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Introduction

The availability of continuous monitoring of metabolic substances is very important in the intensive care units and can be a great aid to patients care. Chirality has been reported as an important issue for different compounds of biological importance. The presence of chiral compounds in human fluids (serum, urine, spinal fluids) as normal metabolites of human metabolism or drug metabolism give the vitality for monitoring levels of these molecules in biofluids. The existence of higher or lower levels of these specific molecules is a marker indicator of human body abnormalities. The normal concentration level changes of molecules in humans mostly referred to the deficiency of some enzymes. Amino acids, carbohydrates and urinary organic acids are excreted in human and their changes can cause different types of diseases such as inborn metabolic disorders and cancer.

Many diseases are caused by substances existing in enantiomeric form and each enantiomer causes a different disease, so to diagnose these illnesses it is very important to find an analytical method that can discriminate between the L- and D-enantiomers. These methods will be very helpful and should exhibit reliable analytical information, fast analysis and could be applied for the continuous monitoring of the enantiomers in biological fluids.

Molecular recognition plays the main role in chiral discrimination around an asymmetric center. Due to the importance of enantiomeric discrimination, there has been substantial need for the discovery of substances used in the enantioanalysis for the diagnosis,

preventation, and treatment of human diseases. These developments have resulted in increased demand for sensitive and specific analytical methods.

The instrumental methods for quantitation which are most commonly used in clinical enantioanalysis are structural analysis, chromatographic and electrochemical methods. Electrochemical sensors are a very good alternative for structural analysis because of their high reliability that is given by high precision, high reproducibility and rapidity. The precision obtained using electrochemical sensors is higher than that obtained using chromatographic methods due to the fact that electrochemical sensors can be used directly for measurements of the compounds in solution.

The aim of this thesis is to construct reliable enantioselective electrodes (amperometric electrodes, amperometric biosensors, enantioselective, potentiometric membrane electrodes) to be applied in diagnosis. Carbon and diamond pastes are proposed as matrices for the sensors' design. Chiral recognition principles based on selected binding as well as on catalyst selectivity must be considered for the selection of the best chiral selector or enzyme.

Differential pulse voltammetry, chronoamperometry and potentiometry can be used for the direct assay of enantiomers in the serum and/or urine samples. An analysis of the performances of the electrodes has shown that the selection of the type of the electrode and matrix of its membrane should be done in accordance with the complexity of the structure of the enantiomer to be determined. Also, the analytical information obtained in

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the enantioanalysis using electrochemical sensors is more reliable than that obtained using conventional or chromatographic methods.

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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

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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].

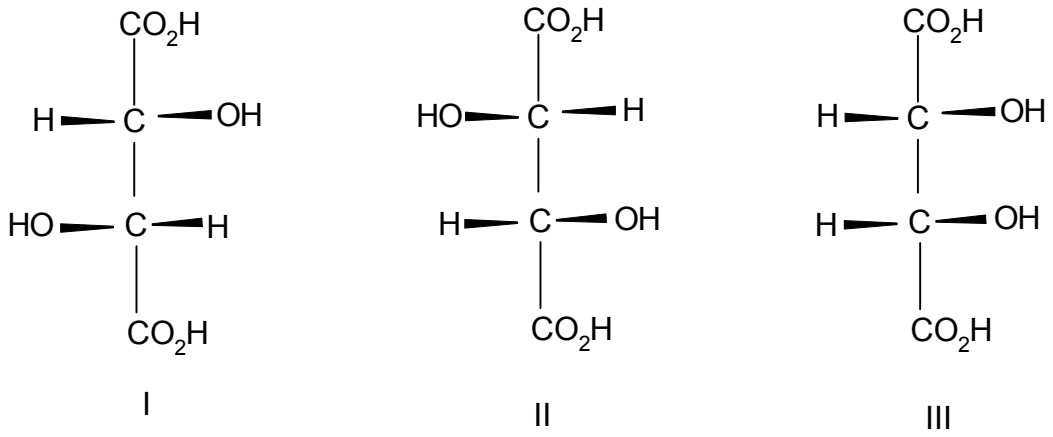

Figure 1.1 Tartaric acid, I and II are enantiomers, I and III are diastereomers, II and III are diastereomers.

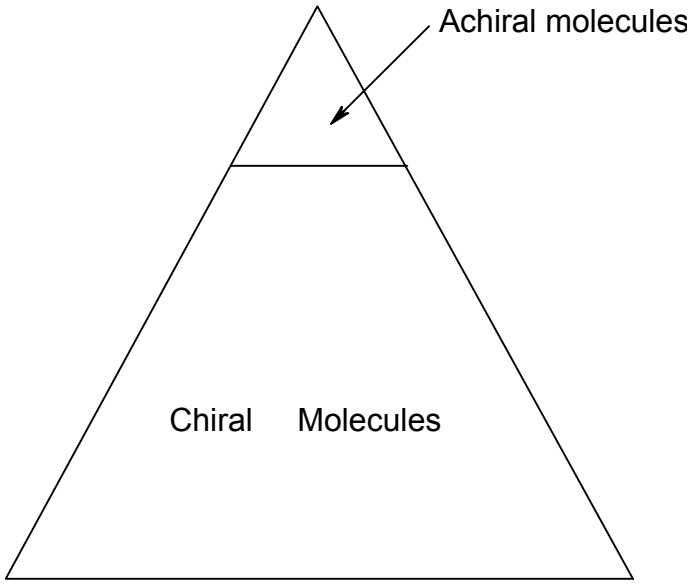

Figure 1.2 Chiral molecules' distribution among pharmaceutical drugs.

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;

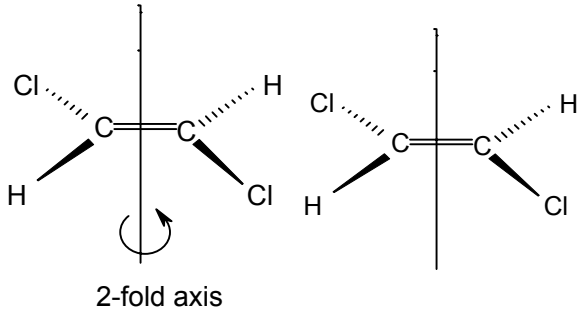

Figure 1.3 (*E*)-1,2-dichloroethane

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.

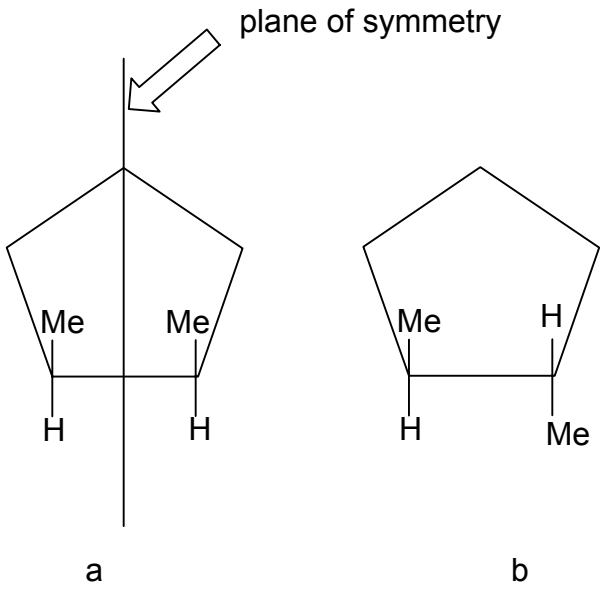

Figure 1.4 Chiral and achiral molecules in plane of symmetry (a) cis-1,2-dimethylcyclopentane(achiral), and (b) trans-1,2-dimehylcyclopentane(chiral)

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.

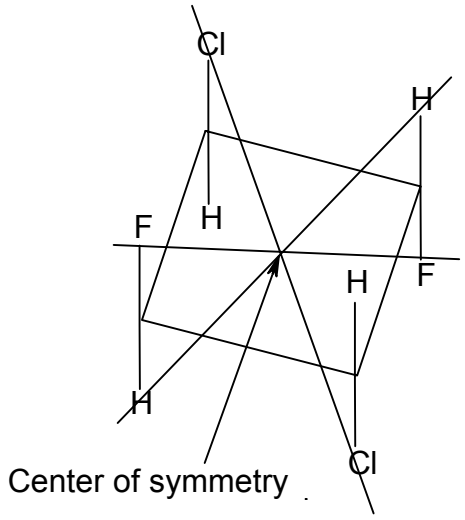

Figure 1.5 1,3-dichloro-2,4-difluorocyclobutane

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),

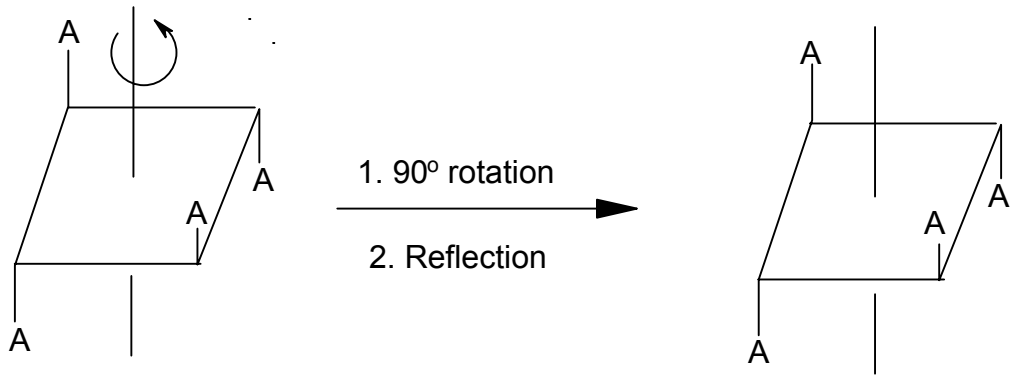

Figure 1.6 Axis of rotation-reflection

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.

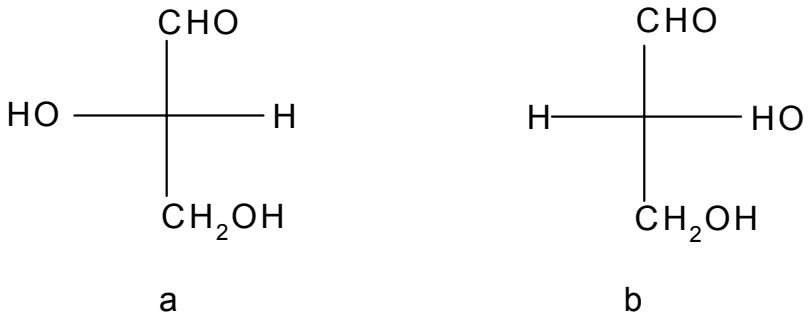

Figure 1.7 Designation of glyceraldehyde (a) L(-)-glyceraldehyde and (b) D(+)-glyceraldehyde

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.

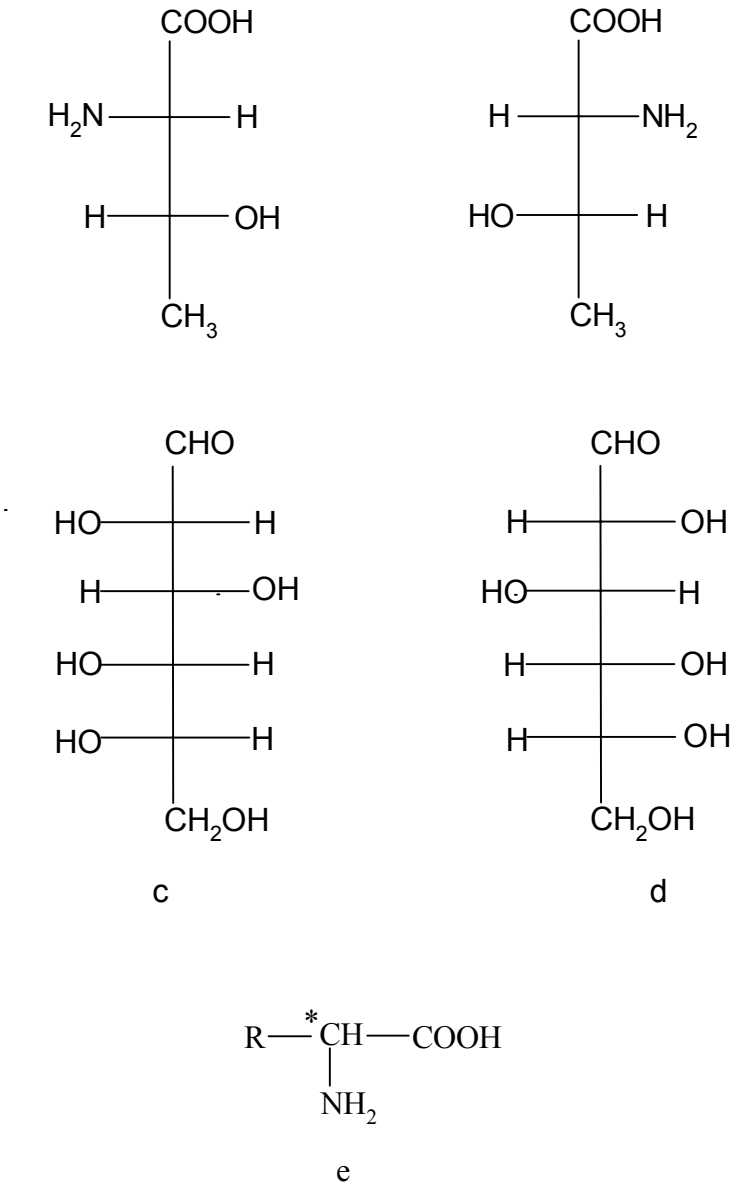

Figure 1.8 Amino acid stereochemistry (a) L-threonine (b) D-threonine (c) L-glucose and (d) D-glucose (e) Asymmetric carbon in amino acids

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].

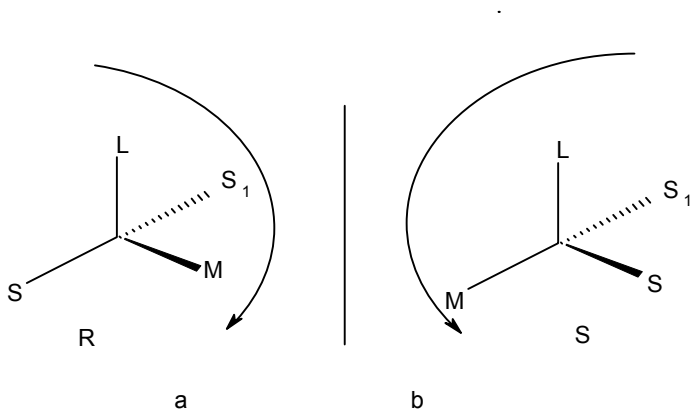

Figure 1.9 The Cahn-Ingold-Prelog designation, (a) sequence clockwise and (b) sequence anticlockwise

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.

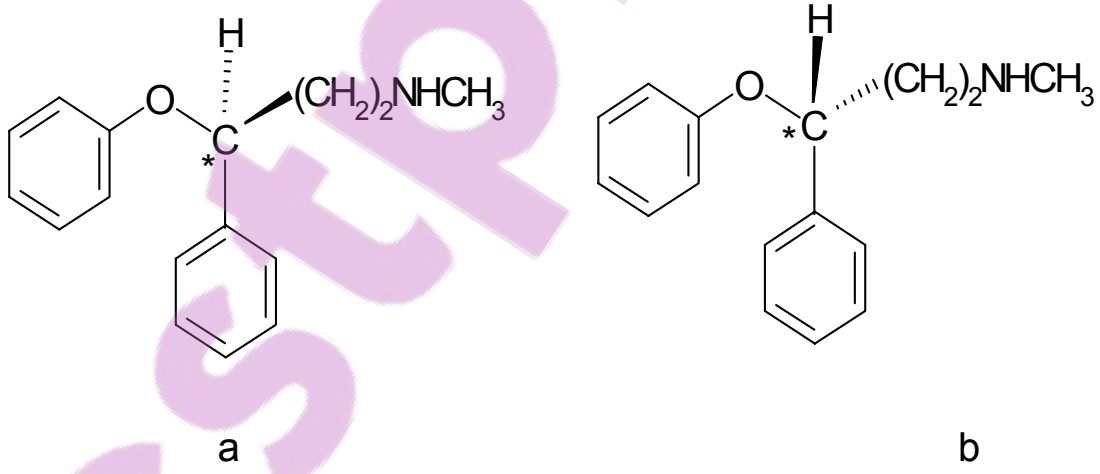

Figure 1.10 Fluoxetine (a) S- fluoxetine and (b) R- fluoxetine

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).

