Chapter 1

Chirality in clinical analysis

1.1. Introduction

The term "Chirality" (from the Greek *kheir* for hand), means handedness, which is the existence of left/right opposites. The concept of "Chirality" has been known in chemistry since the 1870's although a hundred years passed before chemists began using this term. In 1962, the first edition of Eliel's "Stereochemistry of Carbon Compounds" did not mention the word chiral although it would be prominent in later editions [1, 2].

The correlation between structure and activity has been a major tool in contemporary biochemical and biomedical research and in rational drug design and disease discovery [3]. The chemistry of tetravalent carbon, the central atom of organic molecules, allows it to have a planar or a three-dimensional structure, and can thereby generate stereoisomers.

Chiral molecules are molecules whose mirror images are not superimposable upon one another. Conversely, achiral compounds have superimposable mirror images. Stereoisomers are compounds made up of the same atoms connected by the same sequence of bonds, but having different 3-D structures (Figure 1.1).

Chiral phenomena are common in living systems. Amino acids, nucleic acids, lipids, carbohydrates, metabolic intermediates, and many other biomolecules are chiral. Indeed, it is difficult to find molecules of physiological significance that do not possess at least

one chiral center. Nearly 80% of medicinal compounds and most of organic molecules are chiral, with one enantiomer affecting the biological response and the other giving either no response or completely unrelated and possibly undesired (Figure 1.2) [4].

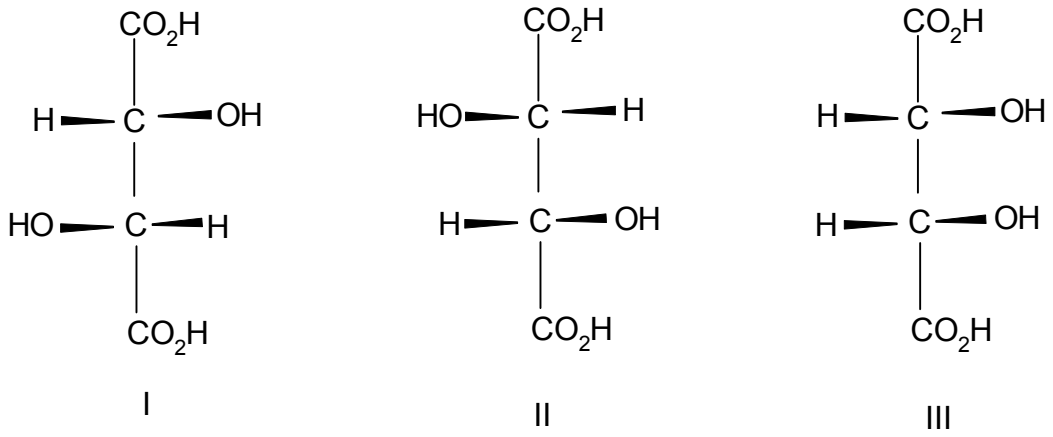


Figure 1.1 Tartaric acid, I and II are enantiomers, I and III are diastereomers, II and III are diastereomers.

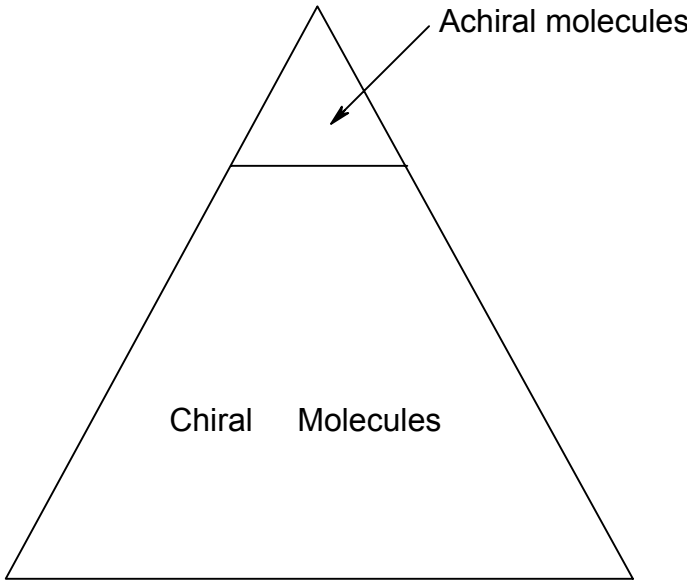


Figure 1.2 Chiral molecules' distribution among pharmaceutical drugs.

1.2. Chirality and configuration

The two mirror image of a chiral molecule are termed enantiomers. To establish whether a molecule is chiral or achiral the evaluation of symmetry elements present in the molecule should be under consideration. The symmetry elements of interest in stereochemistry are [5]:

a) axis of symmetry (C_n)-when operation on an axis C_n, where n = 360°/rotation, leads to a structure indistinguishable from the original. A compound has a simple axis of symmetry if a line can be drawn through its molecular model in such a way that its rotation through a certain number of degree about the line leads to an arrangement which is indistinguishable from the original. For example, in Figure 1.3, (E)-1,2-dichloroethane has a simple axis of rotation that passes through the midpoint of the molecule and is perpendicular to the plane described by the atoms of the molecule;

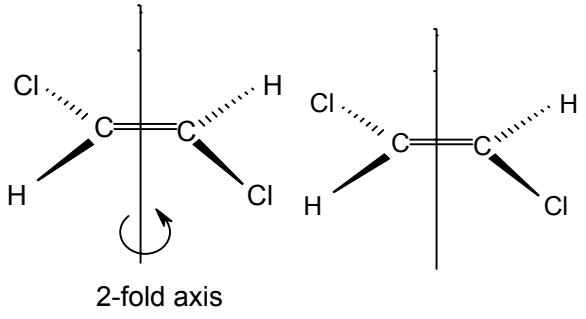


Figure 1.3 (*E*)-1,2-dichloroethane

b) plane of reflection (σ)-corresponds to a plane of symmetry that divides the molecules in two identical halves. It can be visualized also as a mirror plane in which one half of the molecule reflects its enantiomeric image (Figure 1.4). A compound possesses a plane of symmetry if,

1. all the atoms of the molecule are in the same plane;
2. an imaginary double-sided mirror imagined to be inserted through the molecule reflects both the halves so that the new arrangement is similar to the original molecule.

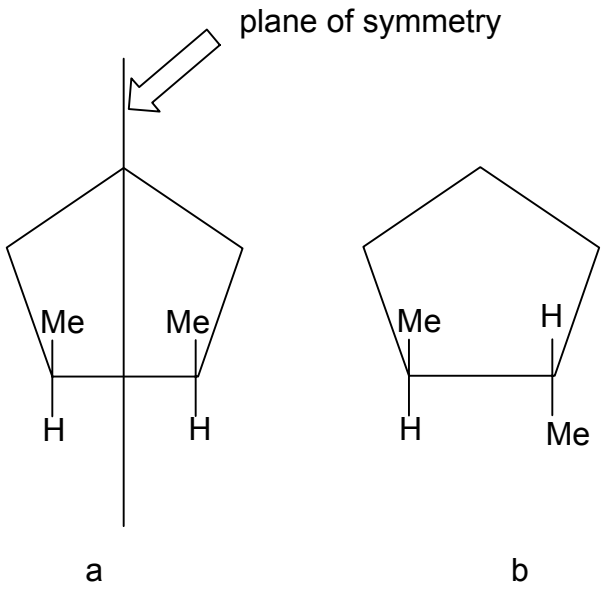


Figure 1.4 Chiral and achiral molecules in plane of symmetry (a) cis-1,2-dimethylcyclopentane(achiral), and (b) trans-1,2-dimehylcyclopentane(chiral)

c) Center of symmetry (*i*) and rotation-reflection axes, S_n (alternating axes) –a formal point in the center of the molecule, in reference to which each atom present finds its equivalent upon extension of an imaginary line of similar length to that joining it to the center of symmetry. As an example, an isomer of 1, 3-dichloro-2,4-difluorocyclobutane has a center of symmetry as its only symmetry element (Figure 1.5). The molecule is possible to have an alternating axis of symmetry if:

1) a rotation through θ degree about an axis is passing through the molecule;

2) the rotated molecule is reflected in a mirror that is perpendicular to the axis of rotation in step 1.

