CHIRAL PHARMACEUTICALS

1. Introduction

The word chirality originates from Greek "cheir," meaning hand, which is a geometric property of any object that "cannot be brought to coincidence" with its mirror image. Chiral compounds are three-dimensional molecules with asymmetry in their structures. Thus, a chiral compound is one whose structure cannot be superimposed on its mirror image and both are entitled as enantiomers (Fig. 1) (1).

The structure asymmetry is often originated by a stereocenter or stereogenic center (*). The most common type of chirality is the tetrahedron carbon with four different groups of substituents or other atoms such as sulfur, phosphorous, and silicon (Figs. 2 and 3), which generate a stereogenic center (2).

However, beyond central chirality, other elements of chirality are described such as planar, axial, and helical, found in structures without stereogenic centers (Fig. 4) (3,4).

Enantiomers have identical thermodynamic and spectrometry properties, making the methodology to accurately quantify and identify them a challenge. Polarimetry through rotation of plane polarized light is the easiest and most conventional mode to differentiate enantiomers. They can be identified by rotation of the polarized light: for the right (clockwise) they are called dextrorotatory, (d) or (+), and for the left (counter-clockwise) they are denominated levorotatory, (l) or (−). Concerning their relative chemical configuration to the spatial orientation of the substituents of the stereogenic center, enantiomers can be (*R*), from the Latin *rectus*, or (*S*) from the Latin *sinister*. The equimolar mixture of both enantiomers is denominated racemate or racemic mixture and does not rotate the polarized light (5,6). Despite the similar thermodynamic properties in achiral context, enantiomers normally have different behavior when they face a chiral environment, such as biological systems or reactions in the presence of chiral catalysis.

Biological systems are structurally chiral, as their essential subunits such as amino acids and carbohydrates, which form proteins, glycoproteins, and nucleic acids, have the so-called intrinsic chirality (7,8). Therefore, molecules that are the basis of biological processes in the living organisms, such as enzymes, receptors, or other binding molecules, can recognize enantiomers as different entities, leading to different biological responses (9). The molecular mechanism by which a chiral molecule, such as a macromolecule in a biological system or a chiral small molecule (in any process) can discriminate enantiomers by selective interactions is called chiral recognition.

Enantiomers can have different pharmacokinetics and pharmacodynamics properties. Pharmacokinetics comprises absorption, distribution, and metabolism as well as excretion, while pharmacodynamics corresponds to the drug–receptor interaction resulting in bioactivity or toxicity. These phenomena can be different for two enantiomers (enantioselectivity) due to the chiral nature of membrane proteins, enzymes, and other chiral molecules, as a consequence of the different dissociation constants from the binding sites. Thus, enantioselective effects can often occur with enantiomers of chiral pharmaceuticals in pharmacokinetics events, in bioactivity, and/or in toxicity (9–12).

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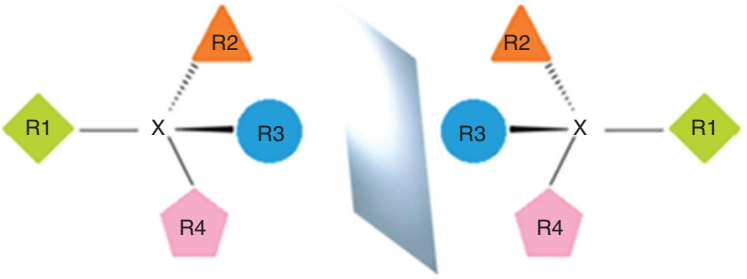
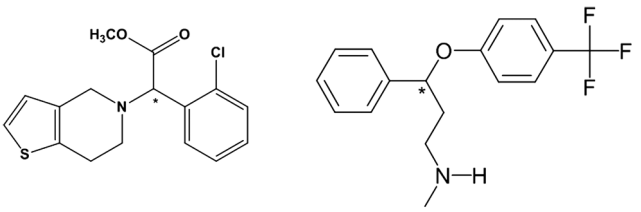


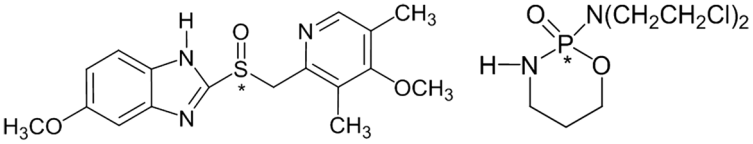
Fig. 1. Stereogenic atom (**X**) with four different substituents (R1, R2, R3, R4) originating two nonsuperposable structures called enantiomers.



(a) (b)

Fig. 2. Structures of two pharmaceuticals with a carbon stereogenic center. (**a**) Clopidogrel. (**b**) Fluoxetine. *: Stereogenic center.

Considering the definition of U.S. Food and Drug Administration, an active pharmaceutical ingredient is "any component that provides pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of man or animals" (13). Pharmaceuticals interact with the binding site of the biological target to form a drug–receptor complex, which is responsible for the pharmacological action (6,14). Chiral pharmaceuticals are administrated as racemates or as enantiomerically pure forms despite the desired pharmacological/biological activity is normally associated with only one enantiomer. The antipode is often inactive, less active, or presents moderate to severe side effects or even high toxicity, and can also differ in pharmacokinetic parameters or present a completely different bioactivity (15).



(a) (b)

Fig. 3. Structures of (**a**) omeprazole, with a sulfur stereogenic center, and (**b**) cyclophosphamide, with a phosphorus. *: Stereogenic center.

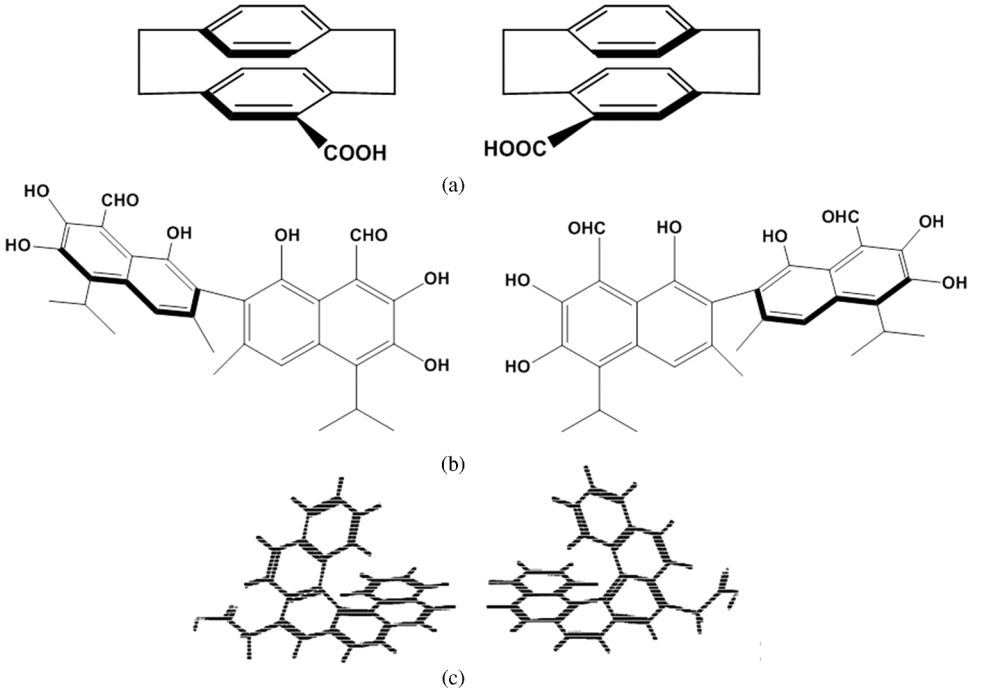


Fig. 4. Different types of chirality. (**a**) Planar: (*pS*) and (*pR*) paracyclophane carboxylic acid. (**b**) Axial: *(aS)* (P) and (*aR*) (M)-gossypol. (**c**) Helical: M(−) and P(+)-hexahelicen-7yl acetic acid. (Reprinted with permission from Reference 4. Copyright 1999, Wiley).