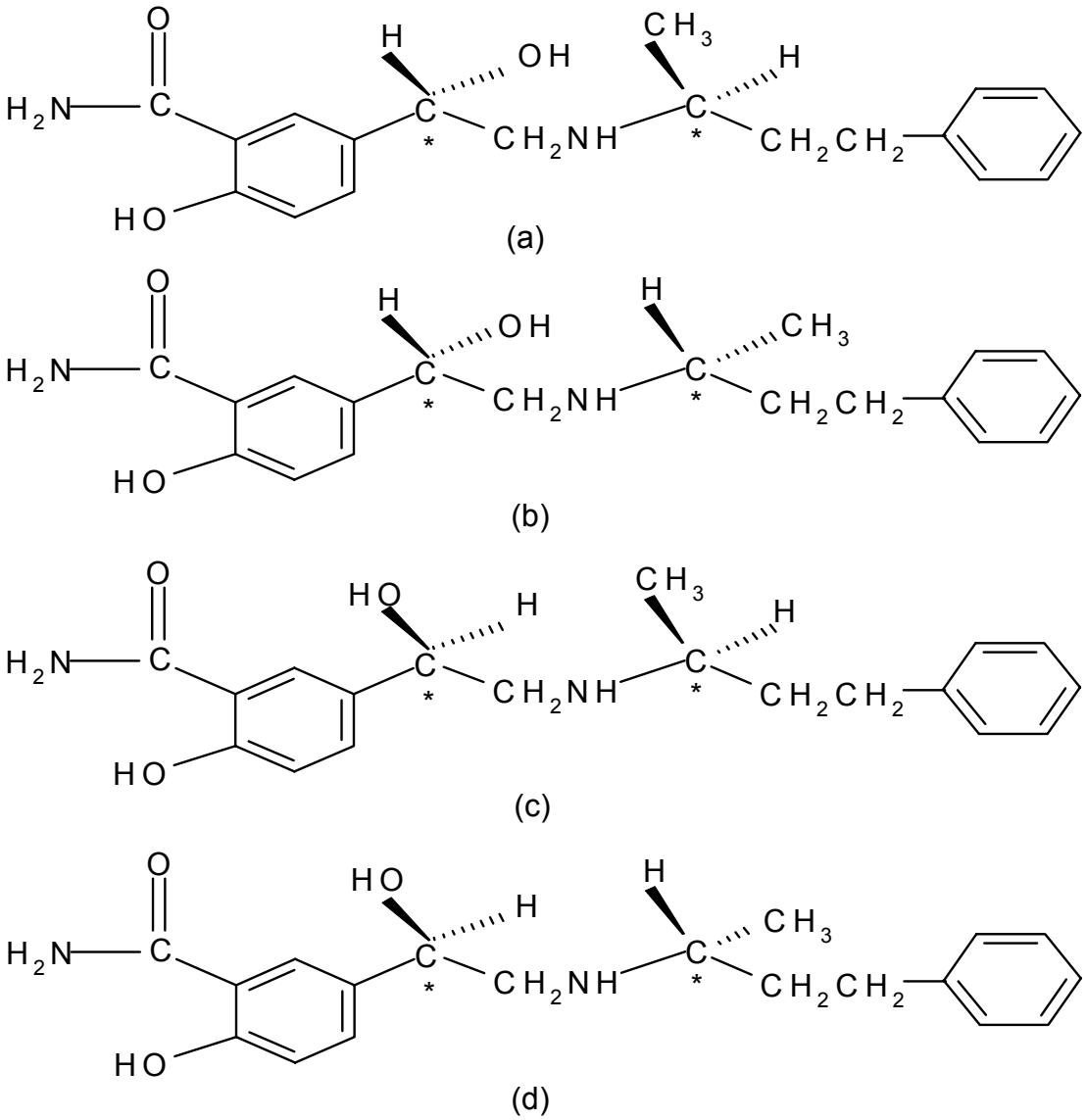

Figure 1.11 Stereochemistry of labetalol (a) S,S labetalol, (b) S,R labetalol, (c) R,S labetalol and (d) R,R labetalol

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].

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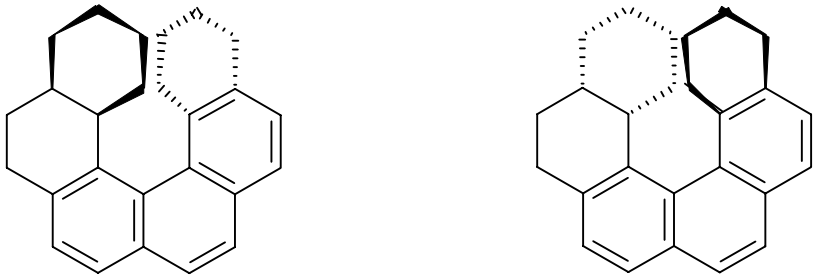

a b

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;

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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];

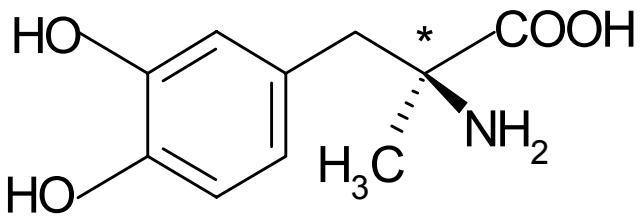

Figure 1.13 (S)-α-methydopa

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

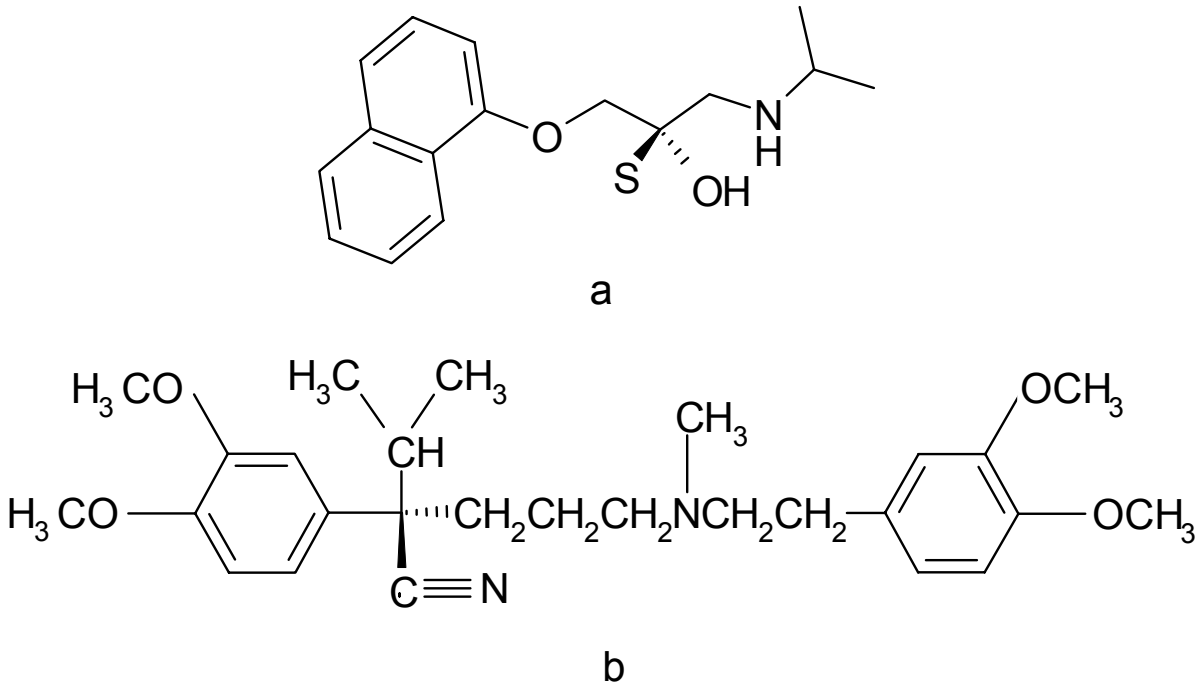

Figure 1.14 (a) S-propanolol and (b) R(+)-verapamil

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);

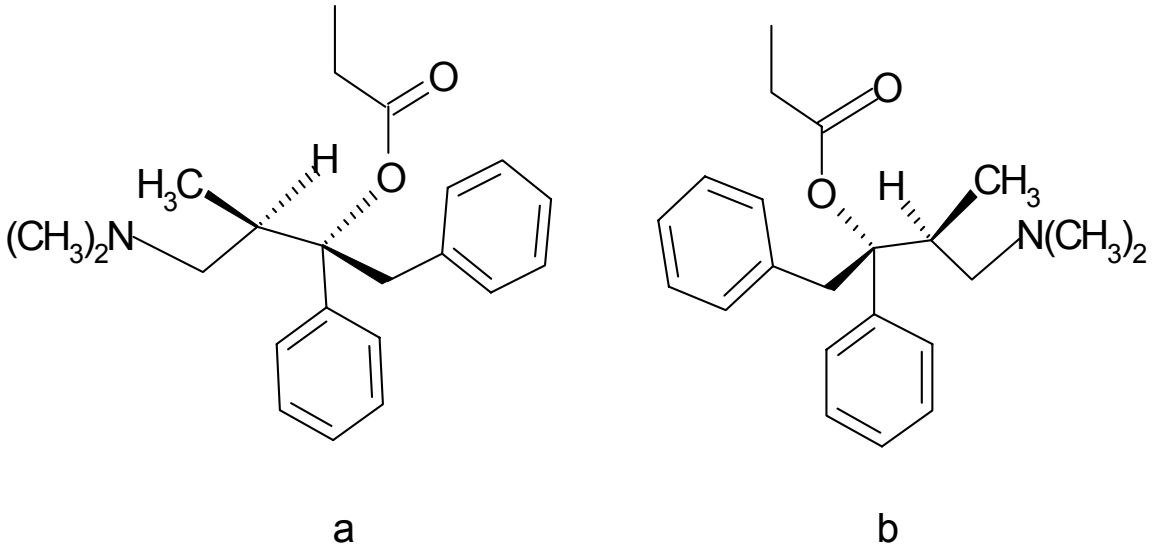

Figure 1.15 (a) (2S,3S)-(+)-Dextropropxyphene and (b) (2S,3R)-(-) Levopropoxyphene

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].

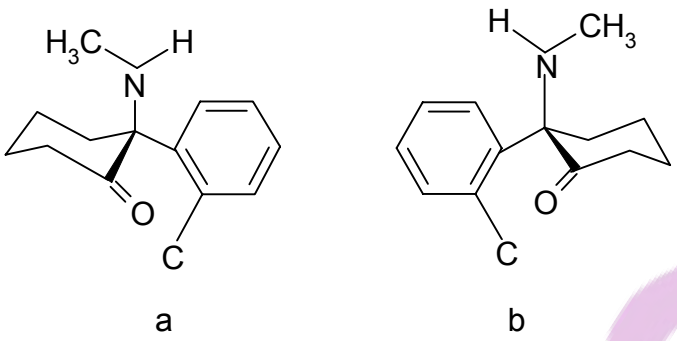

Figure 1.16 Ketamine distomer, (a) S(+)-ketamine and (b) R(-)-ketamine

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.

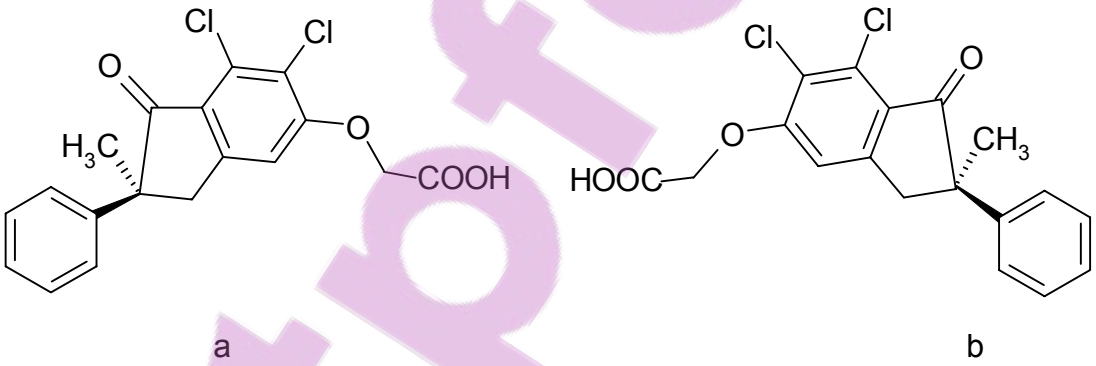

Figure 1.17 Indacrinone (a) S-indacrinone and (b) R-indacrinone

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

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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

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

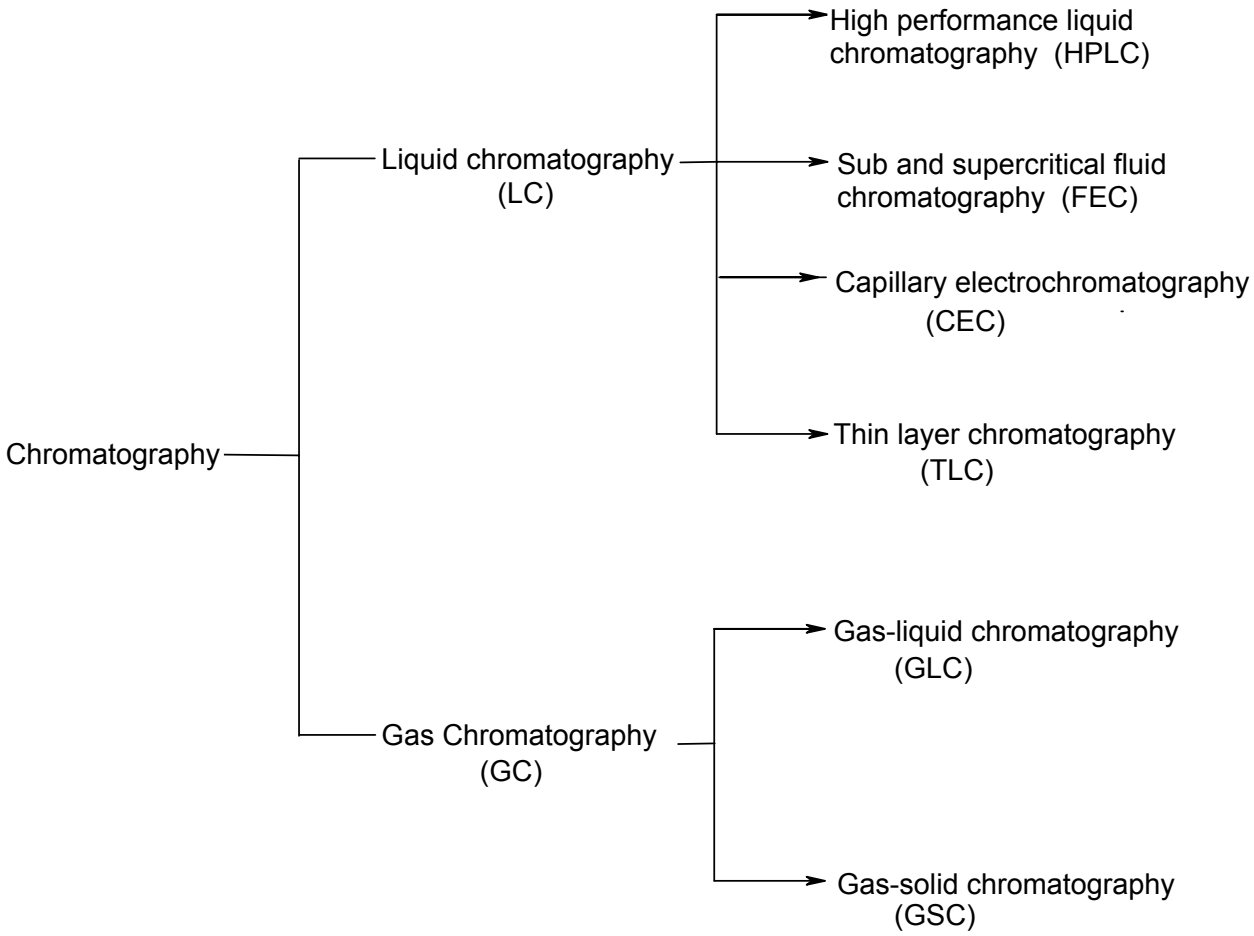

Figure 1.18 Chromatographic techniques for chiral recognition

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

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(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].

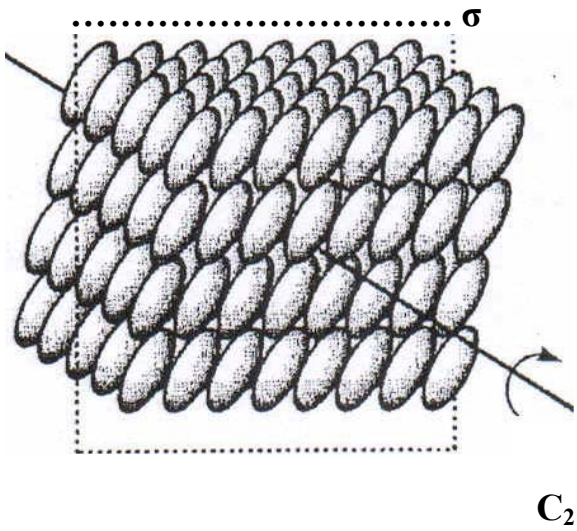

Figure 1.20 Schematic representation of the smectic C phase. The vectors z and n are congruent with the plane of the page, and the C_2 is axis normal to the tilt plane and a reflection plane of symmetry σ congruent to the tilt plane [64].

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

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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.

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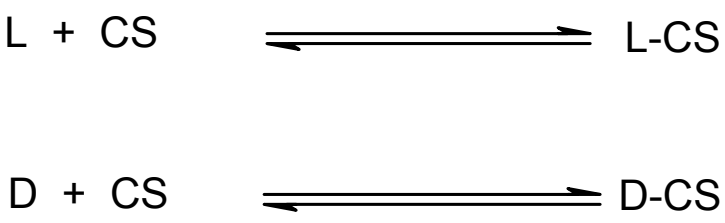

Figure 1.21 Reactions of enantiomers (L and D) with chiral selector (CS)

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.

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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.

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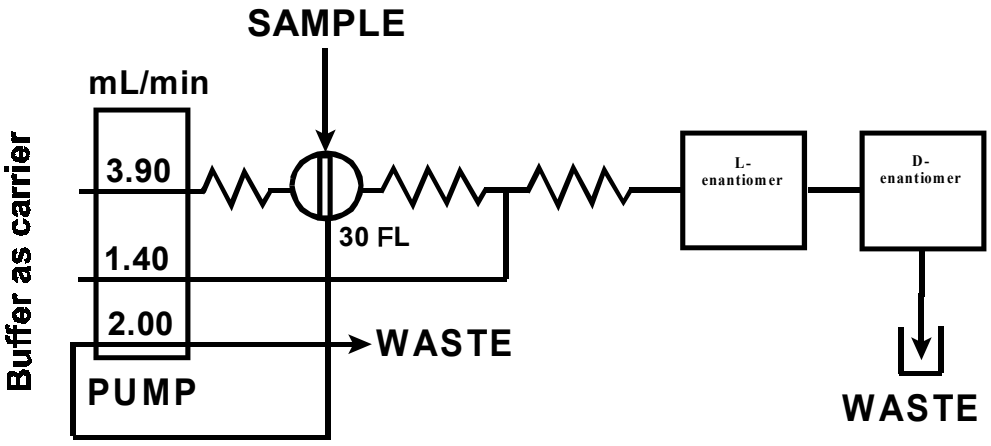

Figure 1.22 Schematic diagram of the FIA system used for the determination of enantiomers.

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.