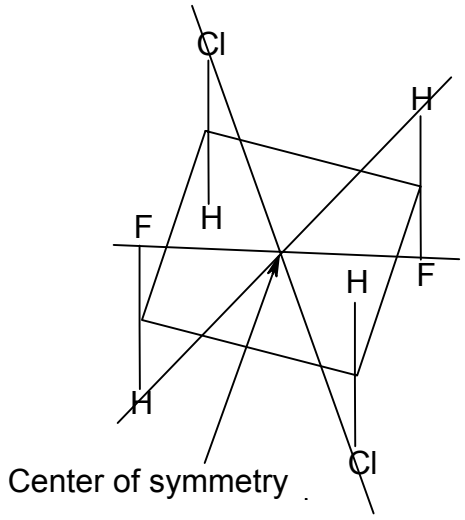


Figure 1.5 1,3-dichloro-2,4-difluorocyclobutane

d) Axis of rotation-reflection (S_n)-a molecule contains a fourfold alternating axis of symmetry if the molecule rotates 90° about the shown axis followed by a reflection perpendicular to that axis leads to an arrangement identical to the original present. For example (Figure 1.6),

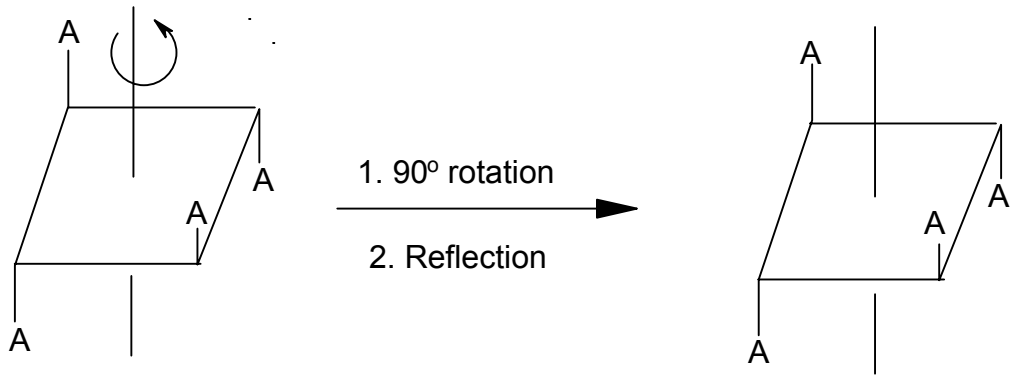


Figure 1.6 Axis of rotation-reflection

1.3. Descriptors of chiral molecules (Nomenclature)

The two enantiomers of a molecule are best identified on the basis of their absolute configuration or their optical rotation. The configuration of an asymmetric carbon (chiral center) is the specification of the relative spatial arrangement of the four groups attached to the chiral center. Absolute configuration determines their order to distinguish the two enantiomers and define their chirality. The most common used conventions are the L and D designations, the Cahn-Ingold-Prelog designation (S and R designations), (-) and (+) designations (*l* or *d*) and helicity *(M or P).*

1.3.1. The L and D designations

This configuration was assigned by Fisher to optically active compounds [6]. It was proposed that the centers of symmetry (stereogenic centers) of the kind C^*, having four different substituents should be delineated in a way that the chiral atom stays in the plane of the paper, the two substituents on the left and right of C^* protrude from the plane of the paper, and the other two above and below C^* lie behind the plane. Dextrorotatory and levorotatory forms were used to differentiate the two enantiomers.

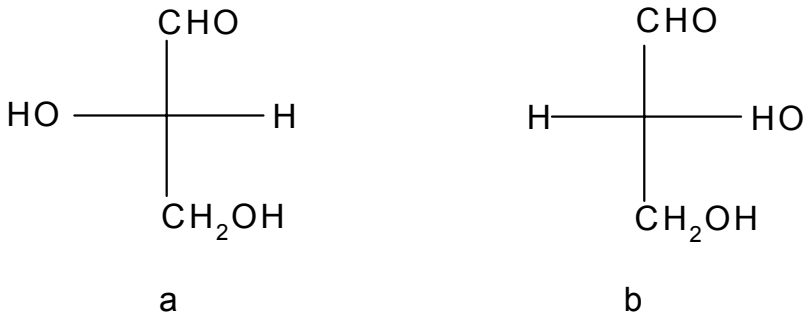


Figure 1.7 Designation of glyceraldehyde (a) L(-)-glyceraldehyde and (b) D(+)-glyceraldehyde

Glyceraldehyde was the standard example used by Fisher (Figure 1.7) where dextrorotatory and levorotatory forms of glyceraldehydes were assigned structures named as L- and D- glyceraldehydes, respectively, depending on whether –OH group is to the right or to the left of the vertical line representing the chain of carbon atoms.

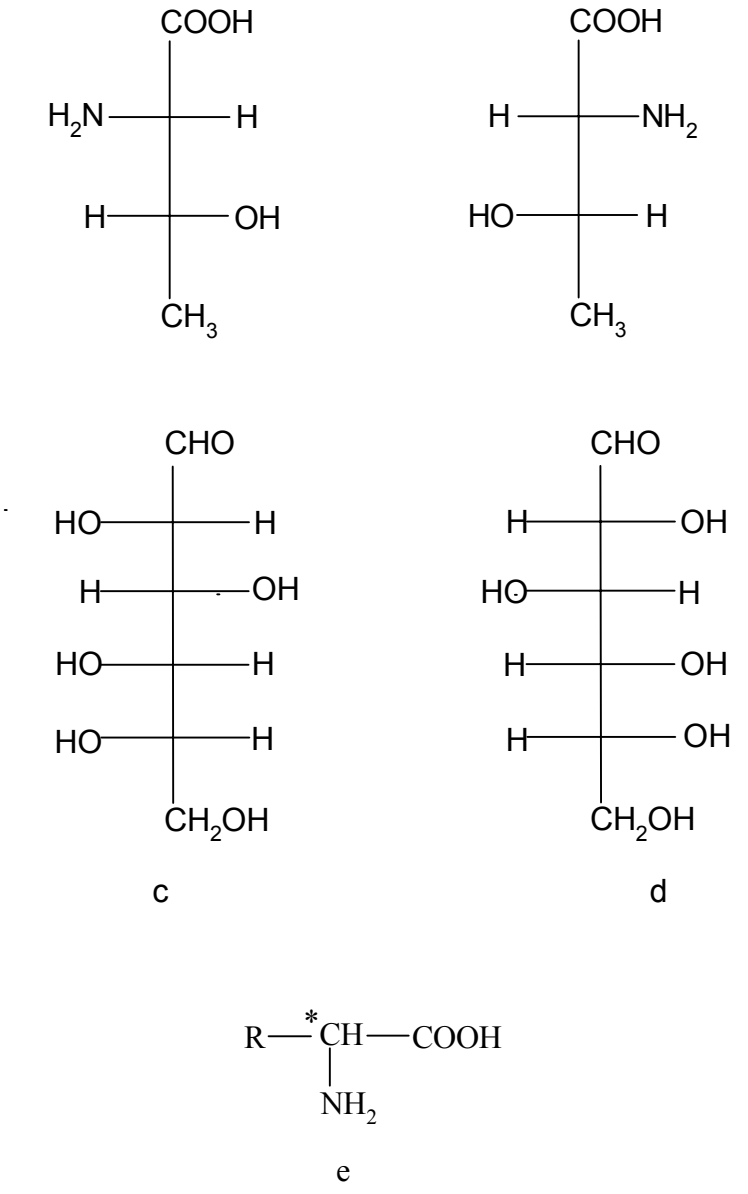


Figure 1.8 Amino acid stereochemistry (a) L-threonine (b) D-threonine (c) L-glucose and (d) D-glucose (e) Asymmetric carbon in amino acids

Amino acids are carboxylic acid with amino groups attached to the carbon atom adjacent to the carboxyl group (the α-carbon). With the exception of glycine (NH_2-CH_2-COOH), all naturally occurring α-amino acids are chiral molecules and exhibit optical activity. The α-carbon in (Figure 1.8e) is asymmetric if $R \neq H$. The position of the α-amino group with respect to the vertical line in a Fischer projection formula determines the configuration of the compound as shown in the case of threonine (Figure 1.8). L and D designations are used for sugars and amino acids, example threonine and glucose (Figure 1.8).

1.3.2. The Cahn-Ingold-Prelog designation (S and R designations)

The absolute configuration at a chiral center is designated as S or R to describe the three-dimensional structure of the compound. S is from the Latin *sinister* for to the left or counterclockwise and R is from the Latin *rectus* and means to the right or clockwise (Figure 1.9). A priority is given to each substituent on the chiral atom (C_{abcd}), which is selected using a precise rules based on atomic number and mass for determining whether a particular chiral center has S or R configuration [7, 8].