The understanding of biological enantioselectivity owes much to the attachment of three-point model interaction of Easson and Stedman (2). The chiral recognition of enantiomers by a receptor depends on their complementary configuration. This model postulated three complementary binding sites in the receptor to which three groups corresponding to one enantiomer simultaneously interact with the receptor (eutomer), the less active enantiomer (distomer) can establish only one or two simultaneous interactions (Fig. 5). Considering a particular biological activity, it is often found that one of the enantiomers is more potent than the other, the ratio of the potencies is termed eudysmic ratio (16).

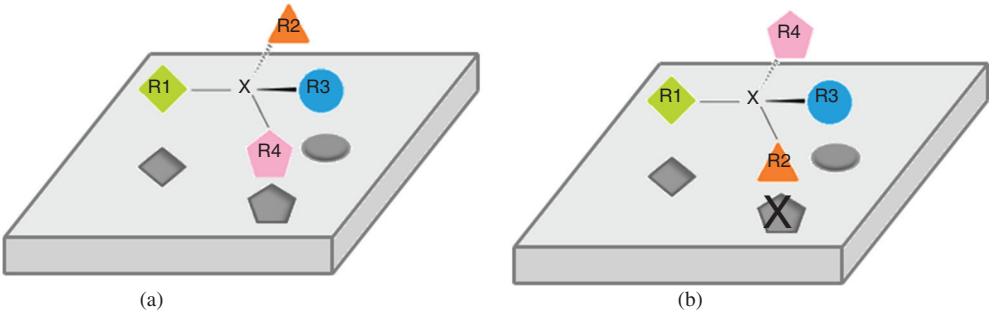


Fig. 5. Schematic process of the complementary chiral recognition between (**a**) the more active enantiomer (eutomer) and the chiral receptor, and (**b**) the less active enantiomer (distomer) and the chiral receptor.

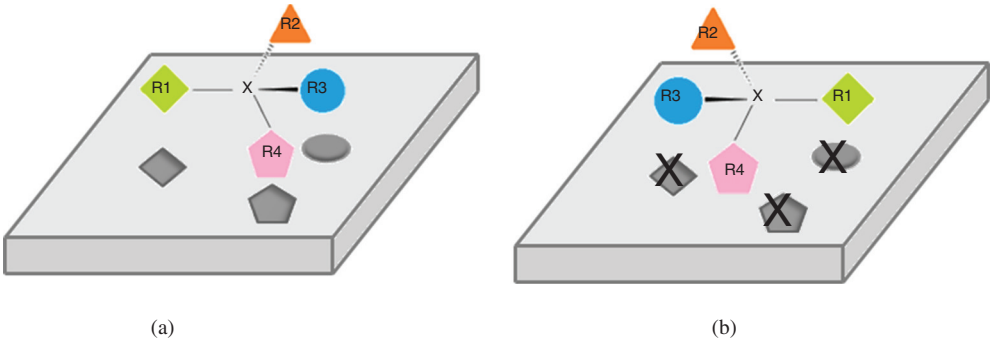


Fig. 6. Schematic process of the complementary chiral recognition between (**a**) the active enantiomer and the chiral receptor, and (**b**) the absence of recognition of the inactive enantiomer and the chiral receptor.

On the other hand, the three interactions can exist for only one enantiomer (Fig. 6). In this case, the antipode is not recognized by the receptor and can be a ligand for other receptor, having a different activity, or causing side effects or toxicity.

However, Easson and Stedman model is valid for small, not flexible tetrahedral objects interacting with planar surface of chiral selectors. Enzyme active sites and drug receptor sites generally do not contain three planar binding positions. In most cases, three contact point model is not enough to explain chiral recognition, and more complex models were later defined by Mesecar and Koshlan (17). A new model to explain the stereospecificity of proteins on the basis of crystallographic data (18) established that four contacts were necessary to explain the mechanism of chiral recognition for enantiomers (Fig. 7).

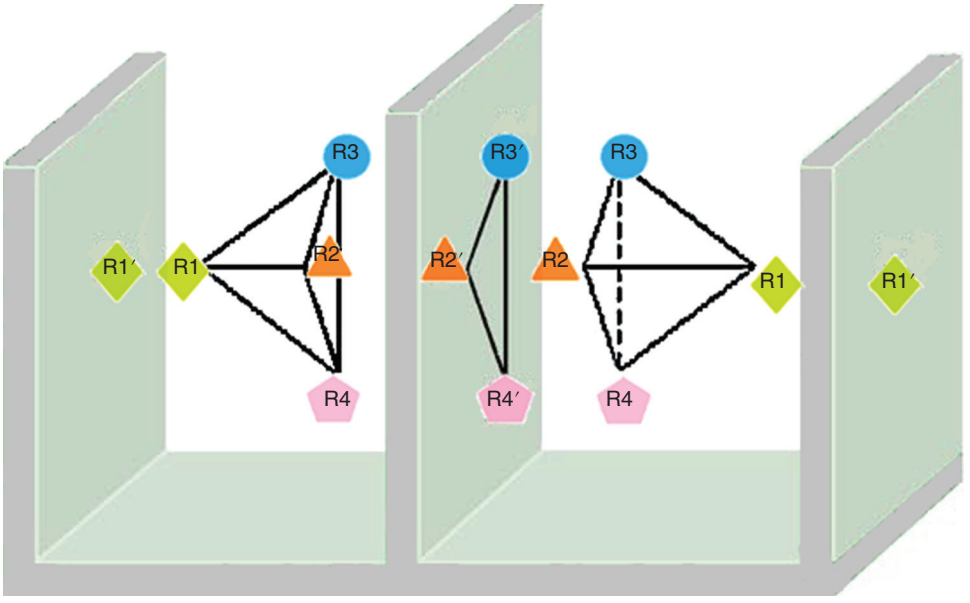


Fig. 7. Four-point location model for stereoselectivity of a protein. (Adapted from Reference 17.)

In summary, types of activities expected from a pair of enantiomers are as follows:

(a) The pharmacological/biological activity of both enantiomers is identical (eg, prometazine, antihistaminic). This effect is due to all interactions of both enantiomers with the biological system having similar dissociation constants; consequently, enantiomers discrimination is not observed due to equivalent pharmacokinetic events and pharmacodynamics properties.
(b) One enantiomer is biologically more active than the other (eg, propranolol, β-blocker). In this case, both enantiomers can reach the same receptor, binding to it at the binding site with different dissociation constants, leading to a stronger recognition of one enantiomer;
(c) One of the enantiomers antagonizes the side effects of the other (eg, indacrinone, diuretic and citalopram, antidepressant).

When enantiomers have different association with receptors, leading to different biological responses, pharmaceuticals should be commercialized as a single enantiomer (10), such as the following examples:

(a) One enantiomer is biologically active and the other does not have known activity (eg, methyldopa, α-adrenergic antagonist and levocetirizine, antihistaminic).
(b) One enantiomer is biologically active and the other is antagonist (eg, picenadol, opioid analgesic) (19).
(c) The pharmacological activity of enantiomers is different (eg, propoxyphene, analgesic and antitussive effects); levothyroxine (synthetic thyroid hormone) and dextrothyroxine (used as a treatment for hypercholesterolemia).
(d) One enantiomer has side effects (eg, L-Dopa, treatment of Parkinson's disease, and ketamine).

Chiral pharmaceuticals from natural resources (natural compounds) are often pure enantiomers such as morphine, epinephrine, hyoscine, levothyroxine, levodopa, L-noradrenaline, (−)brucine, quinine; please see "Chiral Drugs from a Historical Point of View (Old Chiral Drugs: Natural Remedies 3000BC–1900)" in Reference 20. Nowadays, the tendency is to use enantiomerically pure forms; however, there are several pharmaceutical drugs that are commercialized both as racemate and single enantiomer (Table 1).