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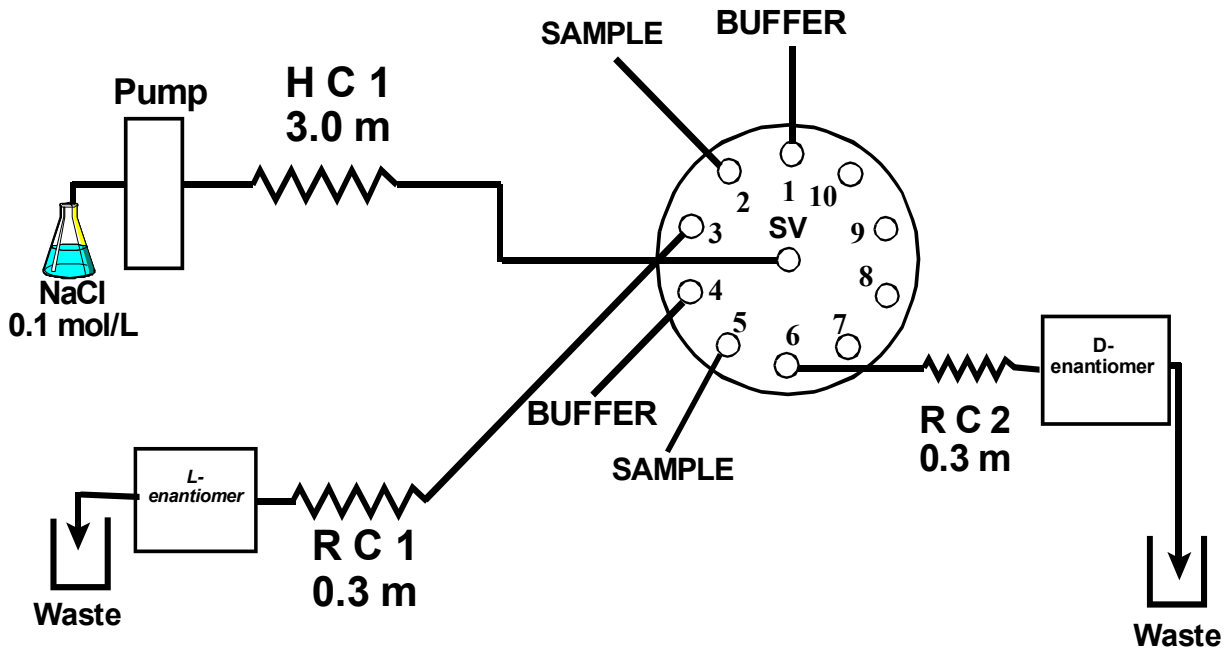

Figure 1.23 SIA system utilized for the simultaneous detection of L- and D-enantiomers.

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.

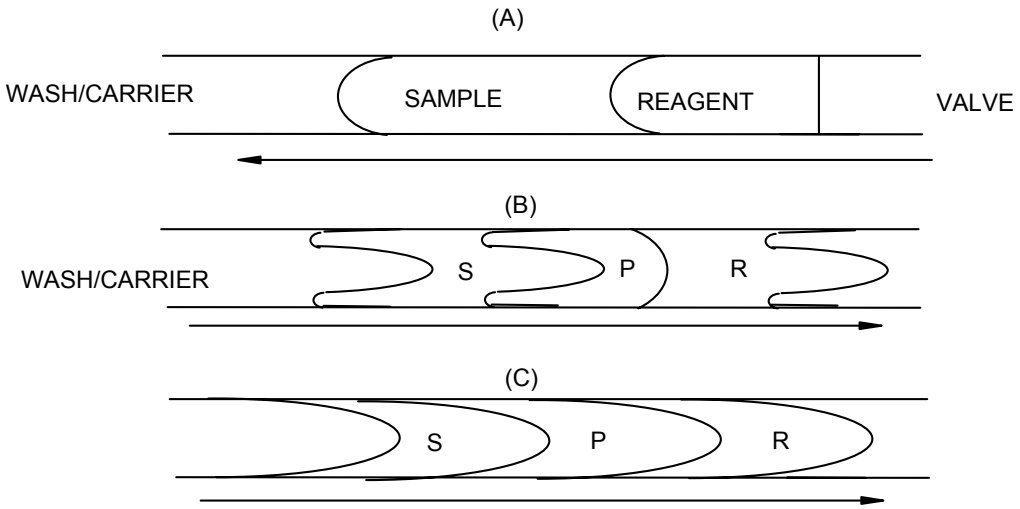

Figure 1.24 Principles of sequential injection analysis. Profile zones after injection (A), immediately after flow reversal (B) and in reaction coil (C), S-sample, R-reagent, and P-product formed.

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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Chapter 2

Enantioselective, potentiometric membrane electrodes

2.1. Introduction

Enantioselective, potentiometric membrane electrodes (EPMEs) were particularly developed for enantioanalysis. EPMEs based on 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrin were proposed for the assay of angiotension-converting enzyme inhibitors [1-5] as well as for L-proline [6].

Rapid development of new electrodes materials and more sensitive and stable electronic components in the last two decades has increased the range of analytical applications utilizing potentiometric electrodes. The fast development of this field is a scale of the degree to which potentiometric measurements satisfy the need of the clinical chemist for rapid, low cost and accurate analysis.

The accuracy obtained when EPMEs were used in clinical analysis made their utilization a valuable alternative for chromatographic techniques [7, 8]. The method is rapid, precise, and not expensive. The high reliability of the information obtained using these electrodes made automation of potentiometric technique possible, by the integration of enantioselective electrodes as detectors in FIA [9, 10] and SIA [11, 12] systems. The type of electrode and chiral selector must be selected in concordance with the complexity of the structure of the enantiomer to be determined. The principle of molecular recognition for EPMEs is the selective binding between a molecule with a special chemical

architecture (chiral selector) and the enantiomer. Therefore, the chiral selector plays the main role in molecular interaction.

$$L + CS \rightleftharpoons L\text{-}CS \qquad K_L$$

$$D + CS \rightleftharpoons D\text{-}CS \qquad K_D$$

where L and D are enantiomers to be determined, CS is chiral selector, L-CS and D-CS are the complexes formed between L(D)-enantiomer and CS, respectively, and K_L and K_D are the stability constants of the complexes formed between chiral selector and enantiomers.

The stability constants (K_L and K_D) of the complexes formed between chiral selectors and L- and D-enantiomers are given by the following equations:

$$K_L = e^{-\frac{\Delta G_L}{RT}} \tag{2.1}$$

$$K_D = e^{-\frac{\Delta G_D}{RT}} \tag{2.2}$$

where ΔGL and ΔG_D are the free energies recorded for the L- and D-enantiomer reactions with the chiral selector, CS. R is the gas constant = 8.31 J/mol K and T is the temperature in Kelvin.

The log K_L is directly proportional to ΔG_L and log K_D is directly proportional to ΔG_D, respectively. This means that a difference in the free energies of the reactions will result in a difference of the stability of the complexes formed between the chiral selector and the L and D enantiomers. Therefore, the stability of the complexes is directly correlated with the response (slope) of the EPMEs [13]. Accordingly, a large difference between the

free energies of the reactions of chiral selector with L- and D-enantiomers will give a large difference between the slopes when L and D enantiomers will be determined. The enantioselectivity of the measurements is given by the difference between the two free energies. Also, the slope is a measure of enantiorecognition. The minimum value tolerable for a 1:n stoichiometry between the enantiomer and chiral selector is 50/n mV/decade of concentration [14].

2.2. Design of enantioselective, potentiometric membrane electrodes

The design of enantioselective, potentiometric membrane electrodes (EPMEs) plays a very important role in the reliability of analytical information. The evolution concerning the design of EPMEs made their utilization a very accurate and precise alternative for structural analysis techniques [15]. The reliability of the response characteristics as well as the analytical information obtained using EPMEs is strictly correlated to the reliability of the electrodes design [13]. Only a reliable design of EPME will give reliable response characteristics and reliable analytical information.

One of the designs proposed for sensors is based on the impregnation of a chiral selector on a conducting layer such as PVC; imprinting polymers, and carbon paste matrices. The repartition of chiral selector in the plastic membrane is not homogeneous and not reproducible. The liquid membrane needs a support characterized by certain porosity that assures reliability in construction. Accordingly, the most reliable design is that of EPME based on carbon paste that is preferred due to the simplicity and reliability of the construction of electrode.

2.2.1. Modified paste electrode design

Graphite powder proved to be a very good material for electrode design. Mixing oil (paraffin or nujol oil) with the graphite powder is forming carbon paste. One of the most reproducible designs for EPME based on carbon paste has been proposed by Stefan et al [16-18]. The paraffin oil and graphite powder were mixed in a ratio of 1:4 (w/w) followed by the addition of a solution of chiral selector (ligand) (10^{-3} mol/L) (100 μL of chiral selector solution is added to 100 mg carbon paste). The plain carbon paste was filled into a plastic pipette peak leaving 3 to 4 mm empty in the top to be filled with the modified carbon paste. The optimum diameter of the designed EPME is 3 mm. Electrical contact is made by inserting a silver wire in the plain carbon paste. The surface of the electrode can be renewed by simply polishing it with alumina paper. Because the electrode response is directly proportional to the complex formed at the membrane-solution interface, different types of chiral selectors were proposed for the design of EPMEs such as crown ether, cyclodextrins and its derivatives, maltodextrins and macrocyclic antibiotics.

2.2.1.1 Cyclodextrins as chiral selector in the EPMEs design

Cyclodextrins (CDs) are oligosaccharides prepared by enzyme degradation of starch and glycosyltransferases of cyclodextrinases producing a mixture of different CDs [19]. The most frequent used CDs as chiral selectors are those consisting of six (α-CD), seven (β-CD) and eight (γ-CD) glucopyranose units with a truncated cone shape providing a hydrophobic cavity (Figure 2.1). Due to the presence of hydroxyl groups (position 2, 3 and 6 of glucopyranose), the outside ring of CD is hydrophilic [20]. The inner diameter

of α-, β- and γ-CDs is increasing as a number of glucose units increases from 0.57 to 0.78 and 0.95 mm, respectively. CDs have a suitable solubility in aqueous medium. β-CD has the lowest solubility (1.85 g/100ml water), caused by the existence of intermolecular hydrogen bonding [21].

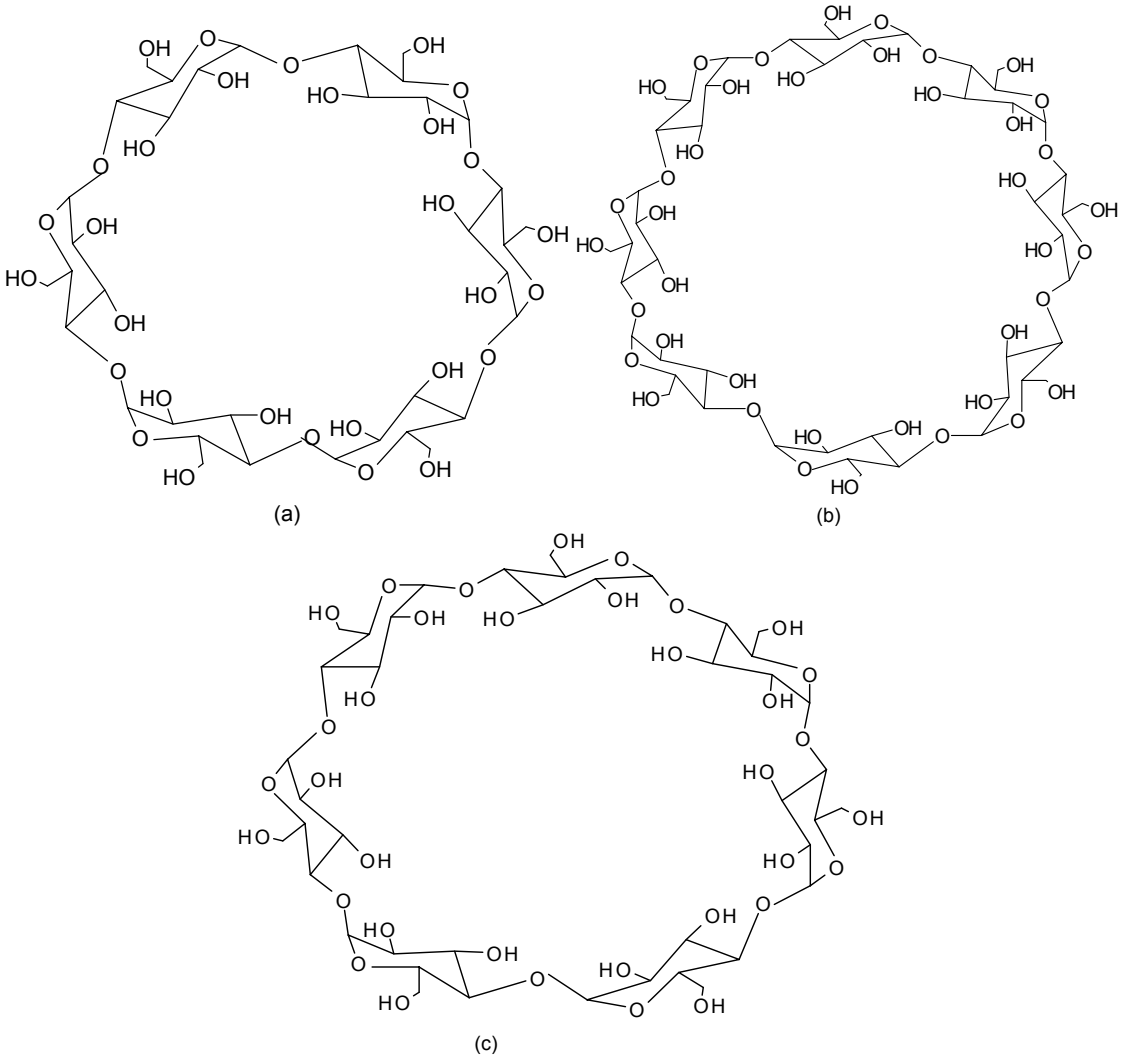


Figure 2.1 (a) α-cyclodextrin, (b) β-cyclodextrin and (c) γ-cyclodextrin

CDs have the enantioselectivity property because of its cavity dimension, providing sufficient interactions with analytes to form the host-guest complex inclusion [22].

Several cyclodextrin derivatives have been developed in order to improve the external enantioselectivity of CDs [23]. EPMEs based cyclodextrin derivatives proved good enantioselectivity and reliable analytical information. Stefan et al. proved the suitability of 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrin based EPME for the enantioanalysis of the S-enantiomer of angiotension-converting enzyme inhibitors [2, 3].

2.2.1.2 Maltodextrins as chiral selectors in the EPMEs design

Maltodextrins proved to be suitable chiral selectors for compounds with acidic moieties [24]. Hydrolysis of starch by means of heat and acid or specific enzymatic treatments or combined acid and enzymatic hydrolysis yields a spectrum of depolimerized oligomers [25]. These hydrolyzates are described in terms of their dextrose equivalent (DE) value, which is a measure of the total reducing power of all sugar present relative on a dry weight basis. Maltodextrins are hydrolyses products of starch with DE lower than 20. They are produced be enzyme-catalyzed conversion using α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) from *Bacillus subtilis* and pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41) [26]. In general, linear maltodextrins consists of D-(+)-glucose units connected through Glu-(1-4)-α-D-Glu linkages (Figure 2.2). Up to now, three types of maltodextrins with different DE values (I (4.0-7.0), II (13.0-17.0) and III (16.5-19.5)) were used as chiral selectors in enantioanalysis. Hygroscopicity, solubility, osmolarity, and their effectiveness to reduce the freezing point increase with increasing DE, while viscosity, cohesiveness and coarse-crystal prevention increase as DE decreases. Enantioselective, potentiometric membrane electrodes based on maltodextrins have been applied for the enantioanalysis of several drugs [27].

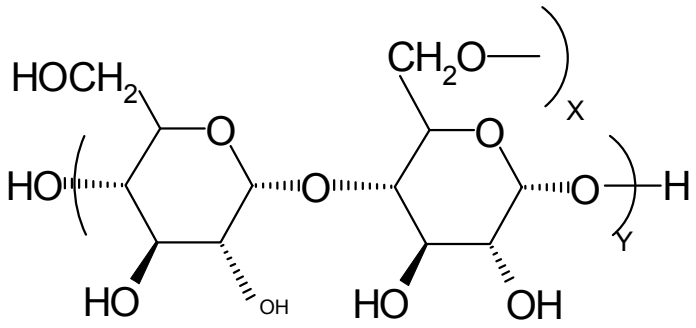
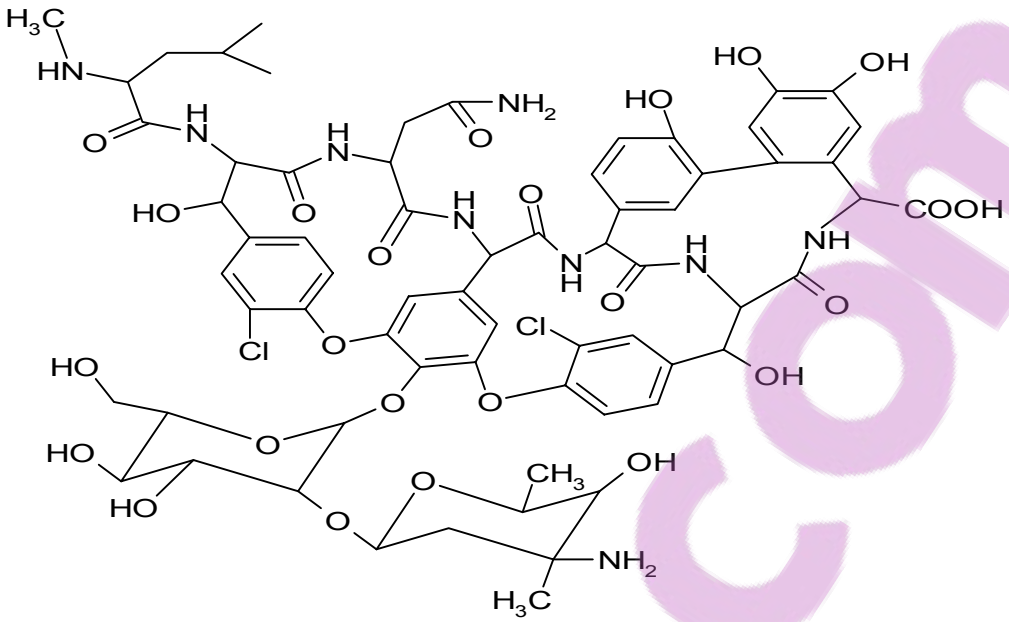


Figure 2.2 Maltodextrin

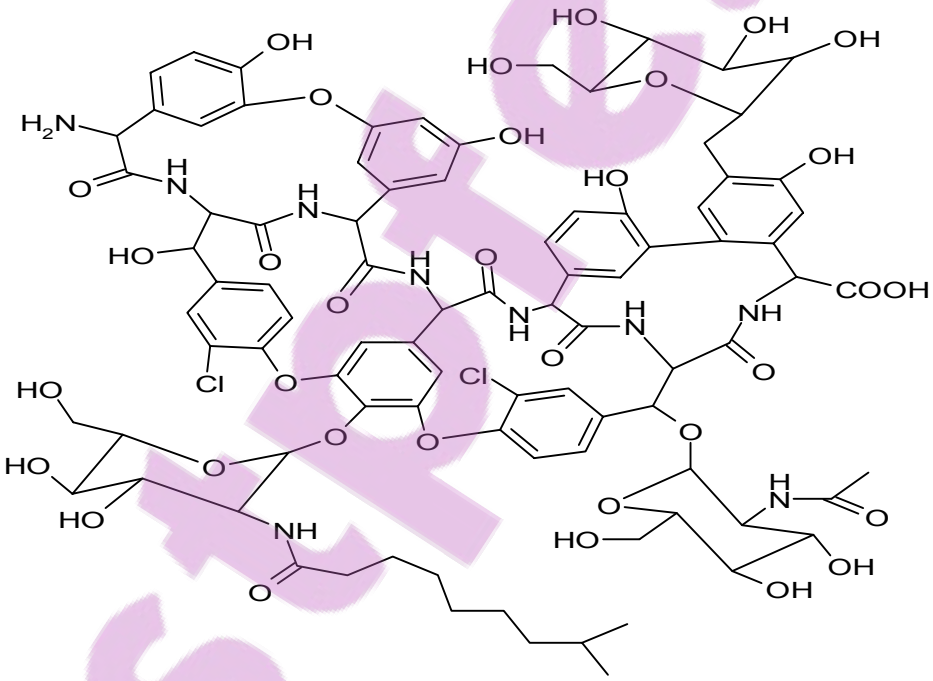
2.2.1.3. Macrocyclic antibiotics as chiral selectors for EPMEs design

Macrocyclic antibiotics have been successfully used as chiral selectors for the enantiorecognition of several classes of pharmaceutical enantiomers of drugs and molecules with biological importance. The high selectivity and efficiency in molecule discrimination make antibiotics a typical chiral selector of biological origin. Macrocyclic antibiotic contains several functional groups responsible for multiple stereoselective interactions. All macrocyclic antibiotics exhibit very similar physico-chemical properties, but they show a different stereoselective power [28]. The most used macrocyclic antibiotics in enantioanalysis are vancomycin and teicoplanin [29, 30].