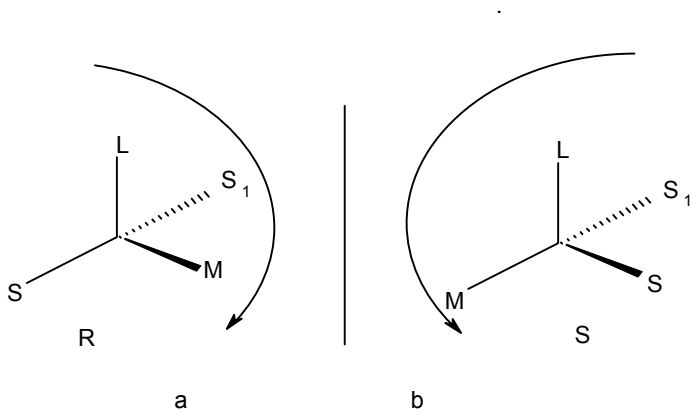


Figure 1.9 The Cahn-Ingold-Prelog designation, (a) sequence clockwise and (b) sequence anticlockwise

The rules for the assignment of priority to the substituent are [5, 9]:

1. atoms of the highest atomic number and directly bonded to the chiral center have the highest priority. For example :

   I > Br > Cl > S > P > Si > F > O > N > C > H

2. if two substituents or more have the same atomic number connected to the chiral center, their substitution pattern must be considered also $-CH_2Br$ according to the atomic number increase;

   $-CH_2Br > -CH_2Cl > -CH_2OH > -CH_2CH_3 > -CH_3$

3. substituent priorities of some groups can be arranged as –CHO > $-CH(CH_3)OH$, phenyl > olefins and triple bond > double bond;
4. priority of the same isotope is increased with the increase of its atomic mass;
5. like pairs [(S,S) or (R,R)] are given priority over unlike pairs [(S,R) or (R,S)];
6. lone-pair electrons are regulated as an atom with atomic number zero.

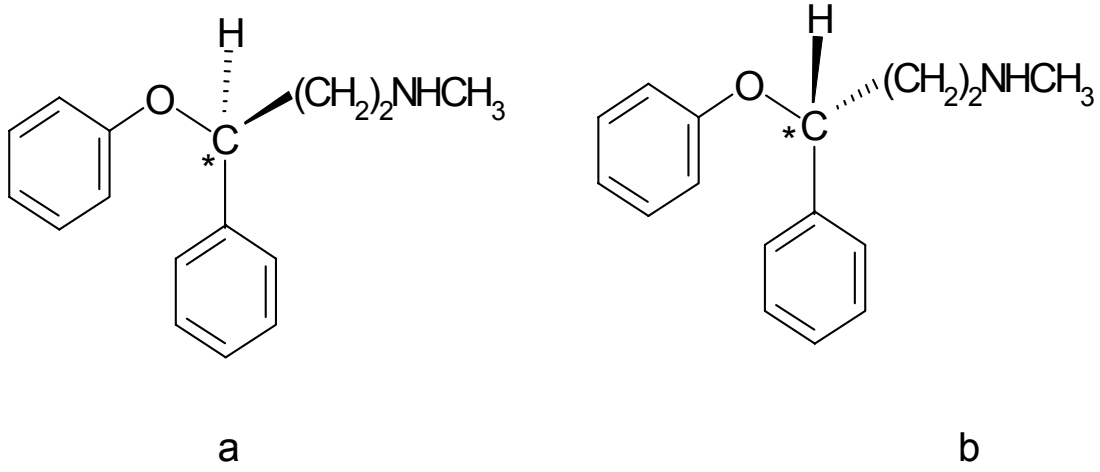


Figure 1.10 Fluoxetine (a) S- fluoxetine and (b) R- fluoxetine

The molecule is viewed with lowest priority group (S_1) pointing away from the viewer as in Figure 1.9. The other ligands are counted in order of decreasing priority, if the path traced is clockwise the (R) absolute configuration is assigned but if the path traced is counterclockwise, the (S) absolute configuration is assigned. This designation is mostly used in the nomenclature of drugs and metabolites due to their different or opposite activity (e.g., fluoxetine (Figure 1.10)).

It is possible for the molecule to have more than one chiral center (e.g., labetalol has two chiral centers) and a maximum of 2n optical isomers or enantiomers (where n is the number of heteroatoms in the molecule) (Figure 1.11) [10]. Accordingly, labetalol has four optical isomers (S,S labetalol; S,R labetalol; R,S labetalol; and R,R labetalol), and two enantiomers (S,S and R,R labetalol and S,R and R,S labetalol) [10].

1.3.3. (-) and (+) designations (*l* or *d*)

These descriptors -/+ or *l/d* are related to laboratory measurements of the ability of a chiral molecule to rotate the plane of polarized light. If a solution of the molecule rotates the plane of the polarized light in the clockwise direction as looked towards the light source, the rotation is + or *d* (dextro), conversely, rotation in counterclockwise direction is – or *l* (laevo).

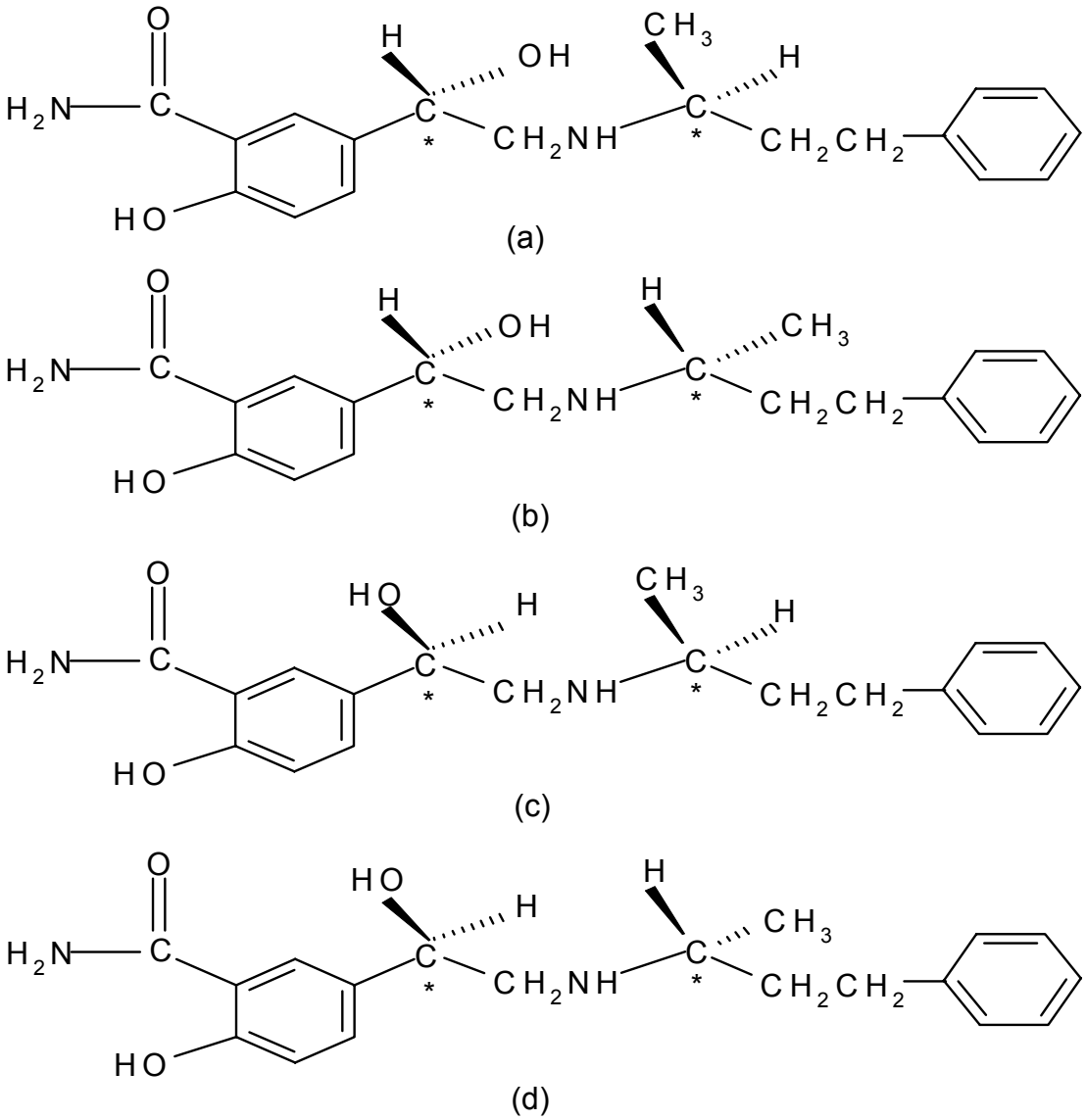
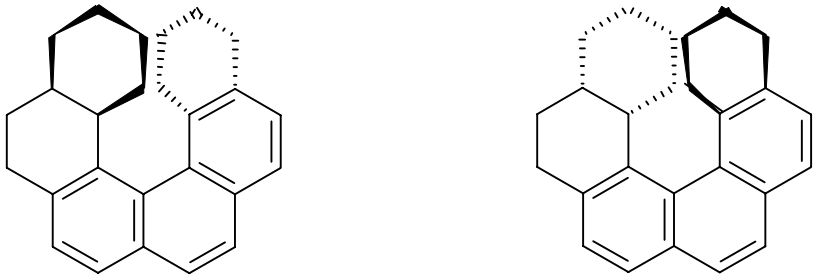


Figure 1.11 Stereochemistry of labetalol (a) S,S labetalol, (b) S,R labetalol, (c) R,S labetalol and (d) R,R labetalol

1.3.4. Helicity *(M or P)*

Helicity is a special case of chirality. Molecules are like screw thread or coiled stairways and shaped as a right or left spiral. Their nomenclature configurations are *(M)* or *(P)* depending on whether the helix approaches the observer in a clockwise or counterclockwise direction, respectively. *(M)* and *(P)*-hexahelicene is an example in which one side of it must lie above the other because of crowding (Figure 1.12) [11].



a b

Figure 1.12 Helicity, (a) (*M*)-hexahelicene and (b) (*P*)-hexahelicene

1.4. Enantiomeric purity

Enantiomeric purity is usually reported in terms of "enantiomeric excess", which can be calculated by the following equation:

$$\text{Enantiomeric excess} = \left| \%R - \%S \right|$$

The R-enantiomer of a drug will not necessarily behave the same way as the S-enantiomer. As well, metabolites may exists in the form of racemate, and each enantiomer may have a different reactivity or may be responsible for a different disease (e.g., excess excretion of glyceric and 2-hydroxyglutaric acid enantiomers which may cause different types of acidemias or acidurias).