The evaluation of the license for enantiomerically pure pharmaceuticals that were sold as racemates in the past (chiral switching) (21,22), the many advantages of the use of single enantiomers, and the innovation techniques to obtain and control enantiomers have been important mechanisms to increase the use of chiral pharmaceuticals as single enantiomers (22–24). Regarding pharmacokinetics, pharmacodynamics, and toxicological effects, some advantages of using enantiomerically pure pharmaceuticals include lower therapeutic doses, higher safety margin, lower interindividual variability, less drug interactions and side effects (23,25,26).

Table 1. **Chiral Pharmaceuticals Used as Racemic Mixture and Also as Single Enantiomers**

Racemate	Trade name	Enantiopure pharmaceutical	Trade name	Therapeutic class
amlodipine	Norvasc®	(*S*)-amlodipine (levamlodipine)	EsCordi Cor®	calcium channel blocker
amphetamine	Benzedrine®	(*S*)-amphetamine (dextroamphetamine)	Dexedrine®	CNS stimulant
bupivacaine	Marcain®	(*S*)-bupivacaine (levobupivacaine)	Chirocaine®	anesthetic
cetirizine	Zyrtec®, Reactine®	(*R*)-cetirizine (levocetirizine)	Xyzal®	antihistaminic
citalopram	Celexa®, Ciprami®	(*S*)-citalopram (escitalopram)	Lexapro®, Cipralex®	antidepressant
fenfluramine	Pondimin®	(*S*)-fenfluramine (dexfenfluramine)	Redux®	serotonergic anorectic
formoterol	Foradil®	(*R*,*R*)-formoterol (arformoterol)	Brovana®	beta-adrenoceptor agonist
ibuprofen	Advil®, Motrin®	(*S*)-ibuprofen (dexibuprofen)	Seractil®	NSAID
ketamine	Ketalar®	(*S*)-ketamine (esketamine)	Ketanest S®	general anesthetic
ketoprofen	Actron®	(*S*)-ketoprofen (dexketoprofen)	Sympal®, Ketesse®, Keral®	NSAID
methylphenidate	Ritalin®	(*R*,*R*)-methylphenidate (dexmethylphenidate)	Focalin®	CNS stimulant
milnacipran	Ixel®, Savella®	(*S*,*R*)-milnacipran (levomilnacipran)	Fetzima®	Antidepressant
modafinil	Provigil®	(*R*)-modafinil (armodafinil)	Nuvigil®	Wakefulness-promoting
ofloxacin	Floxin®	(*S*)-ofloxacin (levofloxacin)	Levaquin®,Tavanic®	antimicrobial
omeprazole	Prilosec®	(*S*)-omeprazole (esomeprazole)	Nexium®	proton pump inhibitor
salbutamol	Ventolin®	(*S*)-salbutamol (levalbuterol)	Xopenex®	beta$_2$-adrenergic agonist
zopiclone	Imovane®, Zimovane®	(*S*)-zopiclone (eszopiclone)	Lunesta®	hypnotic

CNS: central nervous system, NSAID: nonsteroidal anti-inflammatory drug.

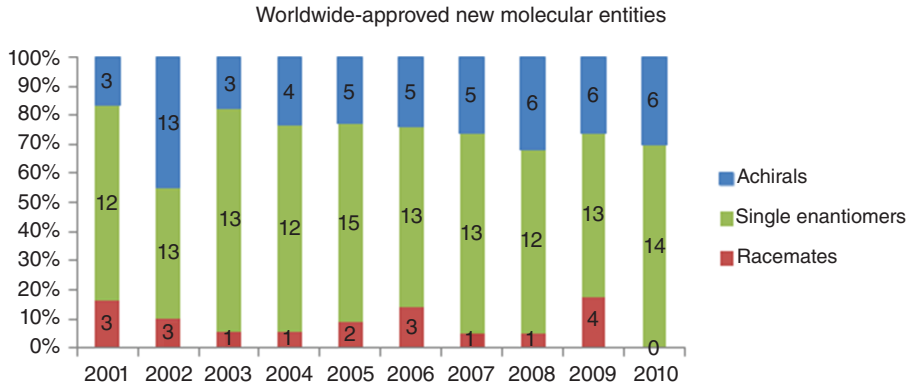


Fig. 8. Annual distribution of worldwide-approved new molecular entities according to chirality in the period 2001–2010 (including diastereomeric mixtures). (Reprinted with permission from Reference 28. Copyright 2012, Elsevier.)

Chiral pharmaceuticals represent 40–50% of the market today and there are several examples for different therapeutic classes (27). The preference to approve enantiomerically pure forms of chiral molecules is evident, and in the year 2010 only single enantiomers were approved worldwide as new molecular entities (Fig. 8) (28).

From the 10 most prescribed pharmaceuticals in the United States (as of May 2015), 8 were chiral small molecules and 7 were commercialized as single-enantiomer trademark drugs (29): Synthroid® (levothyroxine or L-thyroxine), a synthetic thyroid hormone used to treat thyroid hormone deficiency and occasionally to prevent the recurrence of thyroid cancer; Crestor® (rosuvastatin calcium; 3*R*-, 5*S*-, 6*E*-enantiomer), an inhibitor of the enzyme HMG-CoA reductase, used as an antidyslipidemic agent; Nexium® (esomeprazole magnesium; (*S*)-enantiomer of omeprazole), a proton pump inhibitor used as an antiulcer agent; Advair Diskus® (fluticasone propionate; (*S*)-enantiomer + salmeterol xinafoate; racemic form), used as antiasthma, anti-inflammatory, or bronchodilator combination; Vyvanse® (lisdexamfetamine dimesylate), a central nervous system (CNS) stimulant and dextroamphetamine prodrug, used in the treatment of attention-deficit hyperactivity disorder; Lyrica® (pregabalin), used to treat epilepsy, neuropathic pain, fibromyalgia, and generalized anxiety disorder; Januvia® (sitagliptin), an enzyme-inhibiting drug used either alone or in combination for treatment of diabetes mellitus type 2. Moreover, since August 2015, six chiral small molecules in the remaining top 10 pharmaceutical sales were as follows: sofosbuvir (Sovaldi®, Gilead Sciences) a nucleotide analogue used in combination with other drugs for the treatment of hepatitis C; Advair Diskus®, Nexium®, Januvia®, Lyrica®, and Crestor® (30).

2. Methods to Obtain and Purify Chiral Pharmaceuticals

Chirality of a molecule was first reported in 1815 by the French physicist Jean-Baptiste Biot; however, the first chiral separation was reported in 1848 by Louis

Pasteur (31). Because of the hemihedral facets of the crystals of racemic sodium ammonium tartrate, Pasteur was able to separate the mirror image crystals of the isomers using a magnifying glass and tweezers. Furthermore, he advanced the field by studying the influence of one chiral compound upon another and introduced the technique of resolution via diastereoisomers formation in 1858. However, for many years considering drug development, especially pharmaceutical industry, no efforts to study the pharmacological effects of each enantiomer were made. This situation was mainly due to the difficulties related to both obtaining and analyzing pure enantiomers, as well as the scarce knowledge about enantioselectivity in biological activity. Due to the unfortunately well-known pharmaceutical industry's thalidomide (Contergan®) tragedy in the late 1960s and early 1970s (32,33), regulatory procedures of stereoisomeric drugs were introduced (34,35). Since 1992, the regulatory authorities have been encouraging the pharmaceutical industries to provide single enantiomers as new molecular entities, although defining more strict requirements to patent new racemic drugs (36,37).