Vancomycin is "basket" shaped (Figure 2.3a) with three fused macrocyclic rings and two side chains, a carbohydrate dimmer and a N-methyl leucine moiety [31]. It has 18 asymmetric centers and several functional groups such as carboxylic, hydroxyl, amino, amido and aromatic rings [28]. Vancomycin is very soluble in water and can dimerize in aqueous solutions depending on vancomycin concentration [32].



(a)



(b)

Figure 2.3 (a) Vancomycin and (b) Teicoplanin

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Vancomycin solutions are stable at low temperature and in buffered solutions (pH 3.0-6.0) [32, 33]. This antibiotic is very efficient for the enantiorecognition of anionic compounds containing carboxylic groups in their structure, which could be explained by the presence of amine groups [28].

Teicoplanin (Figure 2.3b) has a long hydrophobic tail, which behaves like surfactant properties. The molecular structure of teicoplanin shows a slightly higher solubility in water than vancomycin [30]. The amide and carboxylic groups are the most important functional groups of teicoplanin used for the enantiorecognition of molecules containing carboxylic groups. These groups are ionized over the 3.5-8.0 pH range [34, 35]. Teicoplanin exhibits a very slight basic behavior even at acidic pH. At low pH, teicoplanin favors aggregation and micelle formation [36].The most common teicoplanin glycopeptide (A_2-2) has a molecular weight of 1877 [34]. Addition of organic modifiers to teicoplanin, such as acetonitrile, improves the resolution ability. These modifiers enhance the enantioselectivity by alerting and/or inhibiting aggregation of teicoplanin monomer producing more teicoplanin molecules available to interact with solutes [28].

2.2.2. Plastic membrane based electrode design

Nowadays, polyvinylchloride (PVC) and its derivatives are the most used in sensor technology. PVC membranes have some disadvantages such as:

1- It is not possible to control the uniformity distribution of the electroactive species in the membrane;
2- PVC membranes have low thermal durability and low mechanical strength.

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PVC membranes are prepared by dissolving a polymer (PVC), a large amount of plasticizer, and the sensing compound in an organic solvent. Tetrahydrofuran and cyclohexane can be used as organic solvents. The solvent is allowed to evaporate, leaving a dry membrane attached to the body of the electrode. The ratio between plasticizer and PVC is 70:30 (w/w) [37, 38]. The PVC and plasticizer form the medium of electroactive compound formation. The most used plasticizers are dioctyl phthalate (DOP), dioctyl sebacate (DOS), dinonyl phthalate (DNP) and ortho-nitrophenyl octyl ether (o-NPOE). Crown ethers [39-42] and lipophilic cyclodextrins [43] are used as chiral selectors for the design of PVC membrane based EPMEs

2.3. Response characteristics of EPME

The functional relation between the potential, E measured at I = 0, and the activity, *a*, of the enantiomer gives the electrode function (Figure 2.4).

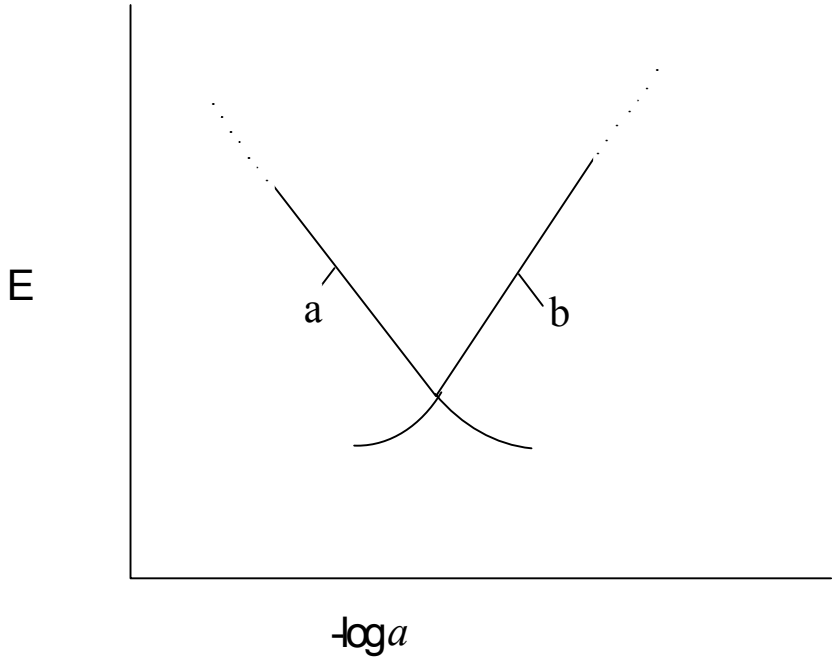

Figure 2.4 EPME function. (a) cation-selective electrode; (b) anion-selective electrode.

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The potential is not dependant on the activity, a of the ion, but on –log a:

$$E = f(-\log a_i) \tag{2.3}$$

Usually, the ionic strength is kept constant by the addition of a strong electrolyte to each solution (e.g., NaCl, KCl), or by buffering the solution with a buffer that can also maintain the ionic strength at a constant value. Accordingly, the activity can be substituted with the concentration, and further more for an ion M^{z+}, pM = - log C_M^{z+} is used, and the electrode function is given by E = f(pM).

2.3.1 Standard electrode potential, E^o

The standard electrode potential is defined by IUPAC as the value of standard emf of a cell in which molecular hydrogen is oxidized to solvated protons at the left-handed electrode [44].

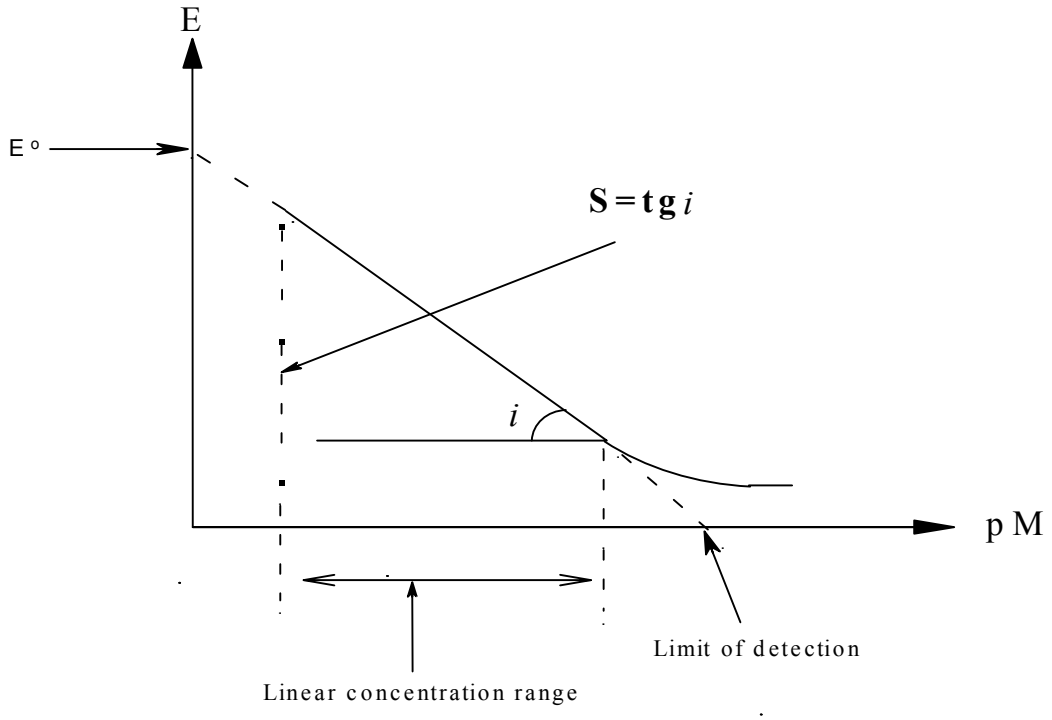

Figure 2.5 Response characteristics of EPME

E^o does not depending on the concentration of the ions in solution and can be determined graphically from the calibration graph of the potentiometric electrode (Figure 2.5).

The value of standard electrode potential is also recommended to be determined using the linear regression method as one of the parameters of the equation of calibration of EPME:

$$E = E^o \pm SxpM \tag{2.4}$$

where E is the potential of the electrode, E^o is the standard potential, S is the slope, and pM = -log C_M.

2.3.2 Response of EPME

The response of EPME is dependant on the slope of the linear part of the calibration graph. It is the main characteristic of the potentiometric electrodes. This value can be computed from the Nernst equation:

$$E = E^o \pm S(\log a) \tag{2.5}$$

where E is the potential of the electrode, E^o is the standard electrode potential, S is the slope, and a is the activity of the ion. The slope of the potentiometric electrode has ideal value given by Nernst (59.16/z mV/decade of concentration) where $S = \frac{RT}{zF}$ (R = 8.31 J/K mol, T = 298 K, z is the charge of the ion that has determined, F = 96500 C). The minimum accepted value of the slope of potentiometric electrodes for bioanalysis is 50/z [45]. Nernstian response implies ideal sensitivity, but not necessarily ideal selectivity since interfering ions may also give Nernstian response when present as the sole potential determining species. The response is dependant on the stability of the compound formed at the membrane-solution interface [13]. The value of the slope can be deducted using the

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equation of dependence of slope on the stability of the compound formed at the membrane-solution interface [13].

$$S = S^o - a + \left(\frac{b}{S^o}\right)\log \beta_S \qquad (2.6)$$

where S is the slope of the electrodes (mV/decade of concentration), S^o is the Nernstian slope (59.16 mV/decade of concentration), β_s is the stability constant of ion-pair complex, and a and b are two coefficients depending on the membrane composition [13]. The slope can be determined experimentally as follows:

1- in figure 2.5, tangent of the angle made by the linear part of the calibration curve and pM axis;
2- as a parameter of the equation of calibration by using the linear regression method.

The response depends on some parameters which characterize the matrix such as polarity of the plasticizer, oil or solvent. The response could be improved by selecting the suitable chiral selector (that forms a compound with higher stability) and matrix.

2.3.3 Limit of detection

The limit of detection is defined by IUPAC as the concentration at which, under specified conditions, the cell potential, E, deviates from the average value by a multiple of the standard error of a single measurement of the cell potential in this region [44]. The limit of detection of EMPE depends on the values of standard electrode potential, slope and the stability of the compounds formed at membrane-solution interface. The internal solution of EPME influences the value of the limit of detection. By using 0.1 mol/L KCl as internal solution, the detection limits obtained for EPMEs are very low.

The value of the limit of detection can be deducted from the calibration graph of EPME, as the concentration (activity) of the ions at the point of intersection of the extrapolated linear calibration curve and activity (or concentration) axis.

2.3.4 Linear concentration range

The definition of linear concentration range is the range of concentration of an analyte over which the sensitivity of the electrode is constant with a specified uncertainty (±5%). It can be determined form the plot of the cell potential difference versus the logarithm of responsive ionic activity (or concentration) (Figure 2.4) The linear response range is very important for EPME because all the solutions required for measurement must have the activity (concentration) of the substances within the linear range. The reproducibility of the linear range is influenced by stirring rate of solution, composition of the solution containing the proposed substance for measurement, pH of the solution, the preconditioning of the electrode, temperature, composition of the solution where the electrode was exposed before the measurement [46].

2.3.5 Influence of pH

The pH can influence the formation of protonated and unprotonated species of the same substance. It is very important to determine for EPME the dependence of their potential on the pH variation. Special care must be taken for the buffering of solutions because a small difference on pH may cause a significant error in the potential value.

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2.3.6 Influence of the temperature on the response of the electrode

The response of the electrode is highly affected by the temperature. The kinetics and thermodynamics of the process that take place at the electrode surface is favored by the increase of temperature, and accordingly the slope will increase. A temperature of 298 K is recommended for electrode characterization. The temperature must be maintained constant during the measurements of standard sample solutions.

2.3.7 Response time

The response time is defined as the elapsed time between the period when the electrodes are immersed in a sample solution and the first time when the slope of the working electrode becomes equal to a limiting value selected on the basis of the experimental conditions and/or requirements concerning the accuracy [44]. EPME response time is influenced by the membrane-solution interface processes. This response time equals the sum between the time required for the ion or molecule to be extracted in the membrane-solution interface and the required time for ions/molecules to reach equilibrium stage of complexation or precipitation or redox. For EPME, the response time depends on the concentration and the stability of the complex formed between the analyte molecules and the chiral selector at the EMPE surface-solution interface. The response time increases with decreasing the concentration of the molecule that has to be assayed. EPME of short response times are preferred to be used in bioanalysis.

2.3.8 Ionic strength and activity coefficients

A source of error can be caused due to the variations of the activity coefficients of the ions in the solution containing the molecules required for analysis. For this reason, in EPME optimization it is necessary to run all the measurements at the same ionic strength. The utilization of a strong electrolyte and some of the buffers in the standard and sample solutions preparation can ensure a constant ionic strength.

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2.4 Selectivity of enantioselective potentiometric membrane electrodes

IUPAC defined the interfering species as substance whose presence in the sample solution influence the measured emf of the cell relative to the specie being determined. Unfortunately, no analytical method is totally selective for the analyte species and unaffected by other species in the assay of analyte. Selectivity depends on ratio between the activities of the main molecule/ion and interfering species in the solution, the composition of the membrane, pH of the solution, developed current, and the complexity of the sample matrix to be analyzed. EPME selectivity is high when utilized for clinical analysis including pharmaceutical analysis. Two classes of interfering substances affect the EPME potential signal. First, electrode/electrochemical interferences include substances whose response is similar to the molecule being determined or electrolyte present at high concentration level. Chemical interferences are the second class where it interacts with the ion/molecule being determined, so as to decrease its activity or apparent concentration (e.g., H^+, HO^-), or substances that interact with the membrane surface. The selectivity degree of EPME is given by the values of the potentiometric selectivity coefficients respectively, as follows:

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(i) if a magnitude order is higher than 10^{-3}, the ion tested for interference does interfere;

(ii) if a magnitude order is 10^{-3}, the ion tested for interference is not a strong interfering species;

(iii) if a magnitude order is less than 10^{-3}, the ion tested for inferences does not interfere.

Nicolsky and Eisenman proposed an equation (2.7) that gives the relation between the potential of the electrode recorded in the presence of the interfering ion and potentiometric selectivity coefficients ($K_{i,j}^{pot}$): *constant*

$$E = Q + \frac{2.303RT}{z_i F}\log\left[a_i + \sum_{j=1}^{N} K_{i,j}^{pot} a_j^{z_i/zj}\right] \qquad (2.7)$$

E is the recorded emf of the cell when the only variables are activities in the test solution; Q is constant; R is the gas constant, 8.314 J/K mol; T is the absolute temperature in Kelvin; F is Faraday constant, 96500 C/mol; a_i is the activity of ion/molecule I, a_j is the activity of interfering species J, N is the number of the interfering species in the solution. Nicolsky-Eisenman equation was modified by substituting the charge number through their absolute values [46], as follows:

$$E = Q \pm \frac{2.303RT}{z_i F}\log\left(a_i^{1/|z_i|} + \sum_{j=1}^{N} K_{i,j}^{pot} a_j^{1/|z_j|}\right) \qquad (2.8)$$

The potentiometric, selectivity coefficient, $K_{i,j}^{pot}$ can be determined experimentally using two methods, mixed solution method and separate solutions method. The potentiometric selectivity coefficient is recommended to be determined at a ratio between main and interfering species of 1:10.