1.5. Sources of chiral compounds

Three primary sources are reported as sources for chiral compounds [12]:

1. isolation of naturally occurring molecules through extraction from plant materials;
2. fermentation of inexpensive available feed stocks using de novo techniques;

3. synthesis using optically active compound obtained from the first two methods or prochiral staring material.

In chemical synthesis, racemic mixtures are prepared and the required enantiomer must be separated from the unwanted enantiomer. Crystallization, kinetic resolution, and preparative high performance liquid chromatography are possible techniques for resolving cremates to obtain pure enantiomers. Biomedical routes using microbes and enzymes as catalyst have had increasing applications in the commercial preparations of chiral molecules. Each technique of resolution of racemic mixtures could be classified into subtitles according to their methodology:

1. crystallization: preferential crystallization (direct), diastereomer crystallization, diastereomer crystallization-resolving agent, diastereomer crystallization-diastereomer introversion, diastereomer crystallization-designer resolving agent;
2. kinetic resolution: this technique is classified into chemical and enzymatic kinetic resolutions;
3. biological techniques: Fermentation and catalysts based reactions.

1.6. Importance of chiral molecules

Chirality has been reported as an important issue for different compounds such as pharmaceutical, agrochemical, environmental, biomedical and metabolites of living systems. The presence of asymmetric center/centers in these compounds gives rise to optical activity that can be responsible for the different properties of the enantiomers. The

different influence of the enantiomer and its antipode increases their applications in some major fields such as pharmaceutical, biomedical and clinical.

1.6.1 Chirality and clinical diagnosis

The presence of chiral compounds in human fluids (e.g., serum, urine, spinal fluids) as metabolites of human metabolism or drug metabolism gives the vitality for monitoring levels of these molecules in biofluids. The existence of higher or lower levels of these specific molecules is a marker of human body abnormalities. The normal concentration level of molecules in humans mostly referred to the deficiency of some enzymes. Amino acids, carbohydrates and urinary organic acids are excreted in humans and their change can cause different types of diseases such as inborn metabolic disorders, cancer, etc. More than 250 organic acids and glycine conjugates are either typically present or may possibly encountered in urine [13]. More than 65 inherited metabolic abnormalities are known to produce a characteristic urinary organic acid pattern, essential for diagnosis and follow-up [13-16]. Possible origins of abnormal excretion patterns of urinary organic acids may be summarized in the following classes:

1. aromatic amino acid metabolism, deficiencies of this group may cause some types of inborn error marks, such as 4-hydroxyphenylacetate (a marker of tyrose-niemai/Zellweger/hawkinsinuria/lactic acidosis) [17], homogetisate (Alcaptonu-ria) [13];
2. branched-chain amino acid metabolism, e.g., D-2-hydroxyisocaproate is a marker for short bowel syndrome [18] and 3-hydroxy-2-methylbutyrate is a marker for Pearson Syndrome [19];

3. fatty acid oxidation; 2-hydroxysebacate may cause peroxisomal diseases [20] and 5-hydroxyhexanoate may cause nonketotic dicarboxyluria [21];
4. Krebs cycle/respiratory chain; abnormality levels of 2-ketoglutaric acid is a marker of different disease phenotypes, e.g., Ketoadipate acidemia and glycogen storage disorder [22];
5. lactic acid, ketone bodies, lactic acid and 2-hydroxybutyrate concentration level changes are markers for primary lactic acidosis [23];
6. lysine, glycine, serine metabolism, D- and L-glyceric acidurias are diagnosed due to the change of D- and L-glyceric acid in urine, respectively [13]. Excess excretion of 2-ketoadipic acid may cause 2-kitoadipic aciduria;
7. other acids and metabolites, e.g., abnormality level of 2-hydroxyglutarate, orotate and mevalonate and/or its lactone cause L- and D-2-hydroxyglutaric acidurias, Lesh-Nyha disease and Canavan disease [24];
8. nutritional, exogenous, or artifactual compounds [25].

Different types of disease are generated due to inborn errors of metabolism such as organic acidemias, fatty acid oxidation defects, primary lactic acidosis, aminoacidopathies, urea cycle defects, disorders of carbohydrate metabolism, lysosomal storage disorders and peroxisomal disorders. Organic acidemias (e.g., methylmalonic or propionic acidemia, glyceric acidurias, 2-hydroxyglutaric acidurias, carboxylase deficiency) are caused by abnormal metabolism of proteins, fats or carbohydrates and are characterized by marked metabolic acidosis with ketosis, often with elevated lactate and encephalopathy, neutropenia and thrombocytopenia. Endogenous D- and L-arabintol (DA

and LA) are present in human body fluids and their serum samples increase in renal dysfunction [26]. Elevated DA/LA or DA/creatine ratios in serum or urine have been found in immunocompremised, usually neutropenic, and patients with invasive candidiasis. Chirality plays a very important role in diagnosis of diseases associated with markers with a chiral moiety (e.g., enantiomers of pipecolic acid, fucose, glyceric acid, 2-hydroxyglutaric acid, vesamicol and lysine), because each enantiomer causes a different phenotype disease associated with different symptoms.

1.6.2 Importance of chirality for pharmaceutical compounds

Chirality has emerged as a key issue in drug design, discovery and development as stereoisomer discrimination is a significant component in many pharmacological events [27-30]. Chirality is an important factor in drug efficacy. About 56% of the drugs currently in use are chiral compounds, and about 88% of these chiral synthetic drugs are administrated as racemates [31, 32]. Although the S or R isomer has the same substituent atoms or groups, qualitatively or quantitatively may have similar or different pharmacological effects, which may relate to their stereoselective pharmacokinetics or pharmacodynamics. The terms “eutomer” for the more potent isomer and “distomer” for the less potent one have been suggested [33]. The differences in the enantiomer pharmacodynamic activity and pharmacokinetic property are related to their different affinity or intrinsic activity at receptor sites. Enantiomeric drugs may classified according to these differences as [31]:

1. all the pharmacological activity may be given by one enantiomer and the other enantiomer is an impurity that may be active with desirable or undesirable activity or inactive, e.g. the antihypertensive active (S)-α-methydopa (Figure 1.13) [34];

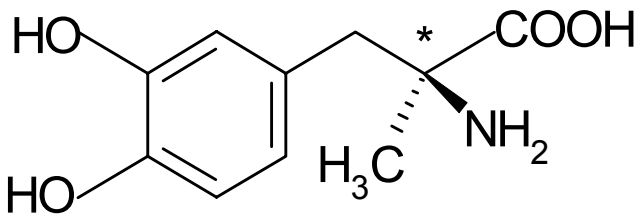


Figure 1.13 (S)-α-methydopa

2. the qualitative and quantitative pharmacological activity of the enantiomers are identical, (e.g. enantiomers of promethazine) [35];