The enantiomerically pure compounds can be obtained either by preparative resolution of a racemate or by enantioselective synthesis of the desired enantiomer: racemic and chiral approach, respectively (Fig. 9). Each of these strategies has advantages and drawbacks. Regarding the early steps of drug development, resolution of a racemate is the preferential approach since it provides both enantiomers with high enantiomeric purity for enantioselectivity studies (38). Resolution of racemate can be performed by several methodologies (39); however, production scale by preparative chromatography using chiral stationary phases (CSPs) is considered as one of the most efficient tools for obtaining enantiomers with high enantiomeric purity (40). Concerning enantioselective synthesis, it can be useful when a large amount of only one enantiomer is required (38). Asymmetric synthesis has become the most powerful and commonly employed method for preparation of single enantiomers (41). Since the 1980s, progresses in many new technologies, particularly in the catalytic asymmetric synthesis, have been observed. The first commercialized catalytic asymmetric synthesis was

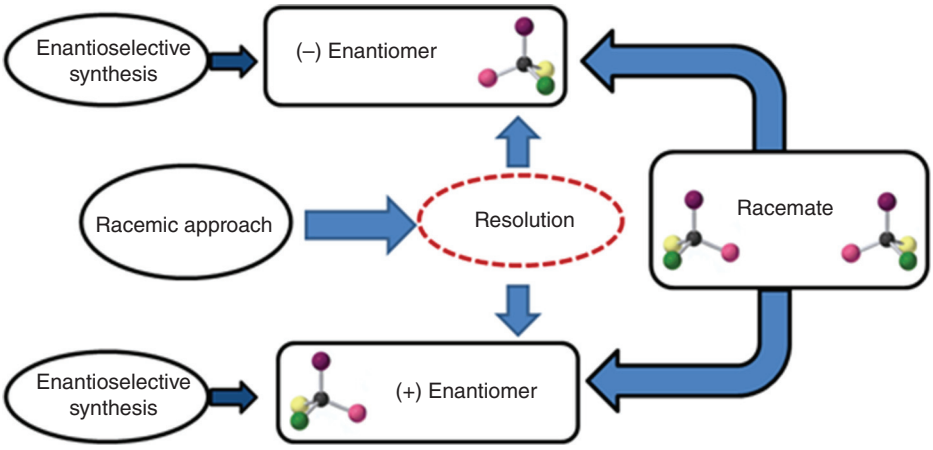


Fig. 9. Racemic and chiral approach (enantioselective synthesis).

established in 1974 in the Monsanto process of L-DOPA by Knowles (42), who was awarded a Nobel Prize in Chemistry in 2001 along with Noyori and Sharpless.

Biological transformations using enzymes, cell cultures, or whole microorganisms are also a powerful means of access to enantiomerically pure compounds from prochiral precursors, even though the scope of some of these reactions is limited, namely, because of the highly specific action of enzymes. Biocatalysts are biomolecules that catalyze or accelerate certain types of chemical reactions in a way similar to that of other kind of small organic molecule catalysts (chemocatalyis). The reactions catalyzed by biocatalysts are referred to as biotransformation, biocatalysis, or enzymatic. Biocatalysis has some advantages over chemocatalytic reactions as it generally provides products with superior chemo-, regio-, diastereo-, and enantioselectivities (43).

Pure enantiomers can also be obtained by kinetic resolution using different reaction rates of each enantiomer, of a racemic mixture, with a chiral catalyst or reagent in order to produce an excess of the less reactive enantiomer. This technique has the limitation of having only maximum theoretical yield of 50% to obtain one of the enantiomers from the racemic mixture. In order to overcome this limitation, dynamic kinetic resolution (DKR) has been developed. DKR is a combination of classical kinetic resolution and chiral substrate racemization. Because certain substrates are not stable and racemization tends to occur in the presence of enzymes or under the chemical conditions, these substrates can be racemized at the time of kinetic resolution. The process of racemization is a dynamic equilibrium, in which slower reactive substrate is converted into faster reactive substrate through the balance. As a consequence, the required compound can be continuously provided. Theoretically, it is possible that DKR can lead to 100% of the product (44). A classical example of DKR is the enantioselectivity esterification of ketoprofen by *Candida antarctica* producing only (*R*)-ketoprofen ester followed by racemization and hydrolyses catalyzed by *Candida rugosa* to obtain (*S*)-ketoprofen (45). Many examples of DKR applied to synthesis of pharmaceuticals can be found in Reference 46.

When both enantiomers are required, two independent syntheses or biosyntheses are necessary and the strategy to obtain pure enantiomers using commercially available chiral reagents in enantiomerically pure form as building blocks (chiral pool strategy) can be an interesting approach to obtain both enantiomers with high enantiomeric purity (47,48).

3. Analysis of Chiral Pharmaceuticals

Along with the development of efficient methodologies to obtain both enantiomers with high enantiomeric purity, the regulatory control had a great impact in the field of chiral analysis. Many countries have issued regulatory guidelines on chiral pharmaceuticals. Regulatory control of chiral pharmaceuticals began in 1992 in the United States with the publication of formal guidelines in a document entitled "Policy Statement for the Development of New Drugs Stereisomeric" (34). In 1993, the EU Committee for Proprietary Medical Products (CPMP) issued formal guidelines called "Investigation of Chiral Active Substances" (49). Later on, the Canadian government announced a "Therapeutic Product Programme" to address

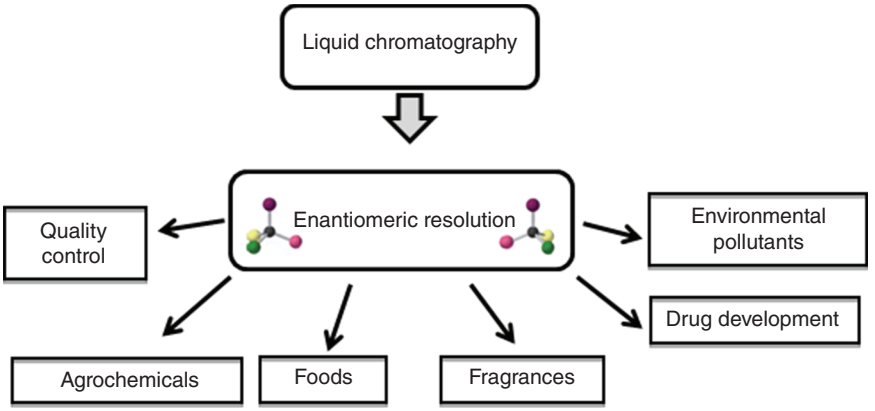


Fig. 10. Examples of diverse fields where chiral LC has a significant role.

stereochemical issues in chiral drug development in 2000 (50). Considering guidelines for Japan, they are essentially consistent with those recommended by the United States and European Union (51). All regulatory guidance emphasize the importance of chirality of active ingredients in the tests of the bulk drug, the manufacturing of the finished products, the design of stability testing protocols, and the labeling of the pharmaceuticals. It states that separation and characterization should be carried out for each enantiomer as well as for bioactivity, pharmacokinetics, pharmacodynamics, and toxicological evaluation of each isomer. Furthermore, drugs that were marketed and patent protected as racemates can extend their years of exclusivity by applying for a "chiral switch."

More information about regulation of chiral pharmaceuticals can be obtained from "Regulatory Aspects of Chiral Drugs" (52) and from the chapter "Regulatory Perspective on the Development of New Stereoisomeric Drugs" (53).

Major advances have been achieved in the field of analytical chromatographic enantioseparation methodologies, such as liquid chromatography (LC) (54), gas chromatography (GC), capillary electrophoresis (CE), supercritical fluid chromatography (SFC), among others (55–57). LC is the method of choice in the analytical laboratories all over the world mainly due to its high speed, sensitivity, and reproducibility.

Accordingly, chiral LC has a strong impact on several economic interesting fields, including the development and analysis of chiral pharmaceuticals (Fig. 10). The resolution of enantiomers can be achieved by LC in two different ways: direct and indirect methods (58,59).

