity as the matrix might be subjected to modification [33].

ESI is the most used interface in MS for environmental analysis. However this interface can be subject to significant signal suppression. Matrix effects can also have negative effects on chiral recognition when using chiral LC-MS(ESI) [33]. As a consequence, an adequate step to concentrate and clean-up the sample, usually carried out through solid phase extraction (SPE) is important. The choice of the SPE sorbent is key to achieve good recoveries and to maintain chiral recognition in selected chiral stationary materials. In fact, in the case of amphetamines, the specific sorbent to extract basic drugs, a mixed-mode cation exchange cartridge (Oasis MCX), was discarded because the use of methanol modified with ammonium hydroxide as an eluting agent resulted in a loss of chiral recognition of amphetamines in the CBH column [68].

A limited number of papers on enantiomeric profiling of amphetamines in wastewater and in the environment have recently been published. A chiral CBH column (100 x 2mm, 5μm) was used to perform environmental chiral analysis of amphetamine-like compounds [77]. In raw wastewater, R-(-)-3,4-methylenedioxymethamphetamine (R-(-)-MDMA) was predominant in respect to the S-(+)-MDMA due to stereoselective human metabolism. EF value increased from 0.68 in raw wastewater to 0.78 (indicating enrichment of MDMA with R-(-)-enantiomer) in treated wastewater due to the treatment of wastewater possibly due to stereoselective microbial metabolic processes. Enantioselective degradation was also observed for amphetamine, leading to an enrichment of R-(-)-enantiomer. In the case of ephedrine, the natural 1R,2S-(-)-enantiomer was detected in raw wastewater, whilst the synthetic 1S,2R-(+)-enantiomer was found in treated wastewater, showing perhaps a chiral inversion process. Receiving waters were also enriched with R-(-)-enantiomers of amphetamine and MDMA, and 1S,2R-(+)-ephedrine. Microcosm experiment evaluated with the CBH column under isocratic conditions (1mM ammonium acetate/isopropanol 9:1) proved that stereoselective microbial metabolic processes lead to enrichment of amphetamine with R-(-)-enantiomer [78]. The enantiomeric composition of 3,4-methylenedioxyamphetamine (MDA)was found to change during the treatment of wastewater. Indeed, EF of 0.28-0.30in raw wastewater (indicating a prevalence of S-(+)-enantiomer) increased to EF=0.38-0.40 in wastewater effluent and further to EF=0.56-0.58 in surface waters, indicating an enrichment of MDA with R-(-)-enantiomer. This phenomenon was observed due to preferential microbial metabolism of S-(+)-MDA [15].

The investigation of illicit drugs at enantiomeric level in environmental samples is principally performed to understand fate of chiral drugs during wastewater treatment and in the environment. It was also proven to be valuable in WBE in differentiating between drug consumption and direct disposal of unused drugs, as well as in verifying the origin of a drug residue. Indeed an application of chiral analysis of illicit drugs in WBE is an attractive one. Originally implemented by Zuccato *et al*. [79] to estimate drug use in studied communities, the WBE concept enables retrieving epidemiological information from wastewater via the analysis of human metabolic excretion products called biomarkers. It consists of several stages: (i) the measurement of the levels of illicit drugs and their metabolites in wastewater, (ii) back-calculation of the mass loads of the parent drugs associated with the investigated population, (iii) and estimation of the consumption of drugs in g day^{-1}, based on drug metabolisms and excretion patterns. The potential of WBE is extraordinary and is currently considered as complementary to other more traditional epidemiological tools. WBE offers several advantages when compared to other traditional epidemiological approaches such as population surveys. These are: (i) near real-time profiling of community health and lifestyle, which is of key importance in for example tracking the emerging trends of new synthetic drug abuse and in verifying changes in usage patterns of "classic" drugs of abuse; and (ii) to give estimates with the possibility to perform retrospective analysis with low-cost studies [80].

WBE has been also employed to evaluate the community-wide use patterns of illicit drugs such as cocaine and its metabolites, amphetamine, opiates and cannabis [81-89]. Temporal and spatial trends were initially studied in19 European cities [90] and then in23 European cities [22]. All these studies utilized achiral methods. Only a few papers reported and correlated the enantiomeric composition of illicit drugs found in wastewater to official statistics. Kasprzyk-Hordern and Baker [15] reported that amphetamine quantified in wastewater was predominantly of illicit origin. This is because amphetamine was enriched with R-(-)-enantiomer in wastewater (only S-(+)-amphetamine, is prescribed in the UK). They also found that the presence of MDA in raw wastewater (which was enriched with S-(+)-enantiomer) was associated with metabolism of MDMA rather than consumption of MDA. Usage patterns of chiral illicit drugs were also studied in the Valencia region by Vázquez-Roig *et al*. [35]. Chiral analysis also helped in understanding an unexpectedly high quantity of ecstasy detected during a monitoring campaign in 2011 in Dutch cities. Indeed, it was confirmed that high levels of MDMA were identified as racemic *via* chiral

chromatography coupled with MS/MS, which indicated direct disposal of unused MDMA possibly as a result of a police raid at a nearby illegal production facility [91].

Recently developed by Castrignanò and Kasprzyk-Hordern, [38] multi-residue chiral method enabled simultaneous analysis of 56 biomarkers including: amphetamine, methamphetamine, MDA, MDMA, MDEA, mephedrone, 4-hydroxy-3-methoxyamphetamine (HMA), 4-hydroxy-3-methoxymethamphetamine (HMMA), paramethoxyamphetamine (PMA), tramadol, venlafaxine, desvenlafaxine, ephedrines, fluoxetine and norfluoxetine (Fig. **4**). This study allowed for the first time for enantiomeric profiling of mephedrone in wastewater.