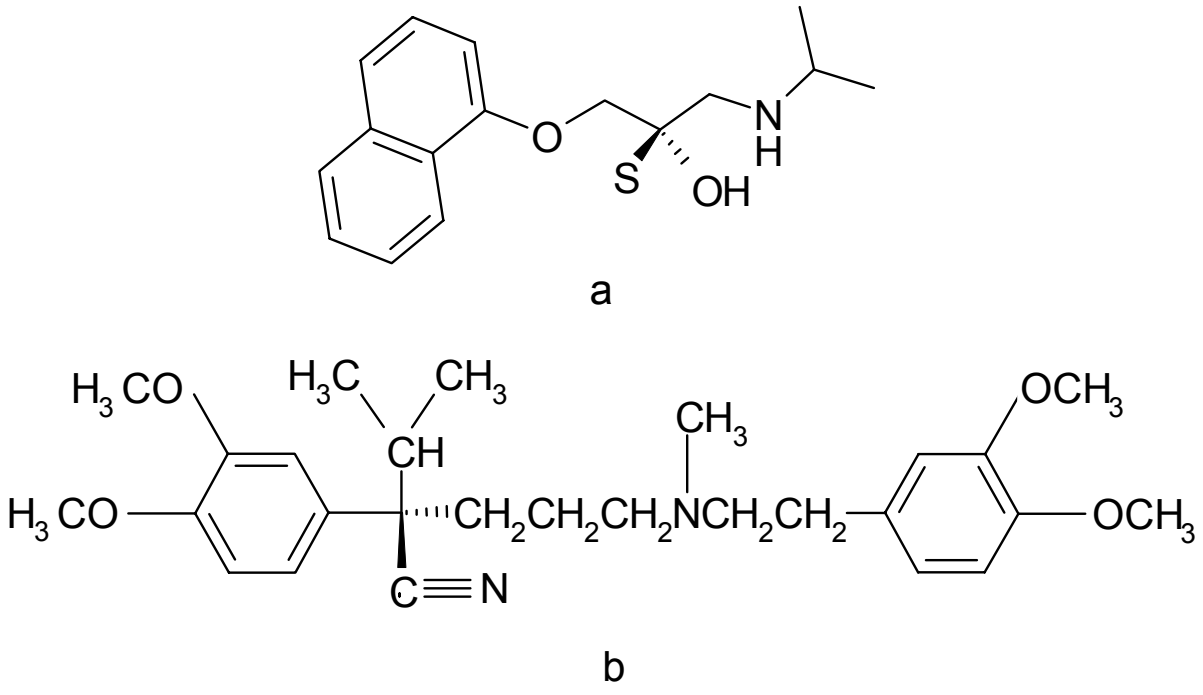


Figure 1.14 (a) S-propanolol and (b) R(+)-verapamil

3. enantiomers may have activity that is qualitatively similar but quantitatively different, e.g. the β-adrenergic blocking agent propranolol [35] and verapamil used in anginal therapy (Figure 1.14) [36];
4. both enantiomers have independent therapeutic potencies, e.g. dextropropoxyphene [37] is an analgesic agent and levopropoxyphene is devoid of analgesic action but is an effective antitussive (Figure 1.15);

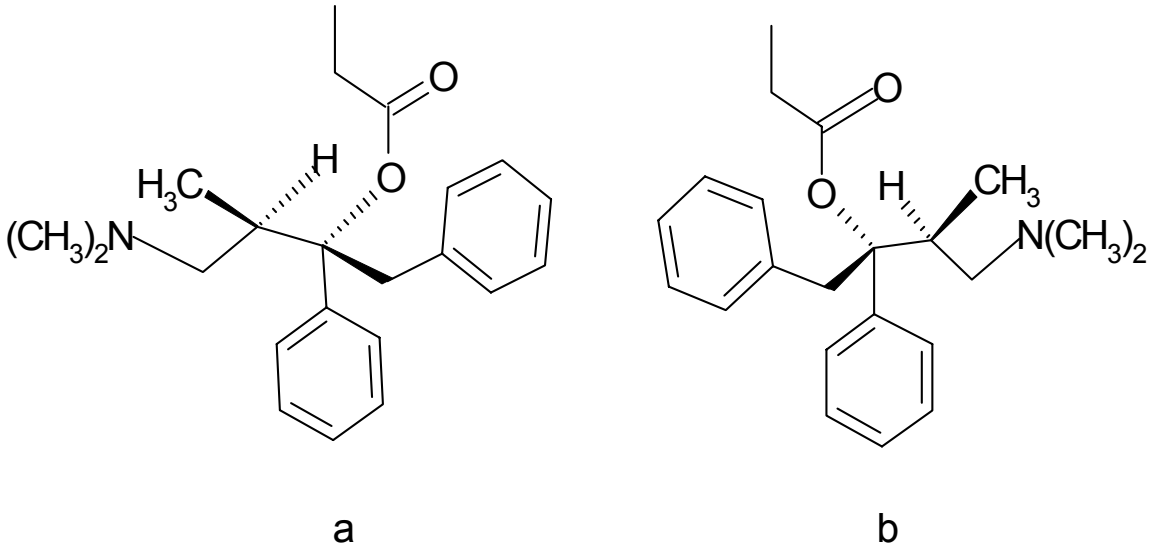


Figure 1.15 (a) (2S,3S)-(+)-Dextropropxyphene and (b) (2S,3R)-(-) Levopropoxyphene

5. the distomer exhibits undesirable side-effects, e.g. S(+)-ketamine is an active anesthetic and analgesic, and undesirable side-effect hallucination and agitation is being associated with the R(-)-distomer (Figure 1.16) [38].

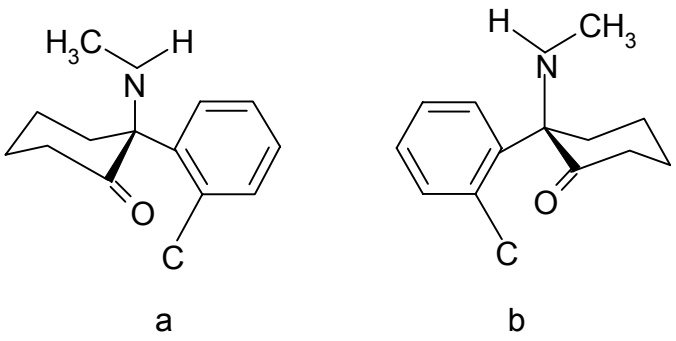


Figure 1.16 Ketamine distomer, (a) S(+)-ketamine and (b) R(-)-ketamine

6. some enantiomers exhibit therapeutic advantages, e.g. R(+)-indacrinone is active diuretic agent (Figure 1.17) [39], but possesses the undesirable side-effect of uric acid retention where S-enantiomer can antagonize the undesirable side-effect of the R-enantiomer by acting as uricosuric.

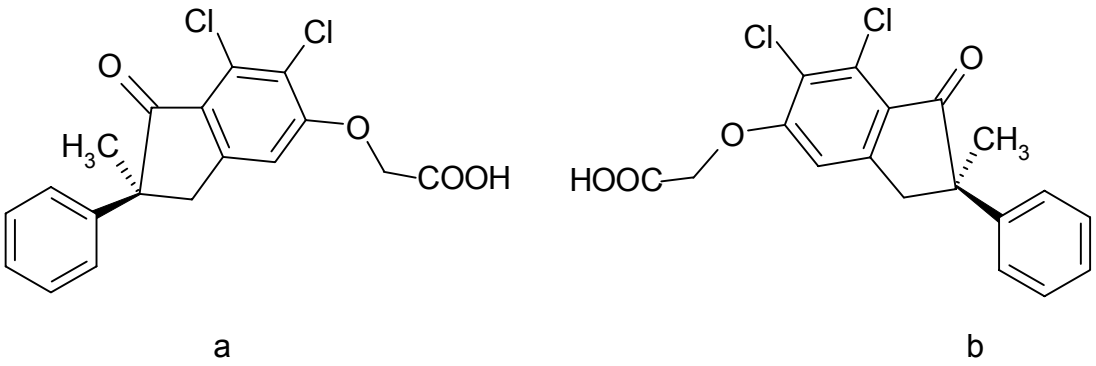


Figure 1.17 Indacrinone (a) S-indacrinone and (b) R-indacrinone

Initial exposure, pharmacokinetics and pharmacodynamic are the main phases of the biological effect of drugs in humans [40]. The initial exposure phase is controlled by the drug activity, affinity for receptors and the activity of metabolites. It is also governed by tissue specificity due to receptor differentiation and distribution of the parent compound

and its metabolites. The second step is the pharmacokinetic phase which involves adsorption, distribution, metabolism and excretion of the drug. Finally, pharmacodynamic involves the interactions of the bioactive agent with the molecular site of action, such as receptors and enzymes, in the target tissue to have the excepted impact. Enantioselectivity has effective role in both phases of pharmacokinetics and pharmacodynamics. A description of the different properties of the enantiomers of some drugs is summarized in Table1.1 as examples of the importance of chirality in drug design, pharmacokinetics and pharmacodynamics.