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2.4.1 Mixed solution method

The potentiometric, selectivity coefficient, $K_{i,j}^{pot}$, is given by the difference between the potentials recorded for a mixed solution (containing the main ion and the interfering ions) and the potential recorded for the solution containing only the main specie, I. The main species must have the same activities in both solutions. $K_{i,j}^{pot}$ can be calculated using the following equation:

$$K_{i,j}^{pot} = \left(10^{(\Delta E/S)} - 1\right) x \frac{a_i}{a_j^{(z_i/z_j)}} \qquad (2.9)$$

where $\Delta E = E_{i,j} - E_i$; $E_{i,j}$, is the potential recorded for mixed solution, E_i is the potential recorded for the solution containing only the main species, i (all recorded in mV); S is the slope of the electrode computed from the equation of calibration in mV/decade of concentration; a_i is the activity of the main species, i; a_j is the activity of interfering species, j; z_i and z_j are the charges of the main species (i) and interfering species (j).

2.4.2 Separate solution method

There are two ways to determine $K_{i,j}^{pot}$ using the separate solution method:

(i) The emf of a cell comprising an ion-selective electrode and a reference electrode is measured for each of two separate solutions, one containing only the main ion of the activity, a_i, and the other containing the interfering species at the same activity, a_j, ($a_i = a_j$). The potentiometric selectivity coefficient is given by the equation:

$$\log K_{i.j}^{pot} = \frac{\Delta E}{S} + \left(1 - \frac{Z_i}{Z_j}\right) \log a_i \qquad (2.10)$$

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where $\Delta E = E_{i,j} - E_i$; $E_{i,j}$ is the potential recorded for mixed solution, E_i is the potential recorded for the solution containing only the main species, i (all recorded in mV); S is the slope of the electrode computed from the equation of calibration in mV/decade of concentration; a_i is the activity of the main species, i; z_i and z_j are the charges of the main species (i) and interfering species (j).

(ii) The activities of two different solutions introduced into the cell comprised of an EPME and a reference cell, are adjusted with each of two different solutions, one is containing only the main species of the activity a_i, while the other is containing only the interfering ion of the activity a_j, as much as required to achieve the same cell potential measured. From any pairs of activities a_i and a_j for which the cell potential is the same, the $K_{i,j}^{pot}$ may be calculated from the following equation:

$$K_{i.j}^{pot} = \frac{a_i}{a_j^{(z_i / z_j)}} \tag{2.11}$$

where a_i is the activity of the main species, i; a_j is the activity of interfering species, j;z_i and z_j are the charges of the main species (i) and interfering species (j).

2.5 Direct potentiometric method

Potentiometric methods are based on the measurement of a potential difference between two electrodes (indicator and reference electrode) immersed in a solution containing the analyte. The indicator electrode is chosen to respond to a particular enantiomer in

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solution. The reference electrode is the one for which half-cell potential is constant. The potential of an electrochemical cell is given by the following equation

$$E_{cell} = E_{ind} - E_{ref} + E_{ij} \qquad (2.12)$$

where E_{cell} is potential of the electrochemical cell; E_{ind} is half-cell potential of the indicator electrode (cathode); E_{ref} is half-cell potential of the reference electrode; E_{ij} is liquid-junction potential.

The interface between two solutions containing different electrolytes or different concentrations of the same electrolyte is called liquid junction. A junction potential arises when the two electrolyte solutions of the different composition are brought together into contact with one another and a potential developed at the interface. The potential arises from an unequal distribution of cations and anions across the boundary due to the difference in the rate at which these species migrate. Calibration procedure of EPME assumes that during the measurements the slope of the electrode is constant and the concentration of the determined enantiomer is proportional to the developed potential. However, because the consumption of the electrolyte to be measured can differ from the solutions used in the calibration process, this assumption is fulfilled very unlikely, if ever. The total relative error expected in EPME measurements with respect to the liquid junction uncertainty can be evaluated by differentiating the following equation [47]:

$$\text{Relative error of measured activity [\%]} = 4n\Delta E_i \qquad (2.13)$$

where n is the charge of the determined species and $n\Delta E_i$ is the uncertainty in liquid junction potential (mV). The minimal $n\Delta E_i$ uncertainty is in the ±1 mV magnitude order, which results in a relative error in the measured activity of ± 4% for univalent ions.

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Direct potentiometry is applied for the enantioanalysis of substance with chiral centers. The EPME must be calibrated before the analysis of samples. The solutions used for calibration are obtained from standard solutions, by serial dilution. All solutions must be buffered. The pH and ionic strength of the sample solutions must be adjusted to the same values of the solutions used for calibration of the electrodes. A curve of calibration is obtained by plotting the emf of the cell comprising the working electrode and reference electrode versus the negative logarithm of the main species concentration. The values of emf for the samples are interpolated on the calibration graph and the unknown concentration of the enantiomer is determined.

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Chapter 3

Amperometric electrodes for enantioanalysis

3.1 Introduction

Amperometric detection has found wide application to measurements in biological media [1-4], environment analysis [5-6] and pharmaceutical industry [7-8]. Amperometric electrodes have higher sensitivity for enantiomer recognition than the best chromatographic technique (e.g., capillary zone electrophoresis in enantiomer recognition (CZE) [9]). The separation step in CZE is not always accurate, therefore the reliability of the analytical information is decreasing. The utilization of amperometric electrodes in molecular recognition of the enantiomers is not laborious if one compares it with structural analysis (IR, NIR, Raman, MS, X-ray Diffraction, and Neutron Diffraction) and with chromatographic techniques [10]. The advantage of using these sensors over the other techniques is the high reliability that is given by high precision, high reproducibility and rapidity [11-12]. High precision can be explained by to the fact that amperometric sensors can be used directly for measurement of the compounds in solution without any prior separation of the enantiomer that has to be determined.

Sensor validation is dependant on the reliable response characteristics and reliable analytical information [13]. The high reliability of analytical information obtained using these electrodes made automation of amperometric techniques possible, by integration of enantioselective sensors as detectors in flow and sequential injection analysis systems [14-15].

Unfortunately, no electrochemical sensor can be reliably used for all types of enantiomers. The type of amperometric electrodes must be selected in concordance with the complexity of the enantiomer structure. The principle of enantiomer recognition using amperometric electrode is catalyst selectivity.

3.2 Design of amperometric electrodes

The design of the amperometric electrode is very important for enantioselective high throughput screening of enantiomers because the reliability of the sensor design determines the quality of the analytical information. Solid membrane electrodes are preferred over liquid membrane electrodes because they can easily be used for environmental, food, and clinical analysis as well as for *in vivo* analysis. Amperometric electrodes design is based on different types of matrices such as molecular imprinting polymers [16], composite polymers [17], sol gel [18-19] and in particular for enantioanalysis, carbon paste [8, 20] and diamond paste [21-22]. Amperometric electrodes based on carbon or diamond paste are the most reliable for enantioanalysis [15].

3.2.1 Design of carbon paste based amperometric electrodes

This design is based on physical mixture of graphite powder with oil (paraffin or nujol oil). These electrodes can be used as they are, or modified with enzyme (amperometric biosensor), or antibody (amperometric immunosensor). The most reproducible design for graphite paste electrode was recommended by Stefan et al. [23]. The carbon paste electrode was prepared by mixing 0.1 g of graphite powder with 20 μL paraffin oil. The

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paste was filled then into a plastic pipette tip. The diameter of the sensing part was 3.0 mm. Electrical contact was made by inserting a silver wire into the carbon paste. Before each use, the electrode surface was smoothed by polishing with alumina paper (polishing strips 30144-001, Orion). When not in use the paste was stored at room temperature.

3.2.2 Design of diamond paste based amperometric electrodes

Diamond has some inherent electrochemical properties such as hardness, high thermal conductivity, high electrical resistance, high electron and hole mobilities, variable conductivity via doping and electrode geometry pattern using selective growth methods. Most of electrochemical studies are performed with diamond film based electrodes prepared by chemical vapor deposition (CVD). Synthetic films often possess a polycrystalline and textured microstructure with a small volume fraction of non-diamond impurity [24].

The application of synthetic diamonds, primarily in the area of active electronic components, did not realize to a great extent because of the poor structural quality of most CVD grown films. The resistively of polycrystalline diamond thin-films made by CVD can be decreased by doping diamond with boron [25]. Resistivity as low as 0.01 Ω-cm has been reported for boron doped films, rendering them conductive enough for electrochemical studies.

Boron doped diamond (BDD) exhibits several superior electrochemical properties that are significantly different from those of other carbon allotropes, which have been widely

used as electrode materials. Its attractive features include a wide electrochemical potential window in aqueous media, very low capacitance and extreme electrochemical stability.

In the crystal structure of diamond, each carbon atom is tetrahedrally (Figure 3.1) surrounded by four equidistant neighbors at 154.45 pm, and the tetrahedral are arranged to give a cubic unit cell with a_o = 356.68 pm. Diamond has the following physical properties: a low density (d = 3.51 g/cm^3); it is the hardest material and the best conductor of heat ever known; transparent to visible light and IR and UV radiation.

The advances in single-crystal diamond have enabled the development of a wide range of monocrystalline diamond products to meet the requirements of different applications [26]. Room temperature drift mobilities of 4500 cm^2/Vs for electrons and 3800 cm^2/Vs for holes have been measured in high purity single-crystal diamond [19]. These values were determined by using the time-of-flight technique on thick, intrinsic freestanding diamond plates and were verified by current-voltage measurements on p-i junction diodes [27].

Monocrystalline diamond paste amperometric electrodes exhibit several superior electrochemical properties if one compares with carbon paste and glassy carbon electrodes [22]. Wide working concentration range and relatively low limits of detection (10^{-8} to 10^{-11} mol/L) obtained by using amperometric electrodes based on diamond paste proved their high sensitivity [22, 28].

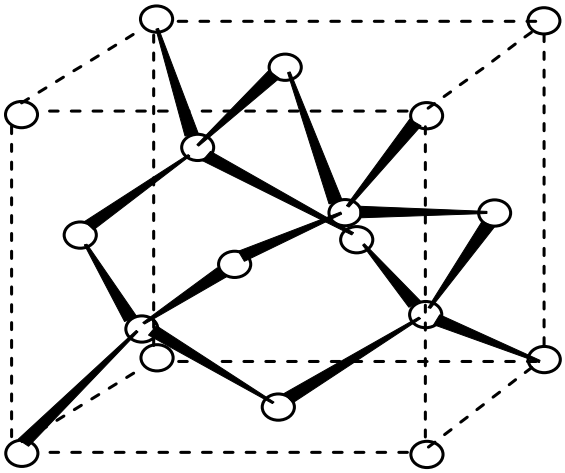

Figure 3.1 Structure of diamond showing the tetrahedral coordination of C.

Three types of monocrystalline diamond powder were used for the design of the diamond paste electrodes used for amperometric sensors, named: natural diamond (1 μm), synthetic diamond (50 μm), and synthetic diamond (1 μm). Amperometric electrodes were prepared by mixing 100 mg of diamond powder with 20 μL paraffin oil. Some of the diamond paste was then filled into a plastic pipette peak. The diameter of the sensing part was approximately 2.3 mm. Electric contact was achieved by inserting a silver wire (0.5 mm in diameter) into the diamond paste. Before each use, the electrode surface was smoothed by polishing with alumina foils (polishing strips 30144-001, Orion). All sensors were stored at room temperature, when not in use.

3.3 Response characteristics of amperometric electrodes

An amperometric electrode is based on the measurement of a developed current in concordance to the controlled applied potential between the working electrode and reference electrode. There are two ways to develop a current in electrochemical cells:

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(i) applying a step potential and recording the developed current as a function of time (chronoamperometry);

(ii) applying variable forms of potentials (e.g., triangle potential wave form (cyclic voltammetry), square voltage pulses of constant amplitude with linear ramp (differential pulse voltammetry).

The functional relation between the intensity of the current (I), measured at the certain potential (E) and concentration of the analyte (C) is given by the linear correlation;

$$I = f(c) \tag{3.1}$$

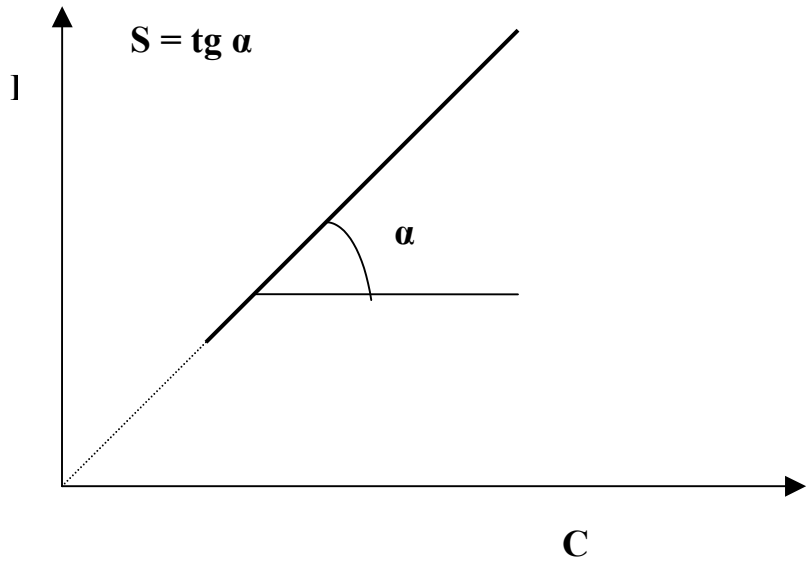

Figure 3.2 Electrode function for amperometric electrodes

The response output of the intensity of current and concentration of the analyte is shown in Figure 3.2. The current of an amperometric electrode is related to the mass transfer of a substrate to the electrode as described by the following equation [29]:

$$i_t = nFAD_E\left(\frac{\partial C_E}{\partial x}\right)_{x=0,t} \tag{3.2}$$

where i_t = current at time t, A; n = number of electrons transferred per molecule; F = Faraday's constant, 96500 C/equiv; A = electrode area, cm^2; C_E = concentration of E, mol/cm^3; D_E = diffusion coefficient of E cm^2/s; t = time, s; X = distance, cm

Thus the current is directly proportional to the slope of the concentration-distance profile at the electrode surface, i.e., to $(\frac{\partial C_E}{\partial x})_{x=0,t}$. This equation can be expressed in terms of the Nernst diffusion layer concept by simply approximating ∂x by δ and ∂C by $C_E - C_E^s$.

$$i_t = nFAD_E \frac{C_E - C_E^s}{\delta} \tag{3.3}$$

The current is determined by the slope of the profile for species E. As the slope increases due to decreasing C_E^s, the current increases. A limiting cathodic current, i_{lc}, is reached when the surface concentration C_E^s becomes effectively zero. Substitution of $C_E^s = 0$ into the previous equation gives the equation of the limiting current [29]:

$$i_{lc} = \frac{nFAD_E C_E}{\delta} \tag{3.4}$$

3.3.1 Response (slope) of the electrode

One of the most important response characteristics for amperometric electrodes is the slope considered for the linear concentration range. The slope is a measure of the electrode sensitivity, where a minimum value of 100 nA/decade of concentration is requested for amperometric electrodes to be considered for bioanalysis. There are two ways to determine the slope of the amperometric electrodes:

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(i) from the graphical method, the slope of the amperometric electrodes is equal to the tangent of the angle formed between the calibration line and the concentration or activity axis. Slope can be represented by the following equation:

$$S = tg\alpha \tag{3.5}$$

where S is the slope of the amperometric electrode; tg is the tangent function; α is the angle between the calibration line and concentration axis (Figure 3.2);

(ii) the slope can be determined by linear regression method (which is more recommended).

3.3.2 Limit of detection

The limit of detection is defined by IUPAC as the concentration at which, under specified conditions, the intensity of the current I, deviates from the average value by a multiple of the standard error of a single measurement of the intensity of the current in this region [30]. An experimental determination of the limit of detection is recommended for amperometric electrodes rather than a statistical treatment of the experimental data obtained for the calibration of the electrodes [15]. The amperometric electrode limit of detection can be considered as the concentration:

(i) where the limiting current intensity value is equal with the one obtained for the buffer solution;

(ii) where the limiting current intensity value is double the one obtained for the buffer solution;

(iii) the concentration below the one where the intensity of the limiting current remains constant [31].

Due to the high sensitivity of the amperometric electrodes, their limit of detection can be of pmol/L magnitude order, or even less.

3.3.3 Linear concentration range

The linear concentration range can be determined according to electrode linearity response in the plot of current versus the concentration of the enantiomer. The linear concentration range can be defined by the range of concentration of the substrate over which the sensitivity of the electrode is constant with a specific variation (usually ± 5%). This response characteristic is very important because the activity of concentration of all the solutions to be measured must lie within the linear concentration range. The reproducibility of the linear range is influenced by the working conditions of the amperometric electrode, like stirring rate, composition of the solution, pH of the solution, composition of the solution where the electrode was exposed before the measurement, the temperature, and the preconditioning of the amperometric electrode [31].

3.3.4 pH range

The pH is an important characteristic of the response of amperometric electrodes. The pH can influence the formation of protonated and unprotonated species of the same enantiomer. It can favor certain redox processes at the electrode. The relation between the intensity of the limited current and the pH variation must be determined for amperometric electrodes. It is very important to work at a certain pH value for all the solution to be

determined and to use a certain composition for the buffer. For some electrode a range where the intensity of the limited current does not depend on the pH value was found. For these electrodes, it is necessary to control the pH and buffer all the standard and sample solutions in the suitable pH range. In the case of the electrodes that did not have any range where the intensity is independent of the pH, special care must be taken to the buffering of each solution.

3.3.5 Ionic strength and activity coefficients

The accuracy of amperometric electrode measurements is influenced by the ionic strength and activity coefficients. It is necessary to work at the same ionic strength otherwise another error may occur due to the variations of the activity coefficients of the ions in the solution [32]. From physiological point of view, the activity is the relevant quantity because equilibrium between different phases depends on activity and not on concentration alone.

3.3.6 Response time

IUPAC defines the response time as the time which elapses between the instant when the electrodes of the amperometric cells are brought into contact with a sample solution, or at which the activity of the ion of interest in solution is changed, and the first instant at which the slope of the working electrode becomes equal to a limiting value selected on the basis of the experimental conditions and/or requirements concerning the accuracy [30]. The response time of amperometric electrodes is a function of the kinetics of the processes that takes place at the surface of the electrode. It increases with decrease in

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concentration of the substrate that has to be determined. Amperometric electrodes with short response times are preferred for the use in bioanalysis. The response time is influenced by the presence of other interferences due to the competitive equilibrium that takes place at the electrode interface.