3.1. Enantioresolution by Indirect Method. The indirect method is based on the reaction of a racemate with an optically active reagent to form a pair of diastereoisomers. The separation of the diastereomers can be achieved taking advantage of their different physiochemical properties and the use of conventional analytical LC column such as C18. After the resolution of the diastereoisomers, the enantiomers can be recovered by overturning the derivatization procedure (58). Despite the large number and range of optically active reagents developed for LC analysis (60), the method has some drawbacks (4,58): it is a time-consuming and labor-intensive procedure that can fail in total separation

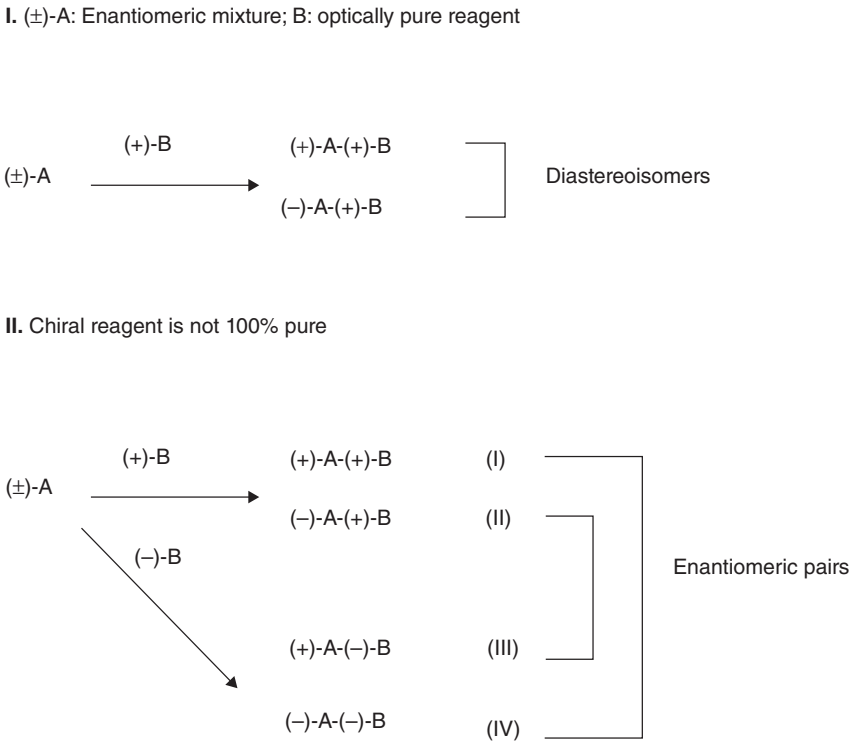


Fig. 11. Representation of enantiomeric resolution by indirect method.

of enantiomers; it requires 100% optically pure reagents to guarantee that the results can be directly representative of the enantiomer composition; it is applicable only to enantiomers presenting a single (or more, but selectively addressable) functional group suitable for derivatization; if the reactions are not complete or are associated with racemization or epimerization, differences in product yields may result in large errors; and it needs a subsequent procedure to recovery the enantiomers (Fig. 11).

Therefore, since the method is time-consuming and there is poor availability of optical pure chiral reagents allied to the possibility of racemization as well as the difficulty in applying it to trace analysis such as biological and environmental samples, this method turns weak for analysis of chiral pharmaceuticals (8,61,62). The remarkable development and application of CSPs has revolutionized the field of enantioseparation and enantioselective analysis, providing a wide range of alternatives to the indirect method.

3.2. Enantioresolution by Direct Method. The direct chromatographic resolution of enantiomers is preferred since it offers many advantages, both for preparative and analytical separations, because there is no need of prior derivatization, it requires much less sample manipulation, and more rapid results are obtained after the preparative chromatography (56,58). Direct resolution of enantiomers by LC is possible using a chiral selector present in the chromatographic system, which must interact preferentially with one of the enantiomers

of the mixture, forming transient diastereomeric complexes. The difference in stability between these complexes leads to different retention times, being the enantiomer that forms the less stable complex eluted first. The chiral selector may be present as an additive in the mobile phase or, alternatively, as component of the stationary phase (58). Many chiral additives are expensive, not available commercially, or must be synthesized; their modes of operation are complex and, after elution, need to be separated from the enantiomers and recovered. Moreover, their applicability in areas such as biomedical, pharmaceutical, and environment is limited due to the degree of purity and detection problems (58,59). For all these reasons, over the years, LC using CSPs has been the most helpful and highly applicable method for the resolution of racemates, determination of the enantiomeric composition, and monitorization of asymmetric reactions, as well as for pharmacokinetic and all enantioselective studies that require quantification/identification of enantiomers (63).

Chromatography and Chiral Stationary Phases. Both GC and LC can be performed by direct method using CSPs. However, there are only few CSPs available for GC, limiting the application of this technique (64–66). One disadvantage of GC is the need of derivatization, in some cases, to increase the volatility, to prevent the peak tailing, and thus to improve detection limits by the peak shaping (67). The high temperature used in GC is a drawback when the analytes are not volatile and when the chiral compound can suffer racemization, enantiomerization, or decomposition (68,69). LC with CSPs has achieved a high reputation in enantioselective separation in both analytical and preparative modes (70–72). CSP consists in a chiral selector adsorbed or covalently bonded to a solid support, forming transitory diastereoisomeric complexes with both enantiomers with different stability (38,72). The major advantage of this method is the ability of the analyte to remain unmodified. There are many different types of commercial CSPs that were recently reviewed in an extensive way (72–76). The most important chiral selectors are Pirkle-type, polysaccharide derivatives, cyclodextrin, protein, macrocyclic glycopeptide antibiotics-based, and others founded on synthetic polymers (72,77,78). The choice of the CSP is based on the experience and on the available literature, often being empirical by trial and error evaluation. The success of an efficient enantioseparation, however, is primarily determined by the chiral discriminative power of the CSP employed. Nowadays, polysaccharide-based, macrocyclic antibiotics-based, and Pirkle-type CSPs are considered as the most useful and broadly applied (55,72,75,76,79). Many authors start the trial and error challenge with polysaccharides, macrocyclic antibiotics-based CSPs, or Pirkle-type Whelk-O1, because they are versatile and suitable for all elution modes (77,80–89). Normal elution mode preference has changed over the last decade to reversed, polar organic, and polar ionic modes (83,84,86–88), mainly due to the compatibility with liquid chromatography–mass spectrometry (LC–MS).

The range of application of polysaccharide-based CSPs is broader than that of macrocyclic antibiotics-based CSPs, concerning the number of compounds enantioseparated by both CSPs (55,75,81). However, macrocyclic antibiotic-based CSPs are complementary to polysaccharide-based CSPs in their ability to resolve important classes of pharmaceutical compounds (55,75). Pirkle-type CSPs are

useful for more specific applications and can offer the chiral selector on both enantiomeric forms (*R* or *S*) (77,90).

Polysaccharide-Based CSPs. Okamoto and co-workers were the first to coat polysaccharide derivatives successfully onto silica gel (91). Since the 1980s, a large number of polysaccharide-based CSPs have been prepared (63,92). This type of CSPs is recognized as the most successful for both analytical (93–96) and preparative separations (38,96–99). Accordingly, about 99% of chiral separations were performed by polysaccharide-based CSPs (100). Recent advancement was made with immobilized polysaccharide CSPs (70,101), which have the advantage of allowing using a wide variety of organic solvents as a mobile phase, increasing the range of their applications. Additionally, these CSPs are available as 3 μm particle size, offering superior speed and column-resolving power (102,103).