Table 1.1 The different physiological effects of some chiral drugs in humans

	Physiological effect in humans	
Drug	(+)-Enantiomer	(-)-Enantiomer
Penicillamine	Antirheumatic (Wilson's disease)	Neurotoxic
Levodopa	Antiparkinsonian	Agranuloctosic
Estrone	Sexual hormone	Inactive
Barbiturates	Excitation	Sedation
Dobutamine	Vasodilatation	Positive inotropic/vasoconstriction
Fluoxetine	Selective serotonin reuptake inhibitor	Minimal effect
Ketamine	Strong anesthetic	Weak anesthetic
Pentazocine	Anxiety	Analgesia, respiratory depress-ion
Propoxyphene	Analgesia	Antitussive
Propanolol	Suppress ventricular arrhythmia without β-adrenergic blockade	Active β-adrenergic blocker
Thyroxine	Inactive	Thyroxemic effect
Verpamil	Minimal effect	Negative dromotropic; negative inotropic and chronotropic effect
Acenocoumarol	Anticoagulant	Minimal effect
Thalidomide	Mutagenic	Sedative-hypnotic teratogenic
Albuterol	Proinflammatory effect	Bronchodilator
Morphine	Minimal effect	Strong analgesic
10-Hydroxy-carbazepine	Antiepileptic	Minimal effect
Methadone	Minimal effect	Strong analgesic
Warfarin	Weak anticoagulant	Anticoagulant

1.7. Methods of chiral recognition

1.7.1. Polarimetry

Polarimetry was the first method used for analyzing chiral molecules [41]. This method depends on the different optical activity displayed by one enantiomer when it exists in excess of the other. Pure enantiomers rotate the plane of polarized light in equal amounts but in opposite directions, that can be measured by the polarimeter. Amount of rotation is defined as the number of degrees from the original plane of polarization. The number of degrees for a molecule rotation of the plane of polarized light is influenced by the length of the sample path, the temperature, the solvent, the concentration of the analyte, the pressure and the wavelength of light.

1.7.2. Chromatographic methods

For enantiomer separation on analytical scale a great variety of methods based on chromatographic techniques have been developed (Figure 1.18). Direct and indirect separations are used in chromatographic methods.

Indirect method is based on the use of chiral derivatization reagents to form diastereomeric derivatives which differ in their chemical and physical behavior and therefore can be separated using an achiral stationary phase. The presence of suitable functional groups in the molecule is a precondition of a successful derivatization. The derivatization procedure has some limitations such as: it is tedious and time-consuming due to the different reaction rates of the individual enantiomers; the suitable chiral

derivatizing agent has to be of high enantiomeric purity, the presence of derivatable groups in the analyte is a prerequisite.

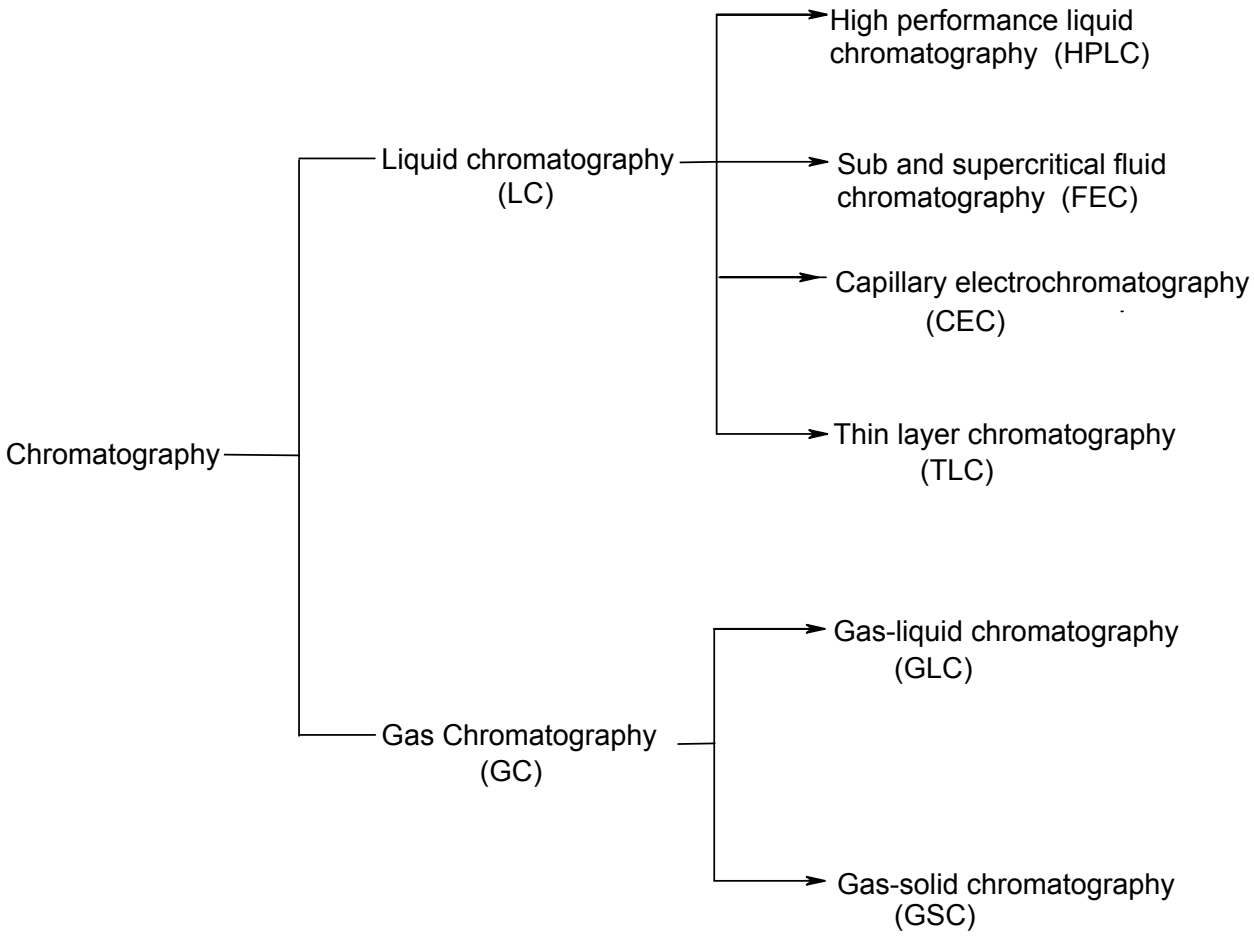


Figure 1.18 Chromatographic techniques for chiral recognition

The direct approach using columns with chiral stationary phases is more convenient and also applicable for separation on preparative scale, but requires a collection of expensive columns to solve a variety of problems. The chiral mobile phase approach represents a simple and flexible alternative which is not always applicable. Since the mobile phase

containing the chiral selector cannot be reused, expensive reagents cannot be utilized for this technique.

HPLC involves passing a liquid phase containing the compounds to be separated through a stationary phase under high pressures. One type of HPLC utilizes a chiral stationary phase (CSP) that interacts with the chiral molecules in the liquid phase [42]. Several reviews have been reported on the use of HPLC approaches to the chiral separation of various drugs classes, drug enantiomers bearing carboxyl groups, β-adrenoceptor blocking drugs, the chiral drug analysis in biological fluids and the application of column switching methods in chiral drug analysis in biological samples [43]. The molecular recognition is based on the utilization of different mobile and stationary phases summarized by the following points [43]: phases based on multiple hydrogen bonds; chiral π-donor and π-acceptor phases; cyclodextrins phases; chiral stationary phases based on polysaccharides; macrocyclic antibiotics; synthetic chiral macrocycles; chiral imprinted polymers; protein-based chiral stationary phases; ligand-exchange chromatography, chiral ion-pairing chromatography; separation on preparative scale [44-50].

Most enantioseparations of pharmaceuticals and metabolites have been carried out in the indirect method by preparing diastereomers and resolved them using TLC. Few researches have been reported on direct enantiomeric separation on chiral TLC plates. Ligand exchange based chiral thin layer chromatographic plates are only commercialized for racemates separation.

Gas chromatography works well for compounds that are readily vaporized without decomposition. The compounds to be analyzed are injected onto a column packed with a chiral stationary phase (CSP), vaporized and traveled down the CSP. Gas chromatographic method cannot be accepted as the method of choice of chiral separation of racemic compounds due to its requirement of the conversion of the racemic compound into volatile species, which is carried out by a derivatization process [51]. The most frequently used chiral derivatizing reagents in indirect methods of gas chromatography (GC) are S(-)-heptafluorobutyryl prolyl chloride, (-)-menthyl chloroformate, S-α-methyloxy-α-rifluoromethylphenyl acetyl chloride, S(-)-trifluoroacetylprolyl choride and R-(-)-2,2,2-trifluoro-1-(9-anthryl) ethanol [52]. Chiral stationary phases based on amino acids and diamides, metal complexes, cyclodextrins, cyclocholates and calixarenes are used for direct separations by GC [43].