3.3.7 The influence of temperature on the response of the electrode

The response of the electrode is highly influenced by the temperature. The increase of the temperature will favor the kinetics and the thermodynamics of the processes that take place at the electrode surface and as a result the slope will increase. During the measurements of standard and sample solution using amperometric electrodes, the temperature must be maintained constant. The recommended temperature for electrodes characterization is 25^{o}C.

3.4 Selectivity of the amperometric electrodes

Amperometric electrodes are affected by two classes of interfering substances: electrode/electrochemical interferences include substances whose response is similar to the molecule being determined or electrolyte present at high concentration level; chemical interferences interacts with the ion/molecule being determined, so as to decrease its activity or apparent concentration (e.g., H^+, HO^-), or substances that interact with the membrane surface. The selectivity degree of amperometric electrodes is given by the values of the amperometric selectivity coefficients, $K_{i,j}^{amp}$, respectively as follows

(i) if a magnitude order is higher than 10^{-3}, the ion tested for interference does interfere;

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(ii) if a magnitude order is 10^{-3}, the ion tested for interference is not a strong interfering species;

(iii) if a magnitude order is less than 10^{-3}, the ion tested for inferences does not interfere.

The following equation was proposed for the correlation of the total current response and amperometric selectivity coefficients by Wang [33].

$$I_t = b + \left(c_i + \sum_{j=1}^{N} K_{i,j}^{amp} c_j \right) \tag{3.6}$$

where I_t is the total current response, c_i and c_j are the concentration of the main and interfering species, respectively, N is the number of interfering species and b is given by the following equation:

$$b = \frac{n_i AFD_i}{\delta_i} \tag{3.7}$$

where n_i is the number of electrons transferred per mole of analyte, A is the surface of the electrode (cm^2), F is Faraday number (96500 C), D_i is the diffusion coefficient of the analyte (cm^2/s), and δ_i is the thickness of diffusion layer (cm). There are two methods for the determination of amperometric selectivity coefficients: mixed and separate solutions methods.

3.4.1. Mixed solution method

The mixed solution method is recommended for the determination of amperometric selectivity coefficients because it shows the actual conditions under which the electrode is used. In this method the current recorded for a solution that contains only the main enantiomer is compared with the total current given by the equation proposed by Wang

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for a solution containing both the enantiomer and the interfering species [33]. The selectivity coefficient, $K_{i,j}^{amp}$, can be calculated using the following equation:

$$K_{i,j}^{amp} = \left[\frac{\Delta I_t}{\Delta I_i} - 1\right] x \frac{c_i}{c_j} \qquad (3.8)$$

where $\Delta I_t = I_t - I_b$ and $\Delta_i = I_i - I_b$, I_t = the current recorded for the mixed solution; I_b = the current recorded for the blank solution; I_i = the current recorded for the solution containing only the enantiomer for which the electrode was designed; c_i = the concentration of the main species; c_j= the concentration of the interfering species.

3.4.2. Separate solution method

The separate solution method is based on recording the intensity of current separately, for the solution containing the enantiomer and the interfering species solution. Both recorded currents and concentrations of main and interfering species are compared with each other to calculate the amperometric selectivity coefficient, $K_{i,j}^{amp}$ [34]:

$$K_{i,j}^{amp} = \frac{\frac{\Delta I_j}{C_j}}{\frac{\Delta I_i}{C_i}} \qquad (3.9)$$

where $\Delta I_j = I_j - I_b$ and $\Delta_i = I_i - I_b$; I_j = the current recorded for the main species; I_i = the current recorded for the interfering species; I_b = the current recorded for the blank solution; c_i = the concentration of the main species; c_j= the concentration of the interfering species.

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3.5 Direct amperometric method

Direct amperometry is a very simple method to be applied for the direct analysis of enantiomers in their sample solutions without any or minmum prior preparations. To obtain the best precision of measurement it is necessary to calibrate the working electrode just before the assay of sample. This can be performed by standard addition method. Standard aliquots of the analyte are added to the solution and plotting steady-state response possibly corrected for the blank signal versus the enantiomer concentration [35]. To avoid any error in measurements, pH and the ionic strength of the samples must be adjusted to the same values as the solutions used for calibration. The values of current intensity obtained for the samples are interpolated on the calibration plot and the unknown concentrations are determined.

3.6. Differential pulse voltammetry

Voltammetry comprises a group of electroanalytical methods in which information about the analyte is derived from the measurement of current as a function of applied potential under conditions that encourage polarization of an indicator electrode. These methods are classified according to the type of potential (AC, DC) and form of applied potential with time (linear scan, differential pulse, square wave, triangle …etc.). Polarography is a particular case of voltammetry that differs in the respect that the working electrode takes the form of a dropping mercury electrode. A plot of current flowing in the cell as a function of the applied potential is called a voltammogram. A residual current flows in the cell is developed at small applied potentials which is caused by the reduction of trace impurities in the sample solution. An electroactive species are initiated by reduction

when a decomposition potential is applied, a developed current (limiting current) increases with applied potential until it levels off a limiting value. The difference between the limiting current and the residual current is known as the diffusion current, i_d. Voltammetry is useful for quantitative analysis (diffusion current is proportional to analyte concentration and the height of a voltammetric wave tells how much analyte is present) and qualitative analysis of an unknown substance (enantiomer) using the half-wave potential $E_{½}$ of the particular electroactive species to compare with known values of $E_{½}$.

Diffusion current is directly proportional to the concentration of electroactive species. If the applied potential exceeds the decomposition potential of the electroactive species, its concentration at the surface of the electrode is immediately dimensioned and a concentration gradient is established and more of that species diffuses from the bulk solution to the electrode surface (Fick's law of diffusion). The resulting current flow is proportional to the rate of diffusion, which in turns is determined by the concentration gradient,

$$i = k\,(C\text{-}C_o) \qquad (3.10)$$

where C and C_o are the concentrations of the electroactive species in the bulk solution and at the surface of the working electrode, respectively. The current flowing in the cell reaches the limiting value by progressively increasing the applied potential; reduction occurs more rapidly and C_o eventually becomes virtually zero:

$$I_d = kC \qquad (3.11)$$

Half-wave potential of the electroactive species are given by the following equation:

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$$E_{1/2} = E^{o} - \frac{0.059V}{n}\log\frac{k}{k_r} - E_{reference} \qquad (3.12)$$

where E^{o} is the standard electrode potential; $E_{1/2}$ is the half wave-potential, $E_{reference}$ is the potential of the reference electrode and n is the number of electrons changed per mole of analyte; k and k_r are proportionality constants relating cell current to the rates of diffusion of oxidized and reduced forms of the electroactive species.

Differential pulse polarography is the most used technique in analysis that differs from normal pulse in that after the potential pulse, the potential does not return to a constant base value, Figure 3.4. The potential pulse is superimposed on a conventional rising linear dc voltage ramp. The pulse is also imposed for about 60 ms near the end of the drop lifetime when the growth of the drop almost ceased.

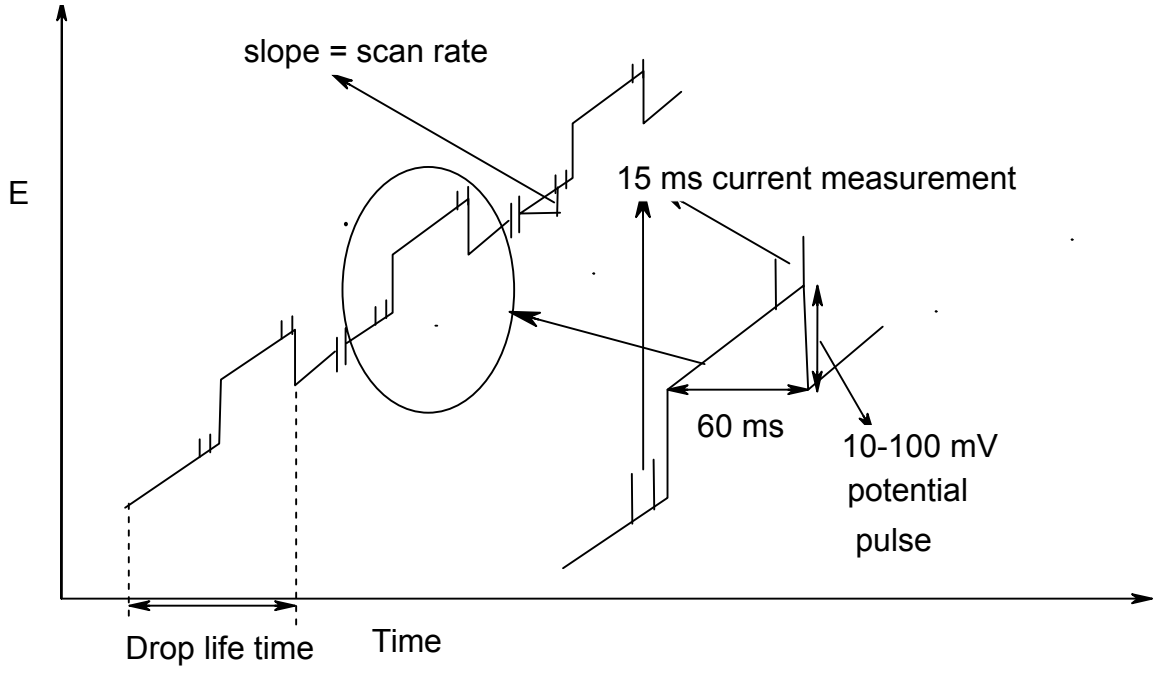

Figure 3.4 The profile of the potential pulse and current measurement in differential pulse polarography [36].

Current is measured once before the pulse and again for the last 15 ms of the pulse. The polarograph subtracts the first current from the second and plots this difference versus the applied potential (measured just before the voltage pulse). The resulting differential pulse polarogram is nearly the derivative for a direct current polarogram as shown in Figure 3.5. The two current represents the current at two potential values separated about 10-100 mV (the pulse amplitude). This technique produces a peak with highest current signal at roughly the half wave potential of the classical dc and normal pulse polarography.

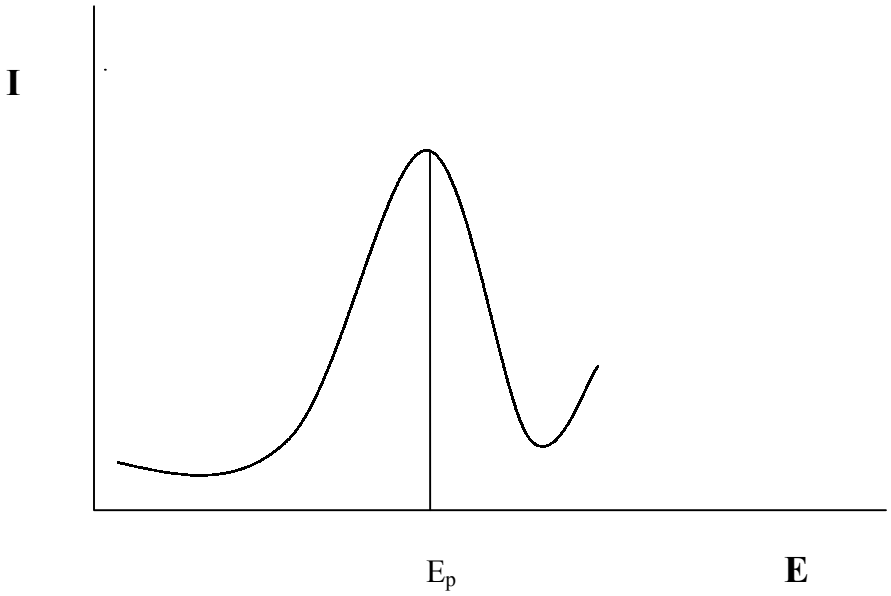

Figure 3.5 Voltamograms for a differential pulse polarography

The potential of the peak E_p is indicative of which species is involved. The concentration of the species controls the current in case the redox mechanism is diffusion controlled. The area under peak and its height are proportional to the concentration of species under reduction (or oxidation) mechanism.

The enhanced sensitivity of differential pulse polarography relative to direct current polarography is due to an increase in faradaic current and a decrease in condenser current. Differential pulse polarography provides better resolution of adjacent waves than does normal pulse polarography, because it is easier to distinguish partially overlapping derivative maxima than partially overlapping polarographic waves. For quantitative analysis, the current at the height of a peak is measured in differential pulse polarogram and subtracted from the residual current at the same potential measured in the absence of analyte. The differential pulse technique has typical limits of detection 10^{-8}-10^{-6} mol/L magnitude order for the normal pulse technique while for the classical dc it would be only about 10^{-4} mol/L magnitude order.

Differential pulse polarography can increase the sensitivity if the signal has been reduced by depletion because it reduce the noise and show better resolution. At low concentration levels the favorable signal to noise ratio of differential pulse polarography gives well-defined peaks where no dc response can be recovered. The current measurement is only taken in very brief pulses at the end of the drop lifetime, although depletion occurs throughout the drop lifetime. A capacitive current will be reduced to its almost constant and lowest level at the end of the drop lifetime. The capacitive and nonfaradaic currents have very little effect due to the potential jump 10-100mV, while a small potential jump will cause a large Faradaic current change specially at the peak potential. Thus the differential pulse mode allows the maximum differentiation of the analytical signals from the background ones.

Differential pulse voltammetry output signal is influenced by many factors like potential pulse amplitude and scan rate. So these variables must be selected carefully to have a good peak resolution that is related directly to the concentration of the enantiomer.

3.6.1. Potential pulse amplitude

Potential pulse amplitude was selected in the range of 10-100mV. The fixed pulse amplitude (usually 50 mV) is the only choice to be made before the analysis. Parry and Osteryoung have derived an equation of the peak current I_p for a totally thermodynamically reversible electrode process controlled by diffusion:

$$I_p = \frac{n^2 F^2 Ac}{4RT}\left(\frac{D}{\pi t}\right)\Delta E \qquad (3.13)$$

where ΔE is the amplitude of the potential pulse, n is the number of electrons changed per mole of analyte, A is the area under the peak, c is the concentration, t is the drop lifetime, R is gas constant, T is the temperature and F is Faraday's number. The equation shows that the peak height is proportional to the concentration and the potential pulse amplitude. The higher potential pulse amplitude produces the greater sensitivity, but increasing the potential pulse amplitude results in peaks broadening and loss of resolution. Two close lying peaks will not be resolved unless the pulse amplitude is significantly smaller than the separation in the two peak potentials. The choice of potential pulse amplitude must be a compromise between a higher value for increased sensitivity and lower amplitude for increased resolution. This is particularly true for thermodynamically irreversible process that produces broader, lower and less well formed peaks than do reversible processes.

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3.6.2. The scan rate

The scan rate (mV s^{-1}) plays an important role in the peak resolution. The suitable scan rate must compromise between the adequate resolution and required time for analysis. High scan rate will be too coarse for adequate resolution and the slowest scan rate gives the best resolution results with longer time of analysis.

3.6.3 Peak area and peak height

Peak height and peak area are directly proportional to the current of electrolysis and hence to the concentration of analytical species to be determined. The area under peak will remain constant for the determination of the same concentration of the enantiomer; in most of the cases even though the adsorption of other inactive species can alter the reversibility and electrode kinetics of the process producing sometimes huge change in the shape of the peak. Because DPV is particularly susceptible to surface active phenomena, the utilization of diamond as electroactive material in the design of amperometric electrodes minimizes the contamination of the surface by adsorptive processes favoring a long term of utilization of the electrode and a higher sensitivity of the measurement.

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Chapter 4

Amperometric biosensors for enantioanalysis

4.1. Introduction

A biosensor is a device that incorporates a biological sensing element connected to a transducer. Possible combinations of sensing element and transducer are summarized in Table 4.1 [1]. The first biosensor was reported in the early 1960's and comprised enzyme immobilized to an oxygen electrode. Continued development of this kind of biosensor led to the commercialization of various devices for different applications e.g., the measurement of glucose in blood.

Table 4.1 Biological elements and transducers used in biosensor's design

Biological elements	Transducers
Organisms Tissues Cells Organelles Membranes Enzymes Enzymes components Receptors Antibodies Nucleic acids Organic molecules	Potentiometric Amperometric Conductometric Impedimetric Optical Calorimetric Acoustic Mechanical Molecular electronic

In previous works different amperometric biosensors were constructed for the determination of chemical species regardless their stereochemical configurations (L- or D-enantiomer).

The evolution concerning the design of enantioselective amperometric biosensors made their utilization a reliable alternative for chromatographic techniques in high throughput enantioselective screening analysis [2-5].

The principle of enantiorecognition used by biosensors is catalyst selectivity. Enzymes are used to catalyze only the reaction of one of the enantiomers. The enzyme can be coupled with different types of electrochemical transducers such as field effect transistor, potentiometric and amperometric. The most used enzymes pairs are L-amino acid oxidase (L-AAOD) and D-amino acid oxidase (D-AAOD) for the assay of L- and D-enantiomers of amino acids.

4.2. Design of amperometric biosensors

The reliability of the response characteristics and the analytical information obtained by using amperometric biosensors is correlated with the design of the biosensor. Different types of membranes have been utilized with amperometric sensors. Distribution of the enzyme in the matrix plays an important role in sensor design and response characteristics. The quality of the matrix of the membrane and the type of immobilization of the enzyme recorded several improvements in sensor design [6-12]. New generations of biosensors are continuously being developed (e.g., amperometric biosensor) based on the direct enzymatic regeneration at the electrode surface [13].

The most reliable matrices used for enantioselective amperometric biosensors are carbon paste and diamond paste. The type of sensor and utilized enzyme must be selected in

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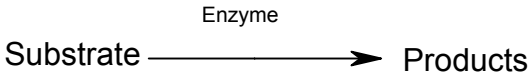
concordance with enantiomer configuration (L- or D-enantiomer). The biochemical reaction is very selective and sensitive: due to the presence of immobilized enzyme, the substrate (enantiomer) is transferred under the action of the enzyme into product that will be detected by the transducer:



Biosensors design is related to two main parts (Figure 4.1):

1. the immobilization techniques for the sensing medium (enzymes, cells, or the biological active substances).
2. the type of transducer that will be used.