Macrocyclic Antibiotics-Based CSPs. Macrocyclic antibiotic CSPs, introduced in 1994 by Armstrong and co-workers (104), became versatile and selective tools for enantioresolution (105–107). The success of these CSPs can be associated with the diversity of their structures that contain a variety of functional groups, multiple stereogenic centers, and inclusion cavities (108). CSPs based on vancomycin (104), teicoplanin (109), ristocetin A (110), and the aglycone of teicoplanin (111) are nowadays commercially available as CHIROBIOTIC V, T, R, and TAG, respectively. In addition to these most important macrocyclic antibiotics, a diversity of other variants of these glycopeptides has been examined as chiral selectors in LC, including avoparcin (110), norvancomycin (112), and eremomycin (113). Additionally, these CSPs show different patterns of enantioselectivity, the possibility of chemo- and enantioresolution of several chiral pharmaceuticals allied with the high strength in the analysis of complex matrices (114–117).

Protein CSPs. Protein-based CSPs have played an important role in the 1980s for analyses of chiral drugs in biological fluids as the main chiral selector used in reversed mode of elution. However, small changes in experimental conditions may change drastically their enantioseparation capacity (118,119). Among a series of commercially protein-based CSPs, the most important are human serum albumin (HSA), α1-acid glycoprotein (AGP), ovomucoid (OVM), and cellobiohydrolase I (CBH). HSA was developed by Wainer and co-workers (120) and is currently useful for drug–protein binding studies (121–124) besides the enantioseparation application, being indicated for acidic chiral compounds. CBH is useful for basic chiral compounds and AGP and OVM give the broadest enantiorecognition to a wide variety of neutral, acidic, and basic pharmaceuticals (72); AGP is also used for drug–protein binding studies (121,125).

Cyclodextrin CSPs. Cyclodextrin (CD)-based CSPs, introduced by Armstrong and DeMond in 1984 (126–128), are cyclic oligosaccharides that differ in the number of glucose units (126–128). CD-based CSPs in LC remain as one of the most important for enantioseparation and various novel CD derivatives have been synthesized and coated or covalently bonded (129) onto silica surfaces and other supports such as monolithic (55). These CSPs can perform separation in normal and reversed modes of elution and also hydrophilic interaction chromatography (HILIC) elution mode (130). Sub-2 μm porous CD-CSPs have been developed in order to track the development and efficiency in LC (129,131).

Pirkle-Type CSPs. Pirkle-type CSPs consist in chiral small molecules chemically bonded to a chromatographic support via a spacer, and are also called brush-type CSPs (54,132). The principle of reciprocity (133) and the studies of the chiral recognition phenomenon, based on chromatographic (134,135) and spectroscopic methods (136,137), were the centerpieces of the evolution of Pirkle-type CSPs. The most relevant characteristics of this type of CSPs include good performance, broad applicability, chemical and thermal inertness, multimodal elution, high sample loading capacities, and availability to invert elution order (63,72,77). Among brush-type selectors, the Whelk-O1 is the most widely used in LC and it was originally designed in the early 1990s for the separation of the enantiomers of naproxen (138). Whelk-O1 is a broad-spectrum CSP for the separation of compounds bearing an aromatic system with a hydrogen-bond acceptor group located near the stereogenic center (88). More recently, as happened with CD-CSPs, the new sub-2 μm porous Whelk-O1 has been developed (139).

LC with different types of CSPs has been used for the determination of the enantiomeric fractions (EF) of many chiral pharmaceuticals in a variety of matrices, including environmental samples (117,140,141). Few examples are shown in Table 2.

4. Chiral Pharmaceuticals as Pollutants

Despite the well-known importance of enantioselectivity in pharmacokinetics, pharmacodynamics, toxicology, and biomedical analyses (15,163,164), the stereochemistry is often neglected in environmental research. Many examples of stereospecificity/stereoselectivity were mentioned above in biological/pharmacological activity; the same phenomena can be observed in the environmental field as the enantiomers of a pollutant molecule can be selectively degraded by the action of microorganisms through the same mechanism that explain the chiral recognition in pharmacology. Therefore, when a racemate reaches the environment, the enantiomers can significantly differ in their environmental fate as well as in toxic impacts. Concerning biodegradation, ecotoxicity, and environmental fate, the recognition of the enantioselectivity is essential to provide a more realistic risk assessment (165–167).

As already mentioned, chiral pharmaceuticals are administrated as racemates or as enantiomerically pure forms (168). Each stereoisomer can suffer metabolism, leading to other stereoisomeric compounds, or can be excreted unchanged. The study of chiral pharmaceuticals in the different environmental compartments may provide valuable insight about the transport, occurrence, transformation, and fate of these chemicals in the environment. Although routes for chiral pharmaceuticals in the environment are the same as described for pharmaceuticals in general, they can be detected in the environment with different values of EF (105), as represented in Figure 12 (117,169,170).

Enantioselectivity have been demonstrated in several ecotoxicological studies. Enantioselective survival and sublethal effects on highly ecologically relevant end points, such as growth, reproduction, and feeding rate, were reported (171). However, only few therapeutic classes of chiral pharmaceuticals have been determined in environmental matrices, concerning the quantification of their

Table 2. **Analytical Methods of Separation of Several Enantiomers in Different Matrices**

Chiral pharmaceutical	Analytical method	Elution mode	Chiral stationary phase	Mobile phase	Matrix application	Reference
omeprazole	2D-LC/UV-vis or IT-MS/MS	RP	tris-(3,5-dimethylphenylcarbamate) of amylose coated onto APS-Nucleosil	ACN/water (35:65, v/v)	waste and estuarine water samples	142
lansoprazole	2D LC–UV	RP	amylose tris(3,5-dimethoxyphenylcarbamate) chiral column	ACN/water (35:65, v/v)	human plasma	143
8 eight β-blockers	LC–UV	NP	CelluCoat	*n*-heptane/EtOH/DEA	screening study	144
fluoxetine and norfluoxetine	LC–FD	RP	Chiralcel OD	75:25 potassium hexafluorophosphate and sodium phosphate/ ACN	human plasma	145
venlafaxine (antidepressant) and 11 analogs	LC–UV	NP	Chiralpak AD and IA	0.5% TFA or DEA in *n*-hexane/2-propanol or EtOH	semipreparative HPLC for *in vitro* pharmacological essays	146
methadone	LC–MS	RP	Chiralcel OJ	65:35 ACN/0.02% TEA in water	human plasma	147
Dechloroethylifosfamide metabolites of Ifosfamide	LC/MS/MS	RP	Chiralpak-AGP	gradient of 10 mM ammonium acetate in water (pH 7.00) and 30 mM (pH 4.00)	blood plasma	148
62 pharmaceuticals	LC–UV	POM	Chiralcel AD-RH, OD-RH, AS-RH, and OJ-R	100:0.1:0.1 ACN or MeOH/DEA/TFA	screening study	149
62 pharmaceuticals	LC–UV	POM	Sepapak-2 and Sepapak-3	100:0.1:0.1 ACN or MeOH or EtOH/DEA/TFA	screening study	149
ketoprofen	LC–UV-vis	RP	Diamonsil C18	MeOH/0.25% TEEA buffer (pH 5.5) + vancomycin (chiral mobile-phase additive)	*in vitro* release test	150
β-blockers, selective serotonine reuptake inhibitors and salbutamol	LC–MS–MS	RP	Chirobiotic V	90:10 MeOH/20 mM NH_4OAc, 0.1% formic acid(pH 4)	WWTP influents and effluents	151

(*continued*)

Table 2. (*Continued*)