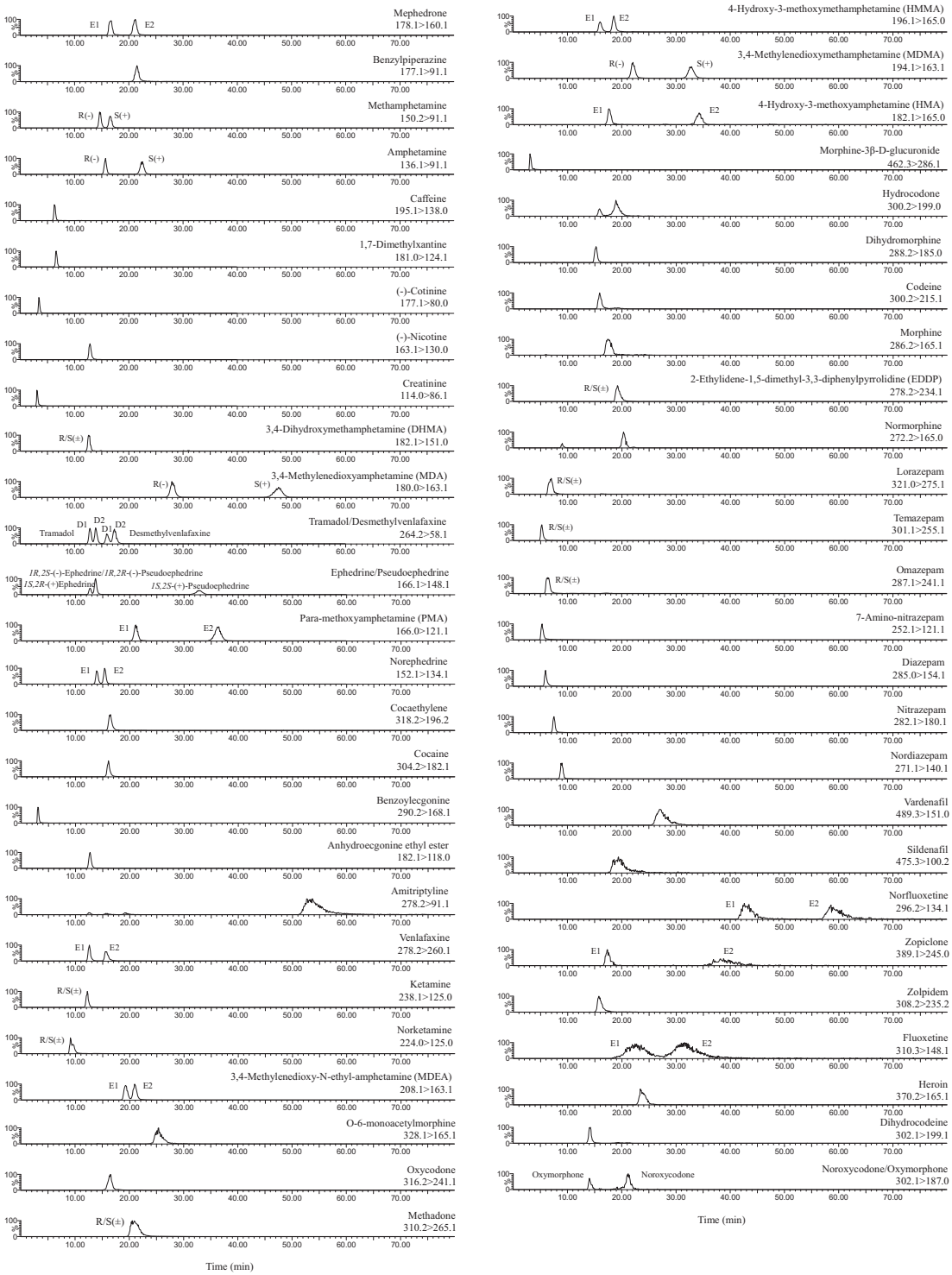

Fig. (4). LC-MS/MS chromatograms using a CBH column for (illicit) drugs of abuse and their metabolites spiked at 500 μg L^{-1} into a influent wastewater sample and extracted by SPE (E1/D1 means first-eluted enantiomer/diastereisomer, E2/D2 means second-eluted enantiomer/diastereisomer).

5. CONCLUSION

Many PACs are chiral. Unfortunately, the phenomenon of chirality, despite its great importance in the pharmaceutical industry, has been mostly ignored in the environmental field. Currently, environmental fate and toxicity of chiral drugs are assessed without taking into consideration their enantiomeric composition. This might lead to a significant under or overestimation of toxicity of chiral drugs and to incorrect ERA. Limited published research concerning fate of cPACs revealed that they are usually present in the environment in non-racemic mixtures of enantiomers. Furthermore, many cPACs undergo stereoselective metabolism in humans and once excreted, they are subject to stereoselective microbial metabolism during wastewater treatment and in the environment. Limited research in this area is associated with lack of analytical methods allowing for enantiomeric profiling of cPACs at trace ppt levels in complex environmental matrices. This review outlined recent advances in the field of environmental chiral analysis of cPACs as well its various applications. An increase in a number of papers published in the area of chiral environmental analysis indicates that researchers are actively pursuing new opportunities to provide better understanding of environmental impacts resulting from the enantiomerism of cPACs.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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