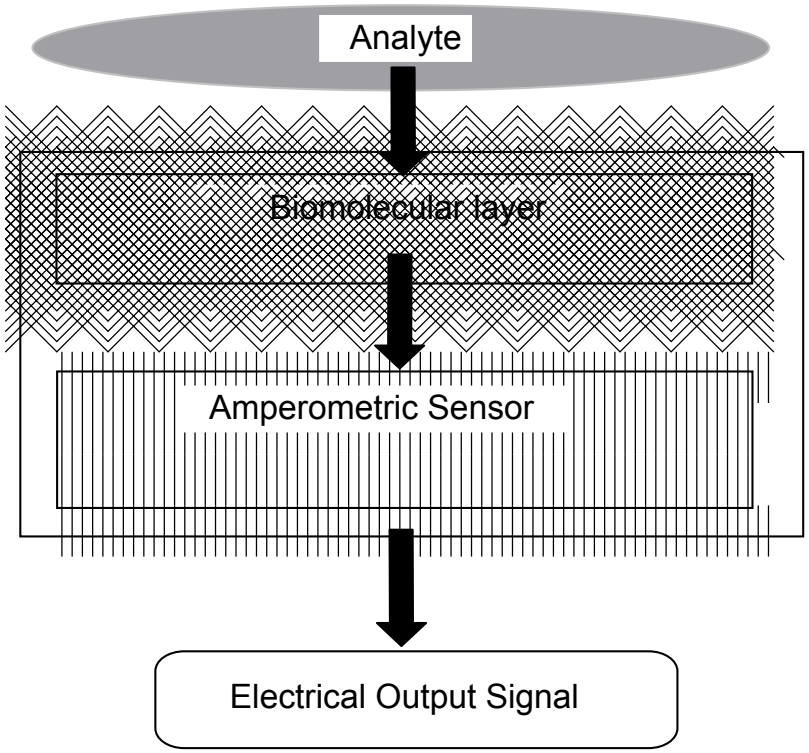


Figure 4.1 Amperometric biosensor design.

The biomolecular layer consists of a biological sensing substance (e.g., enzyme, antibodies, receptors, etc.) impregnated in a matrix (PVC, imprinting polymers, carbon paste, diamond paste). The fast development evolution of transducer influenced the improvement of biosensor design in terms of sensitivity and reliability. The type of transducer must be selected in biosensor design according to the type and quantity of the products formed in the enzymatic reaction, e.g.,

(i) when H^+ is forming, a pH electrode is used as transducer,

(ii) when H_2O_2 is forming, an amperometric transducer for determination of H_2O_2 is recommended.

(iii) when carbon dioxide or ammonia are formed, the gas sensor is utilized as transducer.

The sensitivity of the enzymatic reaction must be correlated to the sensitivity of the transducer or biosensor's design, the best electrochemical transducer is amperometric electrode [4].

The nature of the matrix, as well film technology will influence the behavior of the enzyme because the biotransformation equilibria of the substrate are taking place at the membrane-solution interface. Carbon [4] and diamond [14] pastes proved to be the best matrices for enzymes immobilization. To immobilize an enzyme, three facts must be taken into account: the enzyme activity and its stability with temperature, type of the matrix where the enzyme will be immobilized and finally the possible types of immobilization. These pastes are prepared by mixing graphite powder or diamond

powder with nujol or paraffin oil. There are several principles to add the sensing part in the biosensor: membrane entrapment, physical adsorption, matrix entrapment and covalent bonding (Figure 4.2). A covalent bond of the enzyme to the matrix will decrease its activity, therefore it is necessary to check the enzyme activity after immobilization. This type of immobilization will also decrease the sensitivity of the electrode and its lifetime.

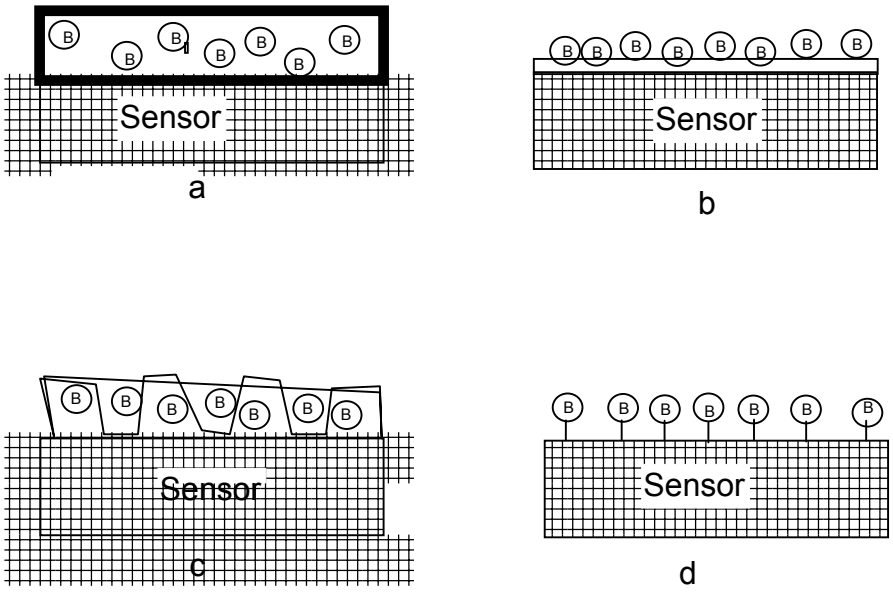


Figure 4.2 Principles of biosensors (a) imembrane entrapment; (b) physical adoption; (c) matrix entrapment and (d) covalent bonding

4.2.1 Physical immobilization

Physical immobilization is based on adsorption, cross linking techniques and electrostatic entrapment. Physical immobilization is including the mixing of biological substance into plastic membrane or solid pastes (e.g., carbon and diamond paste).

4.2.1.1 Biosensors based on plastic membranes

Enzyme adsorption is preferred for the design of plastic membranes based amperometric biosensors. Plastic based biofilms can be prepared by sandwiching a porous cellulose nitrate membrane in a sterile syringe filer holder with a certain volume of the diluted cell broth adsorbed by the membrane by suction [15].

Another technique of physical mobilization on a plastic membrane is the cross-linking technique [16]. The tip of the transducer was cleaned with acetone, then immersed in a solution of cellulose acetate and let to dry at room temperature for two hours. At the end of the procedure, a thin film of cellulose acetate covered the probe tip. The second step was the preparation of an aqueous enzyme solution consisting of 10μL of enzyme 10%, 10μL bovine serum albumine (BSA) 10%, and 6μL glutaraldehyde 5%. Finally the probe is dipped in the solution and left to dry for two hours at room temperature.

4.2.1. 2 Biosensors based on carbon and diamond pastes

One of the most reliable techniques for enzyme immobilization proved to be the mixture of enzymatic solution with the carbon [17-20] and diamond pastes [21]. The utilization of carbon or diamond pastes for physical immobilization of enzymes has the advantages of a reliable construction of the sensor and of ensuring high activity for enzyme. Carbon or diamond paste based biosensor is shown in figure 4.3.

100 mg of carbon or diamond powder is mixed with 20 μL paraffin oil. The plain paste is filled in a plastic tip leaving an empty space of 3-4 mm in the top part to be filled with

modified carbon/diamond paste (containing the different enzymes). 100µL of enzyme solution (1 mg enzyme in the optimum buffer of a certain pH) are mixed with 100 mg graphite or diamond powder and 40µL paraffin oil of IR purity. The diameters of all biosensors were 3 mm. Electric contacts were obtained by inserting silver wires into the diamond paste. The biosensors tips were gently rubbed on fine abrasive paper to produce a flat surface. The surface of the biosensors were wetted with de-ionized water and then polished with an alumina paper (polished strips 30144-011, Orion) before use. The biosensors were stored dry at 4^0C, when not in use. 0.1 mol/L KCl solution was used as electrolyte for these biosensors [2,14].

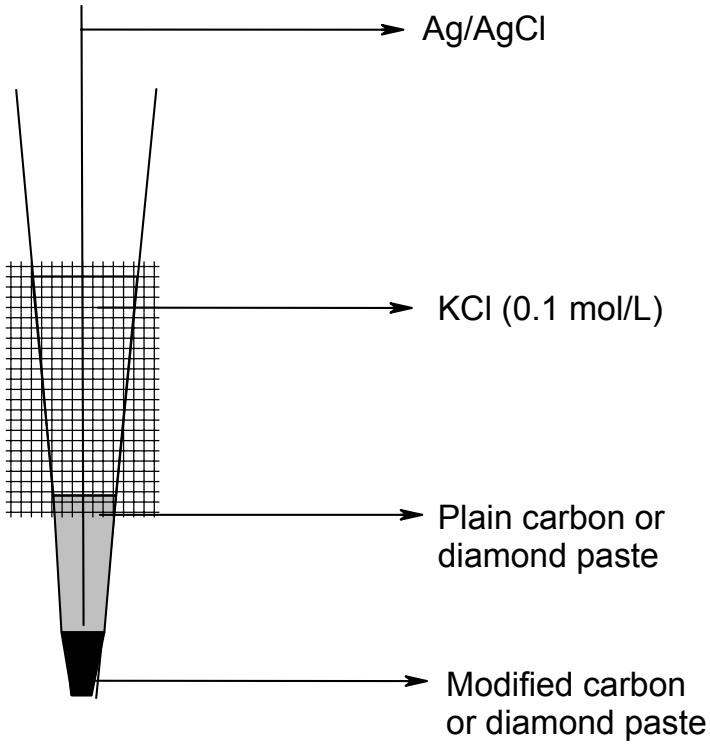


Figure 4.3 Design of carbon or diamond paste based biosensor

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4.2. 2 Chemical immobilization

4.2.2.1 Plastic based amperometric biosensors

Chemical immobilization is based on the covalent bonding of the enzyme to a matrix. This type of immobilization may have decrease as a result of the enzyme activity as well as the sensitivity of the electrodes. Sometimes, the covalent bond of the enzyme to the matrix is done at a temperature higher than the temperature of the body (37^{o}) and part of the activity of enzyme is lost.

The most utilized matrix for covalent immobilization of an enzyme is nylon that must have special treatment before enzyme bounding as follows: the nylon meshes need to be cut into disks that are dipped in methanol, rinsed with water and dried in an air steam prior to use. For enzyme immobilization [21] the following solution were added to each disk: 2.0 µL glutaraldehyde (2.5%), 2.5 µL bovine serum albumin (BSA) (1% w/v), and 5.0 µL of enzyme (1 mg enzyme dissolved in a certain buffer). The mixture was homogenized on the surface of the disk. Gelification of glutaraldehyde and the protein was carried out at room temperature for 30 min. Disk was immersed in a certain buffer solution to inactivate the remaining carboxyaldehyde groups.

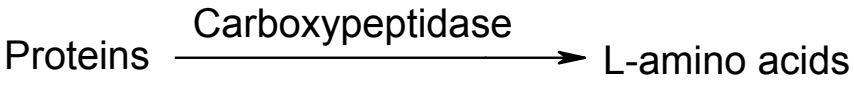
Acrylate polymer was recommended as plastic matrix for enzyme immobilization. A copolymer of 25% glycidyl and 75% methacrylate (25G75M) was chosen as the basic material to provide a polymer with a reactive epoxide group for immobilization. The responsible group of enzyme immobilization is the glycidyl group. 2 mL of 1 mg/mL enzyme were incubated in 100 mg of the copolymer [22].

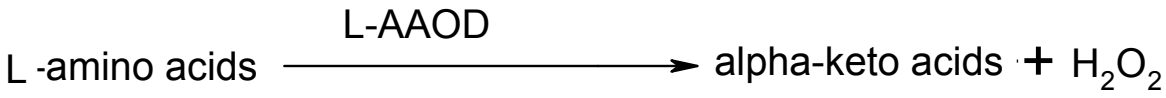
4.2.2.2 Carbon paste based amperometric biosensors

The chemical modified carbon paste was prepared as follows: 0.4 g graphite powder were mixed with 400 µL (0.03 mol/L) solution of 1-ethyl-3-3-(3-dimethylaminopropyl) carbodiimid (EDAC) and heated to 700^{o}C for 60 s in a muffle furnace. After cooling down to ambient temperature, 10 µL polyethylanimine (PEI) and 10 µL of glutaraldehyde were added. 100 µL solution containing 1 mg enzyme was allowed to react at 4^{o}C for 2 hours and then it was mixed with 40 µL of paraffin oil to produce the modified pastes [23].

The design of multienzymatic sensors improved the reliability of analytical information by increasing the sensitivity of biosensors. The second enzyme is added because:

1. the products obtained in the first enzymatic reaction cannot be determined directly by a transducer, e.g., it is necessary to use a bienzymatic sensor for the determination of proteins, when the following reactions are taking place under the influence of carboxypeptidase and L-amino acid oxidase (L-AAOD) [24]:





2. the selectivity and sensitivity of the reaction of product recognition by the transducer is improved, e.g., Horse radish peroxidase (HRP) is added to catalyze the redox transformation of H_2O_2.

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4.3. Response characteristics of amperometric biosensors

Amperometric biosensors function is based on the direct relation of a recorded current, I and the enantiomer concentration. The current is developed due to the electron or mass transfer under the influences of the controlled applied potential between the working and reference electrodes. All the response characteristics (response of the electrode, limit of detection, linear concentration range, ionic strength and activity coefficients, response time and the temperature) are the same like for amperometric electrodes in chapter 3. In this section, particular response characteristics of the biosensors such as pH range, life time (t_L) and Michael-Menten constant (K_M) will be discussed.

4.3.1 pH range

The pH value plays the main role in the biochemical reaction at the electrode-solution interface. The enzymes are working in a specific pH range. Accordingly, it is very important to optimize the pH to find the suitable enzyme catalytic activity of the substrate reaction.

4.3.2. Life time (t_L)

Life time can be defined as the storage or operation time necessary for the sensitivity, with the linear concentration range, to decrease by a factor of 10% (t_{L10}) or 50% (t_{L50}) [25]. The mode of assessment of lifetime should be specified by reference to initial sensitivity, upper limit of detection of the linear concentration range for the calibration curves, accuracy and reproducibility. The biosensor should have a prolonged lifetime; it should be easily replaceable if necessary and not be expensive if it has to be replaced. In

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cases where the sensors to be partially inserted in the subcutaneous tissue, in a needle-like fashion, a lifetime of several days if not weeks, could be accepted [1]. It is obvious, a totally implantable device would require a much longer lifetime.

4.3.3. Michaelis-Menten constant (K_M)

Assume a scheme involves a single substrate, S, which combines with enzyme, E, to give the intermediate enzyme-substrate complex, ES. This complex then undergoes a reaction to produce the product, P, the overall scheme being summarized by:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

where k_1 and k_{-1} are the forward and backward rate constants for complex formation and k_2 is the rate constant for complex decomposition into product. The Michaelis-Menten constant (K_M) can be represented by the following equation [11]:

$$K_M = \frac{k_{-1} + k_2}{k_1} \tag{4.1}$$

where k_1 and k_{-1} are the forward and backward rate constants for complex formation and k_2 is the rate constant for complex decomposition into product. Michaelis-Menten constant (K_M) represents the analyte concentration yielding a response equal to half of its maximum value for the current or potential measured for infinite analyte concentration [25]. When the apparent K_M is much larger than its value for soluble enzyme, it means either that a significant substrate diffusion barrier is present between the sample and reaction layer, or that the rate of reaction to the co-substrate with the enzyme is increased. As for enzyme solution kinetics, the apparent K_M is usually computed using Lineweaver-

Burk reciprocal plots, $\frac{1}{\Delta I}$ versus $\frac{1}{c}$, where $\Delta I = I - I_b$, I and I_b are the value of the current recorded for the analyte and the blank solutions respectively, and c is the concentration of the analyte.

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4.4. Selectivity of the amperometric biosensors and immunosensors.

Selectivity is related to the accuracy and precision of enantiomer measurements in presence of the interfering species. Enzymes are used in amperometric biosensors to catalyze only the reaction of one of the enantiomers. Accordingly, the selectivity of the biosensor is higher than for the other types of electrodes, e.g., EPME. The amperometric selectivity coefficient can be determined similar to the one for amperometric electrodes using the mixed and separate solution methods as described in chapter 3. Equations (3.7) and (3.8) may used for the calculation of the selectivity coefficient, $K_{i,j}^{amp}$, based on mixed and separate solutions method, respectively.

4.5. Chronoamperometry

Chronoamperometry is a potential step technique in which the potential of the working electrode is changed instantaneously from initial to final value and the current-time response recorded. A certain potential-time profile (Figure 4.4) is applied to the working electrode. E_1 should be a potential at which neither reduction of analyte species nor any electrode reactions occur. At zero time, the potential is instantaneously changed to a new value, E_2, at which the reduction of analyte species occurs at diffusion controlled rate. A typical current-time profile for chronoamperometry is shown in Figure 4.5. The form of

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this response can be understood by considering the concentration profiles for analyte and its reduced species (products) during the measurement.

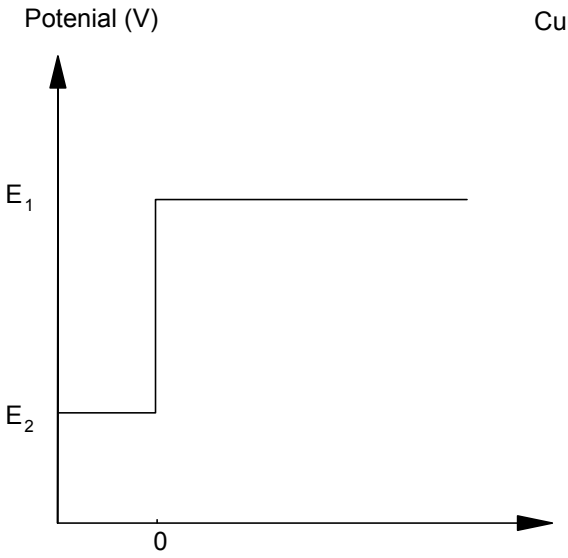

Figure 4.4 Potential-time profile

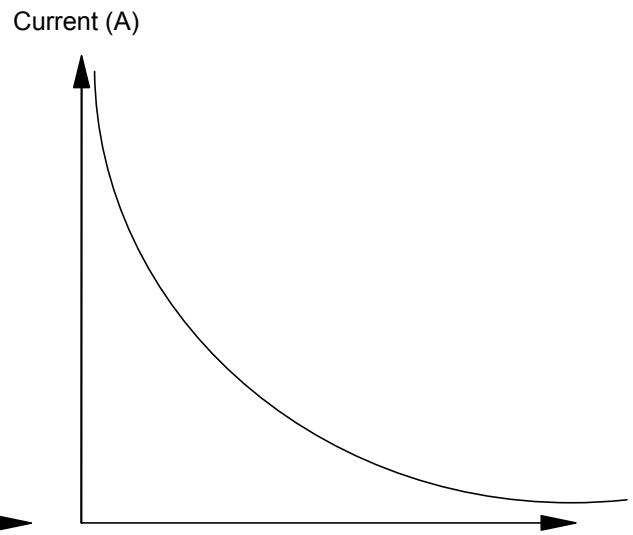

Figure 4.5 Current-time response

If the potential is stepped from a value significantly positive of the redox potential of the couple to one sufficiently negative, then a short time after the step the concentration of analyte species will have changed from its initial value only at points very close to the electrode surface. The concentration profile will be very steep, and over the subsequent time period, diffusion will cause the concentration profiles to relax towards their steady-state by extending into solution and becoming less steep. The current will decrease with time because current is a function of the concatenation of analyte species at the electrode surface.