Chiral pharmaceutical	Analytical method	Elution mode	Chiral stationary phase	Mobile phase	Matrix application	Reference
bufurarol	LC–UV	PIM	Chirobiotic V	MeOH/acetic acid/TEA	plasma samples and pharmaceutical formulations	152
fluoxetine and norfluoxetine	LC–FD	RP	precolumn	tetrahydrofurane/ isooctane	plasma and cerebral cortex	153
fluoxetine	LC–UV	NP/POM	Chiralcel AD, OD, OJ, Cyclobond I 2000 DM and Kromasil CHI-TBB	hexane/isopropanol/DEA or MeOH/0.2% TEAA (triethylamine acetic acid) (cyclobond)	screening study	154
β-blockers	LC–MS–MS	RP	Chirobiotic V	MeOH/0.1% TEEA	WWTP influents and effluents	155
β-blockers (atenolol, metoprolol, nadolol)	LC–UV	NP	Chiralcel OD	hexane/EtOH/DEA/HAc	biological samples and pharmaceutical formulations	156
β-blockers and profens	LC	RP	Chirobiotic V or V2	MeOH/0.1–1.0% TEEA buffer	screening study	157
β-blockers and profens	LC	POM	Chirobiotic V or V2	MeOH/0.005–0.1% HAc/ 0.005–0.1% TEA	screening study	157
β-blockers	LC/UV-vis	RP	synthesized resin *m*-[(+)-α-methyl benzyl carboxamide] XAD-4	60:40 ACN/sodium acetate-HAc buffer (pH 4.1)	screening study	158
bupivacaine	LC–UV	NP	Kromasil CHI-TBB	98:2:0.3:0.05 hexane/2-propanol/HAc/TEA	to develop continuous chromatographic SMB unit	159
fluoxetine and norfluoxetine	LC–UV	RP	Chiralcel OD	65:35 potassium hexafluorophosphate/ ACN	human plasma	160
53 compounds used in pharmaceutical industry	LC–DAD	NP	Chiralcel AD, OD, AS, and OJ	15:85:0.1 EtOH or 2-propanol/hexane/ additive	screening study	82
		POM		50:50 MeOH/EtOH (for neutral compounds)		

53 compounds used in pharmaceutical industry	LC–DAD	POM	Chirobiotic R, V, and T	100:0, 02:0, 01 MeOH/HAc/TEEA (for basic and acidic compounds)	screening study	82
		RP		25:75 MeOH/TEAA (0.1%, pH 6) (for neutral and acidic compounds)		
eight β-blockers	LC–UV and FD	RP	Kromasil C8	Gradient of 0.03% TFA/0.03% in 50:50 water/ACN	corneal permeability studies	161
36 pharmaceuticals	LC–DAD	NP	Chiralcel AD, OD, and OJ	*n*-hexane/2-propanol or n-hexane/EtOH	screening study	80
fluvastatin	LC/FD	RP	Chiralcel OD-R	40:60 ACN/phosphate buffer pH 2.5	blood plasma	162

Note that these methods are widely used in pharmaceutical, biomedical, and environmental applications.
ACN: acetonitrile, DEA: diethylamine, EtOH: ethanol, HAc: acetic acid, MeOH: metanol, NP: normal mode, PIM: polar ionic mode, POM: polar organic mode, RP: reverse mode, TEA: triethylamine, TEAA: triethylamine acetate, TFA: trifluoroacetic acid.
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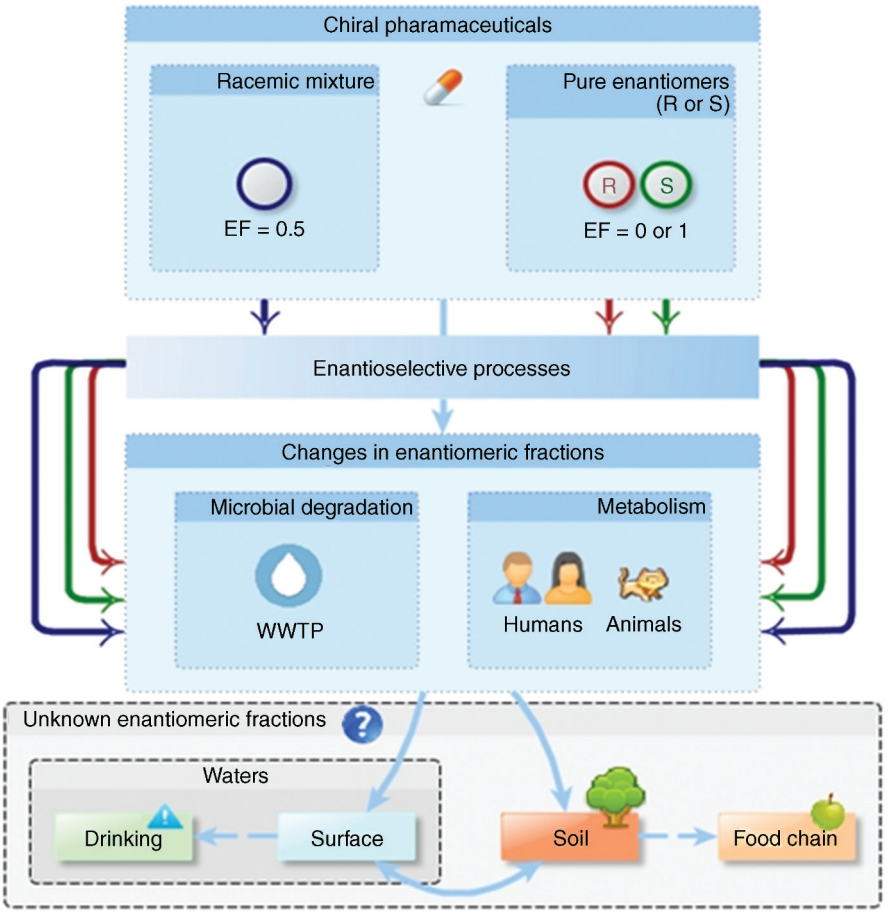

Fig. 12. Representative pathways of chiral pharmaceuticals in the environment.

enantiomers (117,169,170,172–178). Additionally, the biodegradation in biotic media, as occurring in secondary treatment of wastewater treatment plants (WWTPs), adds complexity to this issue since it is also expected to be enantioselective (179–181). Recently, some authors have reported the enantioselectivity occurring during biodegradation and the need to monitor enantiomers in this circumstance. Reports with the anticoagulant warfarin (165), the beta-blockers alprenolol, propranolol (114), atenolol, and metoprolol (115), and the antidepressants venlafaxine (182,183), fluoxetine, and its metabolite norfluoxetine (116) have demonstrated the importance of accurate quantification of enantiomers during biodegradation.

4.1. Toxicity in Aquatic Organisms. Although there are few works about ecological toxicity of isolated enantiomers of chiral pharmaceuticals, several studies reported the toxicity of such pharmaceuticals to aquatic organisms, showing the need of research regarding the effects of single enantiomers.

Some examples of enantioselectivity in ecotoxicity were described in the last years (Table 3). Evaluation of propranolol using the marine bacterium *Vibrio*

Table 3. **Ecotoxicological Data of Chiral Pharmaceuticals as Racemates and as Enantiopure Compounds, and the Effects Caused in Aquatic Organisms**

Drug	Test organism	Test	Effects	Observations	Reference
propranolol	*Oncorhynchus mykiss* (rainbow trout)	10-days $NOEC^{growth}$; 10-days $LOEC^{growth}$	1.0 mg/L; 10 mg/L	the growth was retarded after 10 days of exposure of 10 mg/L; however, after more than 30 days of exposure, the effect was recovered, suggesting a possible adaptation of this species to propranolol	191
fluoxetine	*Cyprinodon variegatus* (sheepshead minnows)	96-h LOEC; 96-h NOEC	2 mg/L; 1.250 mg/L (levels above the environmental concentrations)	the effects on neurotransmitter pathways were observed at concentrations one order of magnitude higher than those reported in the environment. These effects can lead to behavioral changes in the organisms that can potentiate the inability toward a predator or the failure on food providing	192
		sublethal effects (serotonergic system)	effects at 0.300 and 0.030 mg/L		
fluoxetine	sexually mature female zebrafish	E2 ovarian levels	E2 levels decreased threefold when exposed to 32 μg/L of fluoxetine (7 days)	the results suggested no alteration in the ovulatory pathway, due to the decrease of aromatase responsible for the decreased E2 levels. This effect together with the reduced FSHr and LHr gene expression may lead to the decline in spawned eggs	193
		egg production	decline in spawned eggs when exposed to 32 μg/L of fluoxetine or to 50% municipal effluent		
		gene expression	prostaglandins gene expression constant; ovarian aromatase, FSHr, and LHr gene expression were reduced		