1.7.3. Capillary electrophoresis

Capillary electrophoresis (CE) is an analytical technique widely applied in different areas of research such as pharmaceutical, biological and environmental. The different separation used modes used are capillary gel electrophoresis (CGE), micellar electrokinetics chromatography (MEKC), isotachophoresis (ITP) and capillary zone electrophoresis (CZE). The separation of two enantiomers is a difficult task in CE because the two analytes possess similar physico-chemical properties unless a chiral environment is used in order to selectively improve their electrophoretic mobilities [53]. Direct and indirect separations are used in CE for the chiral recognition. Different types of chiral selectors have been applied for enantiomers discrimination, e.g. cyclodextrins

(CD) and their derivatives, carbohydrates, mono-, oligo- and polysaccharides, chiral crown ethers, calixarenes, macrocyclic antibiotics and proteins [54]. The indirect enantiomeric separation approach is based on a chemical reaction of the two antipodes with a chiral compound before the electrophoretic analysis with a production of a mixture of two diastereomers (Figure 1.19) [54]:

$$\underset{a}{(D,L)\text{-}C} + \underset{b}{(L)\text{-}R} \longrightarrow \underset{c}{[(D)\text{-}C..(L)\text{-}R]} + \underset{d}{[(L)\text{-}C..(L)\text{-}R]}$$

Figure 1.19 Indirect enantiomeric reaction C: racemic compound, R: chiral reagent, D- and L- are dextro and levo rotatory, a and b are enantiomers, c and d are diastereomers.

This method has been applied for the enantiomer separation of amino acids. In direct chiral separation, the chiral selector can be added to the background electrolyte or bound to the capillary wall or included in/bound to the capillary wall [55-57]. The chiral recognition of the direct method is based on inclusion-complexation, affinity electrophoresis and micelles enantioselectivity. CZE is widely used in enantiomer analysis than other techniques [54], and it became a fact due to its high accuracy recorded for enantiomers quantification.

1.7.4. Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy can also be used to evaluate the enantiomeric purity of chiral molecules. There are two basic approaches that have been applied for enantiomer recognition [58]:

1. diastereomers are prepared from the mixture of enantiomers. Different resonance peaks can be identified for each of the diastereomers, and a relative percentage of each can be determined;
2. addition of lanthanide shift reagents to the sample, forms complexes with different enantiomers and allow the resonances of the two isomers to be detected [59].

1.7.5. Circular dichroism

Circular dichroism is an optical property caused by the interaction of polarized light with chromophores that are either inherently chiral or placed in an asymmetric environment. As a result, the sample exhibits different absorption coefficients for left and right circularly polarized light. The absorption difference transforms the linear polarized light into elliptically polarized light. It can be measured in a wavelength dependent manner yielding the circular dichroism [60]. Circular dichroism spectroscopy can measure the concentration difference between the enantiomer pair in a mixture and has been used to determine the enantiomeric excess in conjunction with UV spectroscopy that can measure the total concentration of the two enantiomers [61]. The absolute configuration of the chiral sulfoxide 1-(2-methylnaphthyl) methyl sulfoxide was determined using vibrational circular dichroism spectroscopy [62].

1.7.6. Ferroelectric liquid crystals

A liquid crystal is an ordered fluid that is intermediate between the three-dimensionally ordered crystal phase and the disordered liquid phase, referred to as a mesophase and its

constituent molecules as mesogens. Thermotropic and lyotropic liquid crystals are formed from materials that form a mesophase in the absence and presence of solvent (lipids, soaps, and other surfactants). Calamitic liquid crystals are rod-shaped compounds composed of a rigid aromatic core and alkyl side chains. They can be classified into two main classes of liquid crystal phases, the nematic and smectic phases [63]. Smectic liquid crystals are ferroelectric possessing a macroscopic electric polarization that is oriented perpendicular to the smectic tilt plane (defined as the director n and the layer normal z, Figure 1.20). The vectors z and n are congruent with the plane of the page, and the C_2 is axis normal to the tilt plane and a reflection plane of symmetry σ congruent to the tilt plane [64].

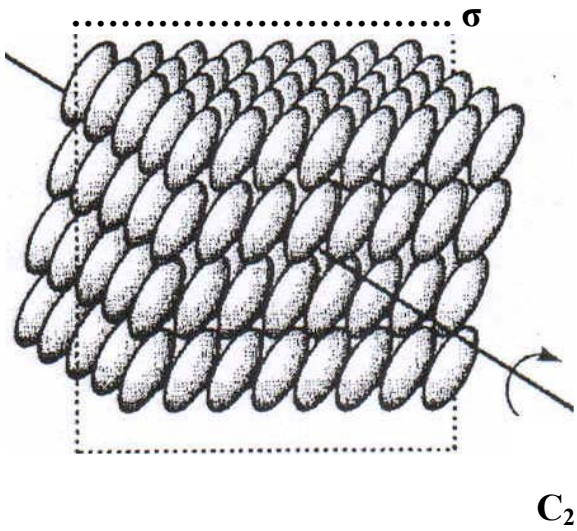


Figure 1.20 Schematic representation of the smectic C phase. The vectors z and n are congruent with the plane of the page, and the C_2 is axis normal to the tilt plane and a reflection plane of symmetry σ congruent to the tilt plane [64].

Liquid crystals are easily processed into thin films, which have the ability to rotate plane-polarized light. The film can effectively work as an ON/OFF light shutter between crossed polarizers y electrically switching the liquid crystal film between two different

molecular orientations relative to the polarizers. Chirality transfers in ferroelectric liquid crystals were studied by Lemieux [64] and detection of chiral perturbations in ferroelectric liquid crystals induced by an atropisomeric biphenyl dopant was reported [65].

1.8. Molecular recognition of enantiomers using electrochemical electrodes

In molecular recognition of the enantiomers, electrochemical electrodes are a good alternative for structural analysis (IR, NIR, Raman, MS, X-Ray diffraction, and neutron diffraction) and chromatographic techniques. The advantage of using these electrodes is the high reliability that is given by high precision, high reproducibility and rapidity [66, 67]. Electrochemical sensors can be used for the measurements of the enantiomers directly, resulting in a high precision than obtained by chromatographic methods [68].

1.8.1. Molecular recognition of enantiomers using enantioselective, potentiometric membrane electrodes (EPME)

The reaction between enantiomers and chiral selectors (CS) is the main part in molecular interactions of enantiomers and plays a main role in their enantioanalysis (Figure 1.21). Different types of chiral selectors have been reported for the molecular recognition using EPME. EPMEs based on crown ethers [69-72], cyclodextrins and their derivatives [66, 73-75], macrocyclic antibiotics [76, 77], and maltodextrins [78, 79] have been reported for enantioselective molecular recognition of different pairs of enantiomers.

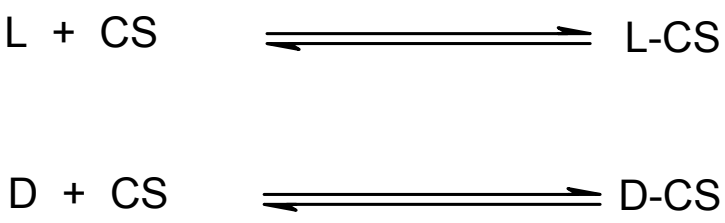


Figure 1.21 Reactions of enantiomers (L and D) with chiral selector (CS)

1.8.2. Molecular recognition of enantiomers using amperometric biosensors

The assay of the enantiomers of amino acids and their derivatives is very important in different fields specially drugs design and clinician laboratory requirements for disease diagnosis. Numerous papers are reported to the assay of enantiomers of substances by using enantioselective amperometric biosensors based on L-amino acid oxidase (L-AAOD) and D-amino acid oxidase (D-AAOD) for the analysis of L- and D-enantiomers, respectively [80]. Introducing the multiplexer for electrochemistry gives the opportunity for a simultaneous screening of L- and D- enantiomers [81].

1.8.3. Molecular recognition of enantiomers using amperometric immunosensors

The molecular discrimination of enantiomers using amperometric immunosensors is based on antigen-antibody reaction. The antibody can recognize the chirality center of the antigen. This reaction is enantiosepecific because the antibody is only reacting with one enantiomer. This advantage can be used to screen a particular enantiomer in a racemic mixture. Immunosensors can be used for trace analysis of enantiomers; as well they are highly sensitive.

1.9 Electrodes as detectors in flow or sequential injection analysis (FIA or SIA)

FIA is a concept of continuous flow analyzer of discrete samples. The process could be summarized as follows (Figure 1.22): when the sample is injected into the carrier stream, a well defined sample-plug is formed in the stream; as the sample-plug is swept downstream through the reaction manifold system of narrow bore tubing the plug disperses freely into and, thus mixes the carrier stream under laminar flow conditions to form a gradient [82]. The magnitude of this dispersion is dependant on the operating parameters applied to the system, which include sample volume, carrier stream flow, reagent stream flow, pressure in the system, type of tubing, tube length, tube bore size, coil diameter and viscosity differences. Using FIA system with electrodes as detectors gives the possibility of on-line monitoring of enantiomer in the flowing samples. The advantages of flow injection are: speed of analysis (FIA methods has a sample frequency between 50-120 samples per hour) [83]; manifolds used to construct FIA system are relatively inexpensive and easily assembled and/or exchanged; low reagent consumption (up to 20-200 times lower than the manual techniques); versatility of adapting to a variety of applications in analytical chemistry; free from air bubbles; low carry over; no glassware, replaced by tubes, pumps and valves and the analyst is not exposing to toxic reagents.