(*continued*)

Table 3. **(*Continued*)**

Drug	Test organism	Test	Effects	Observations	Reference
fluoxetine	*Physa acuta* (*Gastropoda*, *Pulmonata*)	mortality and reproduction effects	increased mortality and decreased reproduction effects	the adsorption of fluoxetine to biomass and sediments must be considered for the effects on reproduction of freshwater molluscs	194
fluoxetine and atenolol	*Daphnia magna* (crustacean)	acute immobilization test—EC50	(*R*)- and (*S*)-fluoxetine: 8.1 and 6.9 mg/L; (*R*)- and (*S*)-atenolol: 1450 and 755 mg/L	Both enantiomers of fluoxetine were toxic to *D. magna*. Atenolol enantiomers showed stereoselectivity but did not show a high toxicity. (*R*)-Fluoxetine was considered harmful to *P. subcapita*. (*S*)-Fluoxetine was more toxic on an order of magnitude. (*R*)-Atenolol was more toxic than the (*S*)-enantiomer	190
	Pseudokirchneriella subcapitata (microalga)	growth inhibition test—EC50	(*R*)-Fluoxetine: 34 mg/L; (*R*)- and (*S*)-atenolol: 190 and 143 mg/L		
	Tetrahymena thermophila (protozoan)	growth inhibition test—EC50	(*R*)- and (*S*)-fluoxetine: 30.5 and 3.2 mg/L; (*R*)- and (*S*)-atenolol: 13.7 and 55.7 mg/L		
atenolol	*Pimephales promelas* (fish)	early life stage—NOEC/LOEC	4-days embryo: $NOEC^{hatching}$—10 mg/L; $LOEC^{hatching}$—>10 mg/L; after 28 days $NOEC^{growth}$—3.2 mg/L; $LOEC^{growth}$—10 mg/L,	atenolol has been reported to be low toxic to fish than the chronic toxicity at low concentrations found in the environment	189
		reproduction effects—NOEC/LOEC	short term: $NOEC^{reprodution}$—10 mg/L; $LOEC^{reproduction}$—>10 mg/L; increase in male condition index (CI) $NOEC^{CI}$—1.0 mg/L; $LOEC^{CI}$—3.2 mg/L		
ibuprofen	*Oncorhynchus mykiss* (rainbow trout) cell lines	cytotoxicity and cytostatic action	ibuprofen at 15 μg/mL is cytotoxic to fish cells. The cytostatic effect was verified between 15 and 150 μg/mL	the cytotoxicity of ibuprofen occurred at concentrations 10,000 times greater than the higher value reported in the environment, thus being the bioaccumulation an important factor to be studied	195

fluoxetine	*Pimephales promelas* (fish) *Daphnia magna* (crustacean)	sublethal and behavior effects—LOEC feeding and growth effects	(*R*)- and (*S*)-fluoxetine: 170 and 101 μg/L (*S*)-fluoxetine revealed an LOEC of 195 μg/L	(*S*)-fluoxetine was more toxic for both organisms. The primary metabolite (*S*)-norfluoxetine is more potent than (*R*)-norfluoxetine in mammals. Thus, the aquatic vertebrates can predict better the mammalian behavior than the invertebrate organisms	196
ibuprofen and naproxen	*Desmodesmus subspicatus* (green algae) *Daphnia magna* (crustacean); *Ceriodaphnia dubia*	growth inhibition test—EC50 immobilization test—EC50	342.2 mg/L for ibuprofen; 625.5 mg/L for naproxen 101.2 mg/L for ibuprofen; 166.3 mg/L for naproxen	ibuprofen and naproxen were not classified as harmful to aquatic organisms since the EC50 values were superior to 100 mg/L. These concentrations are unlikely to cause acute effects in aquatic organisms	197

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EC50: half-maximal effective concentration, FSHr: follicle-stimulating hormone receptor, LHr: luteinizing hormone receptor, LOEC: lowest-observed-effect concentration, NOEC: no observed effect concentration.

fischeri, the water flea *Daphnia magna*, the green alga *Desmodesmus subspicatus*, the duck weed *Lemna minor*, the freshwater crustacean *Thamnocephalus platyurus*, and a fish species *Oryzias latipes* classified it as very toxic (184–186). Long-term exposure of *Ceriodaphnia dubia* and *Hyalella azteca* to the same pharmaceutical showed that it affects sex hormone blood plasma concentrations and reduces fecundity (187). The enantiospecific toxicity of propranolol to *D. magna* and *Pimephales promelas* was also demonstrated (188). Winter and co-workers (189) reported the chronic toxicity of atenolol to a freshwater fish, the fathead minnow (*P. promelas*), at concentrations normally found in the environment. Regarding enantioselectivity, (*R*)-atenolol was reported to be more toxic than the (*S*)-enantiomer (190).

Fluoxetine was subjected to several ecotoxicity studies and enantioselectivity was confirmed. Briefly, accumulation of fluoxetine was reported in brain, liver, and muscle tissues of fishes (198). The induction of spawn in zebra mussels (*Dreissena polymorpha*) at low concentrations was also observed (199). The acute effects of fluoxetine in three aquatic species were achieved with concentrations above the levels found in the environment (200). Low concentrations also slowed female and male sexual development in *Gambusia affinis* (201), and a chronic exposure to *D. magna* increased fecundity (202) as well as increased production of egg masses accompanied by higher mortality in adults of a mollusc species (194). In Japanese medaka, this antidepressant led to a developmental abnormality in offspring, increasing female circulating plasma estradiol levels (203). Bioaccumulation in fish (204) and enantioselectivity in sublethal responses to model aquatic vertebrates and invertebrates were demonstrated (196). (*S*)-Fluoxetine presented higher toxicity to *P. promelas* concerning to survival, growth, and reproduction endpoints and also to *D. magna* regarding the feeding and growth endpoints (196).

Acute toxic effects of ibuprofen in the freshwater crustacean were reported (186). Ibuprofen demonstrated cytotoxic and cytostatic effects to rainbow trout (*Oncorhynchus mykiss*) cell lines (liver and hepatocellular carcinoma), affecting the viability and proliferation of fish cells (195). This anti-inflammatory was suggested to affect population growth rate, survival, and reproduction of *D. magna* (205). Effects of ibuprofen on reproduction of Japanese medaka (*O. latipes)* were described, by induction of a less frequent spawning and more eggs production when spawn occurred (206).

5. Conclusions and Future Trends

In addition to the enantioselectivity studies of chiral pharmaceuticals in pharmacology and bioanalysis, enantioselectivity in ecotoxicity and in environmental analysis is important for the development of safe drugs and risk evaluation. The importance of stereochemistry in the different fields of biomedical research, clinical, and pharmacology is evident and well demonstrated. On the other hand, in environmental field, this recognition is not well established yet. Many efforts have been made by some research groups to demonstrate its importance in monitoring enantiomers in the environment, as well as in biodegradation and ecotoxicity assays.

The analytical tools such as chromatographic enantioseparation with high resolution and sensitivity in short analysis time are crucial to boost the development of enantioselectivity studies, particularly in bioanalysis and in environmental field due to the complexity of those matrices. The use of ultrahigh performance liquid chromatography (UHPLC) requires narrow diameter particles ($\leq 2\,\mu m$) to enable efficiency gain and increase sensitivity and peak capacity per time unit. The number of CSPs commercially available, with particle size lower than $2\,\mu m$, is very limited and more development is needed in order to reach the same efficiency of conventional columns used for analysis of achiral compounds.

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