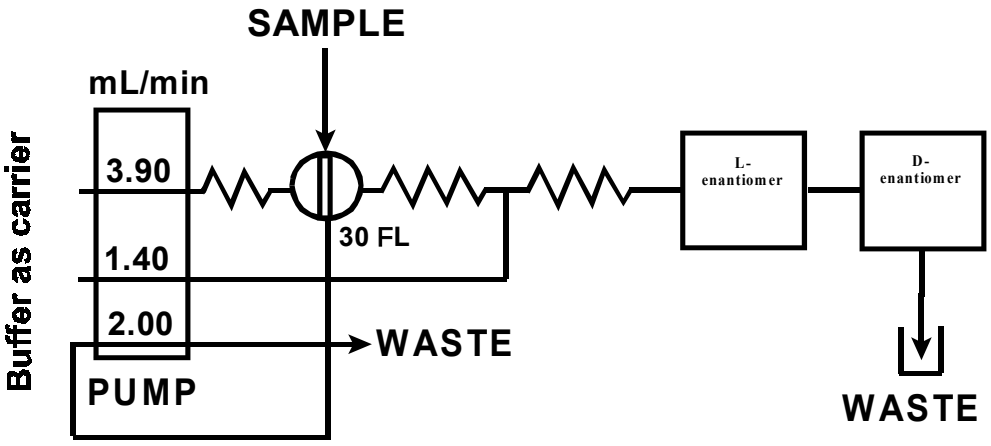


Figure 1.22 Schematic diagram of the FIA system used for the determination of enantiomers.

SIA is based on discontinuous flow where reagents consumed only when the sample is treated by exploiting a combination of stopped flow, reversed as well as forward flow in micro-liter scale [84]. The SIA system is assembled from a pump, a multi-position selection valve, a holding coil and appropriate detector (Figure 1.23). The principle of SIA is based on a sequential aspiration of a sample zone and reagent zone into a holding coil through a selection valve. The aspiration of zones is controlled by means of a pump, which is capable of controlled stop-go-forward-reverse movement. A stack of well-defined zones adjacent to each other are formed in the holding coil (Figure 1.24). The selection valve is then switched to the detector position and flow reversal creates a composite zone in which the sample and reagent zone mutually disperse and penetrate each other due to combined axial and radial dispersion.

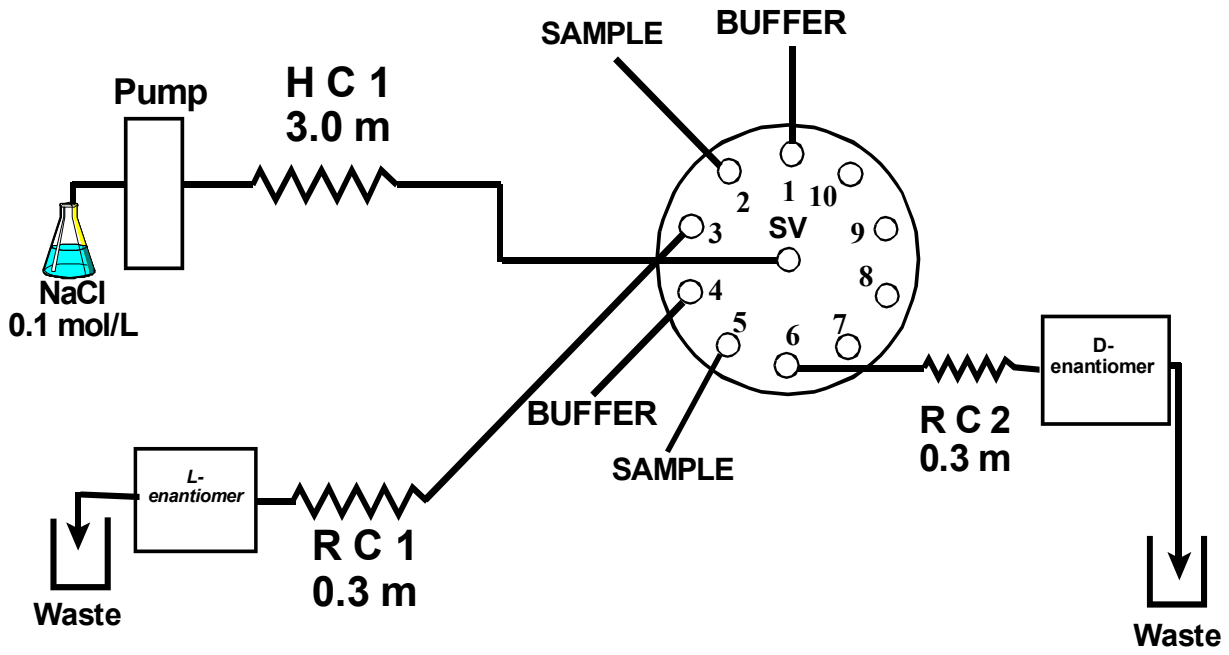


Figure 1.23 SIA system utilized for the simultaneous detection of L- and D-enantiomers.

A product zone is formed and monitored by the detector in the form of a peak whose height or width is related to the concentration of species determined. It is possible to control the amount of dispersion within the system by varying the different operational parameters in a SIA flow conduit [85]. The degree of mixing and length of the reaction time as well as the sample and reagent volumes may change without physical re-configuration of flow channel by means of programming the piston and valve movements. The holding coil is considered the heart of the system as it substitutes the mixing chamber and its volume could be adjusted by changing the length and diameter as needed.

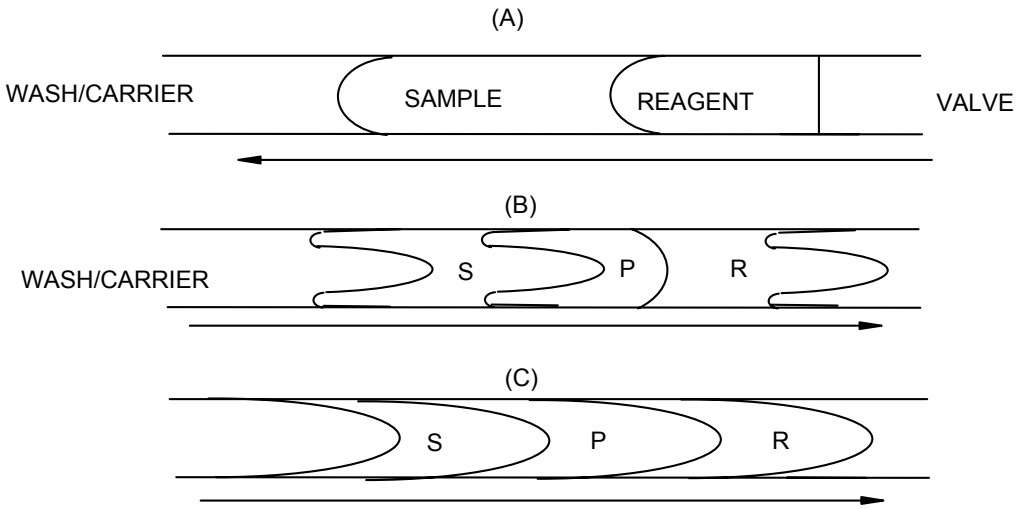


Figure 1.24 Principles of sequential injection analysis. Profile zones after injection (A), immediately after flow reversal (B) and in reaction coil (C), S-sample, R-reagent, and P-product formed.

Peristaltic pumps are preferred than syringe and sinusoidal flow types due to the following advantages:

i) the configuration is easier and simpler to design, initiate, and operate;

ii) they are widely available and easy to handle;

iii) the sampling cycle is considered shorter as there is no need for the aspiration of a wash solution.

EPMEs, amperometric biosensors and immunosensors can be easily incorporated in the conduits of a flow system (FIA and SIA) to form a simple low cost analyzer [86]. High reliable electrochemical electrode/flow system is obtained by controlling the hydrodynamic conditions. The use of electrodes in flow systems has advantages for electrochemical detection itself [87], due to the following reasons:

1- it is no contamination between the samples;

2- the linear concentration range, the sensitivity, the limit of detection and the response time of the enantioselective electrode are improved in flow systems due to establishment of a small thickness diffusing layer at the electrode surface.

3- the ionic species flowing out of the reference electrode cannot influence the response of the indicator electrode because the reference electrode is usually placed down-stream.

4- reference electrodes with flowing inner solution can easily be employed to overcome problems arising from the alteration of liquid junction potential.

5- the intensity of current or potential is measured in non-equilibrium conditions but always at the same moment after the sample injection.

The advantages of using SIA over FIA are : decreasing of the consumption of samples and buffers, a cheap electrolyte can be used as carrier (e.g. NaCl), lower cost, high precision, reliability and accuracy of the analysis.

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