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4.6 Direct amperometry

The direct amperometric method is applied for analysis using amperometric biosensors, and has been discussed in Chapter 3.

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Chapter 5

Enantioanalysis of L- and D-pipecolic acid in biological samples

5.1 Introduction

Piperidino-2-carboxylic acid, more commonly known as pipecolic acid, was first identified in the ruminant animals by Onodera and Kandatsu [1]. Pipecolic acid, a cyclic imino acid, is a minor metabolite of lysine, and includes D- and L-enantiomers [2]. Lysine metabolism in mammal is known to occur via sacharopine in the liver and via pipecolic acid in the brain [3-4]. The former is considered to be the major pathway and the latter the minor one for the entire body, although the latter may also be a major pathway in the brain. L-pipecolic acid may be derived from L- and D-lysine by mammalian and bacterial enzyme [5]. However, D-pipecolic acid is considered to originate from D-lysine only by bacterial D-amino acid oxidase [5]. Pipecolic acid is taken up into synaptosomes [6-7], is released form brain slices in a Ca^{2+}-dependaent manner [8], and depress excitatory synaptic transmission [9-10], suggesting that pipecolic acid serves as a modulator of synaptic transmission. In addition, pipecolic acid may facilitate GABAergic transmission by stimulating GABA release [11], inhibiting GABA uptake [12], or enhancing $GABA_A$ receptor responses [13].

L-pipecolic acid (L-2-piperidine carboxylic acid, L-PA, Figure 1.1) is an important biomedical marker for peroxisomal disorder diagnosis, as its concentration in plasma is

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increased in neonatal adrenoleukodystrofy (Zellweger syndrome) and infantile Refsum disease [14-16]. Hyperpipecolic acidemia in patients with Zelleweger syndrome is thought to be caused by a disturbance in peroxisomal pipecolic acid oxidase activity [17]. L-PA is a minor intermediate in the L-lysine catabolic pathway in the peroxisomes, where it is oxidized to α-aminoadipic acid. The assay of L-PA is considered as a supplementary test for peroxisomal disease following the analysis of very long chain fatty acids (VLCFAs), bile acids, phytanic acid, and pristanic acid in plasma. D-pipecolic acid (D-PA, Figure 1.1) is a marker of liver cirrhosis and chronic hepatic encephalopathy and it originates from the metabolism of intestinal bacteria and from dietary sources [18-21].

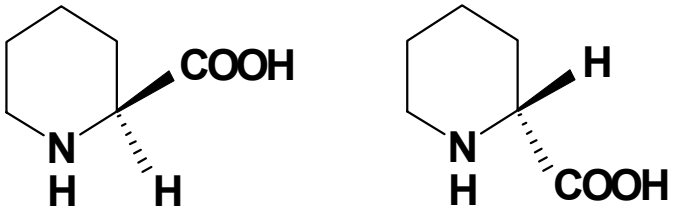

Figure 1.1 Pipecolic acid, (a) L- pipecolic acid and (b) D-pipecolic acid

Different methods were proposed for the determination of pipecolic acid [22-34]. The enantioselective analysis of pipecolic acid was performed using liquid chromatography [22-24], gas chromatography [25], capillary zone electrophoresis [26] and thin-layer chromatography [27]. Chromatographic methods, e.g., HPLC [28-30], and gas chromatography [31-34] were also used for the assay of the total concentration of L- and D-pipecolic acid.

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In this chapter, different electrochemical sensors are proposed for the enantioanalysis of L- and D-pipecolic acids: amperometric electrodes and biosensors and enantioselective, potentiometric membrane electrodes.

Amperometric electrodes and amperometric biosensors and enantioselective, potentiometric membrane electrodes represents a good alternative for the chromatographic methods for the enantioanalysis because of the combination of their sensitivity and selectivity. These electrochemical sensors are capable of direct enantioanalysis of pipecolic acid without prior or minor pretreatments of the sample. A feature of the proposed sensors is *in vivo* enantioanalysis of pipecolic acids.

Monocrystalline diamond paste based electrodes proved to be a good alternative for the classical glassy carbon and carbon paste electrodes used in differential pulse voltammetry method [35-38]. Furthermore, the utilization of these electrodes in clinical analysis is increasing the reliability of the analytical information. A monocrystalline natural diamond paste electrode is proposed for the assay of L- and D-pipecolic acids.

Three enantioselective, potentiometric membrane electrodes were proposed for the enantioanalysis of L-pipecolic acid. These EPMEs are based on carbon paste impregnated with maltodextrins as chiral selectors [39].

Enantioselective amperometric biosensors based on physical or chemical immobilization of L-amino acid oxidase (L-AAOD) and/or D-amino acid oxidase (D-AAOD) in

monocrystalline natural diamond paste or in carbon paste are proposed for the direct enantioselective analysis of L- and D-pipecolic acid in serum samples.

5.2 Reagents and chemicals

Natural diamond powder with particle size ca. 1 μm was purchased from Aldrich. Graphite powder, 1-2 μm was supplied by Aldrich (Milwaukee, WI, USA). Paraffin oil was obtained from Fluka (Buchus, Switzerland). De-ionized water from a Modulab system (continental water systems, San Antonio, TX, USA) was used for all solution preparations: L-amino acid oxidase (L-AAOD) (E.C.1.4.3.2. Type I crude Dried Venom from Crotalus adamanteus, 0.53 units/mg solid (Sigma)) solution, D-amino acid oxidase (D-AAOD)(E.C.1.4.3.3.Type I: from porcine kidney, 1.3 units/mg solid (Sigma)) solution, horseradish peroxidase (HRP)(EC 1.11.1.7 Type I from Horseradish, 87 purpurogallin units/mg solid (Sigma)) solution and solutions of L-and D-pipecolic acid (10^{-4} mol/L), respectively. L- and D-pipecolic acids were purchased from Aldrich (Milwaukee, WI, USA). Maltodextrins (DE 4.0-7.0, 13.0-17.0, 16.5-19.5) were purchased from Aldrich (Milwaukee, WI, USA). Phosphate buffer (pH=7.00) and citrate buffer (0.1mol/L, pH = 2.5) were supplied by Merck (Darmstadt, Germany).

De-ionized water from a Modulab system (Continental water systems, San Antonio, TX, USA) was used for all solution preparations. The L- and D-PA solutions were prepared from stock L- and D-PA solutions ($1x10^{-3}$ mol l^{-1}), by serial dilutions.

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5.3 Amperometric electrode for enantioselective analysis of pipecolic acid

5.3.1Apparatus

A 663 VA Stand (Metrohm, Herisau, Switzerland) in combination with an Autolab 20 and software (Ecochemie version 4.9) were used for all differential pulse voltammetry measurements. A platinum electrode and an Ag/AgCl (0.1 mol/L KCl) electrode served as the counter and reference electrodes in the cell.

5.3.2 Electrode Design

Diamond paste electrode was prepared by mixing 0.1 g of diamond powder with 20 μL paraffin oil. A portion of the paste was then filled into a plastic pipette tip. The diameter of the sensing part was 3mm. Electric contact was made by inserting a silver wire in the paste. Before each use the electrode surface was smoothed out by polishing with alumina paper (polishing strips 30144-001, Orion). When not in use, the diamond paste was stored at room temperature.

5.3.3 Recommended procedure: Direct DPV assay

The technique used for the direct voltammetric assay was differential pulse voltammetry with the applied potential pulse amplitude of 25mV vs. Ag/AgCl. The diamond paste electrode together with the reference and auxiliary electrodes were dipped into a cell containing the buffer and supporting electrolyte (sodium pyrophosphate or NaCl) in a ratio of 3.5:1, as well as the synthetic mixture between L- and D-pipecolic acids. All

solutions were deoxygenated for 5 min before the measurements with N_2. The peak height measured at 900 and 300 mV vs Ag/AgCl for L- and D-pipecolic acid, respectively, was plotted versus the concentration of L- and D-pipecolic acid, respectively. The unknown concentrations of L- and D-pipecolic acid were determined from the corresponding calibration graphs.

5.3.4 Results and Discussion

5.3.4.1 Electrode Response

The electrode response was determined using DPV technique. The response characteristics and the equations of calibration are shown in Table 5.1. Different buffers (citrate buffer, pH=5.0 and phosphate buffer, pH=7.0) and two electrolytes (sodium pyrophosphate (I) or NaCl (II)) were used in order to establish the optimum working conditions for the enantioselective determination of each enantiomer. Accordingly, the highest sensitivity and the lowest limit of detection were recorded when citrate buffer (pH=5.0) was used with sodium pyrophosphate as supporting electrolyte. But, taking into account that the concentration of L-PA in the serum is in the μmol/L magnitude order, the best working conditions will be assured by the utilization of phosphate buffer (pH=7.0) with NaCl as supporting electrolyte.

For the assay of D-PA, the optimum working conditions will be given by utilization of citrate buffer with sodium pyrophosphate as supporting electrolyte, because the electrode has very good sensitivity, a wide concentration range covering the limits within which D-PA is found in the serum samples, and it has the lowest limit of detection. The signal to

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background ratio is very high when compared to classical glassy carbon (GC) and carbon paste electrodes. The reproducibility of peak current was excellent (RSD% values recorded were less than 0.1%), when the measurements were done everyday for a period of 6 months.

Table 5.1 Response characteristics of the amperometric electrode for L- and D-pipecolic acid assay.

Analyte	Electrolyte	Buffer	Linear conc. range	Detection limit	Equation of calibration*	r
L-PA	I	Citrate, pH=5.0	0.01-10 pmol/L	1 fmol/L	$^{1,a}H = 5.21 + 0.230C$	0.9980
		Phosphate, pH=7.0	1-100 pmol/L	0.1 pmol/L	$^{1,a}H = 1.63 + 0.003C$	0.9999
	II	Citrate, pH=5.0	1-100 nmol/L	1 pmol/L	$^{1,b}H = 2.22 + 0.014C$	0.9997
		Phosphate, pH=7.0	0.01-10000 nmol/L	1 pmol/L	$^{2,b}H = 204.5 + 1.27C$	0.9966
D-PA	I	Citrate, pH=5.0	0.0001 - 100 nmol/L	10 fmol/L	$^{1,b}H = 1.39 + 0.03C$	0.9997
		Phosphate, pH=7.0	0.1-100 nmol/L	1 pmol/L	$^{1,b}H = 1.39 + 0.01C$	0.9995
	II	Citrate, pH=5.0	0.01 – 1 nmol/L	0.1 pmol/L	$^{1,b}H = 4.03 + 0.11C$	0.9997
		Phosphate, pH=7.0	1-1000 nmol/L	10 pmol/L	$^{2,b}H = -2.17 + 0.49\ C$	0.9970

*H is the peak height in $^{1}\mu A$, ^{2}nA and C is the concentration of L- and D-pipecolic acid, respectively, in ^{a}pmol/L; ^{b}nmol/L.

5.3.4.2 Selectivity of the diamond paste based electrode

The selectivity of the electrode was checked using the mixed solution method. The amperometric selectivity coefficients were determined using the equation proposed by Wang [40].

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Table 5.2 Enantioselectivity of the amperometric electrode designed for L- and D-pipecolic acid

Analyte	Interfering enantiomer	Electrolyte	Buffer	pK_{amp}
L-PA	D	I	Citrate, pH=5.0	2.97
			Phosphate, pH=7.0	2.24
	D	II	Citrate, pH=5.0	3.00
			Phosphate, pH=7.0	3.00
D-PA	L	I	Citrate, pH=5.0	2.18
			Phosphate, pH=7.0	2.96
	L	II	Citrate, pH=5.0	2.99
			Phosphate, pH=7.0	2.17

All values are the average of ten determinations.

The ratio between the concentrations (mol/L) of the main analyte and interferent was 1:10. The pK_{amp} values (Table 5.2) show a good enantioselectivity of the electrode in the proposed working conditions. Accordingly, the electrode can be used for the enantioselective analysis of pipecolic acid. Inorganic ions such as Na^+, K^+ and Ca^{2+} did not interfere with the analysis of pipecolic acid.

5.3.4.3 Analytical Applications

The differential pulse voltammetry proved useful for enantioanalysis of pipecolic acid in serum samples.

In order to prove the suitability of the proposed electrodes and method (DPV) for the enantioanalysis of pipecolic acids, recovery tests were performed for the solutions containing different ratios between the two enantiomers. The results (Tables 5.3 and 5.4) show that L- and D-pipecolic acid can be successfully recovered one in the presence of the other.

Table 5.3 Determination of L-pipecolic acid in the presence of D-pipecolic acid

Electrolyte	Recovery L-pipecolic acid, (%)					
	L: D					
	Buffer	2:1	1:1	1:2	1:4	1:9
I	Citrate pH=5.0	99.87 ± 0.03	99.88 ± 0.03	99.85± 0.04	99.89 ± 0.03	99.90 ± 0.04
	Phosphate pH=7.0	99.93 ± 0.04	99.90 ± 0.02	99.92 ± 0.03	99.95 ± 0.03	99.93 ± 0.03
II	Citrate pH=5.0	99.93 ± 0.02	99.90 ± 0.03	99.91 ± 0.03	99.90 ± 0.02	99.89 ± 0.02
	Phosphate PH=7.0	99.93 ± 0.02	99.92 ± 0.03	99.91 ± 0.02	99.92 ± 0.02	99.92 ± 0.02

All values are the average of ten determinations.

No significant differences in the recovery values were recorded for the ratios between L:D or D:L enantiomers varying from 1:9 to 1:99.99.

Table 5.4 Determination of D-pipecolic acid in the presence of L-pipecolic acid

Electrolyte	Recovery D-pipecolic acid, (%)					
	D: L					
	Buffer	2:1	1:1	1:2	1:4	1:9
I	Citrate pH=5.0	99.82 ± 0.04	99.78 ± 0.03	99.76± 0.03	99.80 ± 0.04	99.79 ± 0.03
	Phosphate pH=7.0	99.94 ± 0.03	99.48 ± 0.03	99.54 ± 0.02	99.51 ± 0.03	99.55 ± 0.03
II	Citrate pH=5.0	99.42 ± 0.04	99.69 ± 0.03	99.65 ± 0.03	99.67 ± 0.04	99.66 ± 0.03
	Phosphate pH=7.0	99.56 ± 0.03	99.50 ± 0.02	99.58 ± 0.02	99.56 ± 0.03	99.59 ± 0.02

All values are the average of ten determinations.

Samples were collected from patients suspected of peroxisomal disorders (1-3) and hepatitis B (4-6). L- and D-pipecolic acids were assayed from serum samples using a standard method (41) as well as the proposed diamond paste based electrode (Table 5.5).

Table 5.5 Determination of L-and D-pipecolic acid in serum samples

PA	Sample No.	Standard Method	Proposed Method			
			Sodium pyrophosphate		NaCl	
			Citrate buffer pH=5.0	Phosphate buffer pH=7.0	Citrate buffer pH=5.0	Phosphate buffer pH=7.0
		(: mol/L)	(: mol/L)			
L	1	1.00	0.99 ± 0.02	0.98 ± 0.03	1.01 ± 0.02	0.99 ± 0.04
	2	5.00	4.99 ± 0.04	4.97 ± 0.02	4.98 ± 0.02	4.97 ± 0.03
	3	6.50	6.49 ± 0.02	6.48 ± 0.02	6.49 ± 0.03	6.49 ± 0.02
		(nmol/L)	(nmol/L)			
D	4	14.20	14.18 ± 0.03	14.15 ± 0.03	14.17 ± 0.04	14.19 ± 0.03
	5	11.00	10.94 ± 0.04	10.99 ± 0.02	10.95 ± 0.04	10.98 ± 0.03
	6	28.00	27.93 ± 0.04	27.92 ± 0.02	27.96 ± 0.03	27.98 ± 0.02

All values are the average of ten determinations

Good correlations between the results were obtained using both standard and new developed method.

5.4 Enantioselective, potentiometric membrane electrodes for the determination of L-pipecolic acid in serum

5.4.1 Apparatus

A 663 VA stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 100 and software (Eco Chemie version 4.9) was used for all potentiometric measurements. An Ag/AgCl (0.1 mol/L KCl) electrode was used as reference electrodes in the cell.

5.4.2 Electrode design

Paraffin oil and graphite powder were mixed in a ratio of 1:4 (w/w) followed by the addition of the aqueous solution of maltodextrin (DE 4.0 -7.0 (I), 13.0 - 17.0 (II), or 16.5

- 19.5 (III)) (10^{-3} mol/L) (100 µL chiral selector solution to 100 mg carbon paste). A certain quantity of carbon paste free of maltodextrin was prepared and it was placed into a plastic pipette peak leaving 3 to 4 mm empty in the top to be filled with the carbon paste that contains the chiral selector. The diameter of the EPME was 3 mm. Electric contact was obtained by inserting a Ag/AgCl wire in the carbon paste. A solution of 0.1 mol/L KCl was utilized as internal solution.

The surface of the electrodes was wetted with deionised water and polished with alumina paper (polishing strips 30144-001, Orion) before using them for each of the experiments. When not in use, the electrode was immersed in a 10^{-3} mol/L L-pipecolic acid solution.

5.4.3 Recommended procedure. Direct potentiometry

Direct potentiometry was used for potential measurement of each standard solution (10^{-10} -10^{-3} mol/L). The electrodes were placed in stirred standard solutions. Calibration graphs were obtained by plotting E(mV) versus p(L-PA). Unknown concentrations were determined from the corresponding calibration graph.

5.4.4 Results and discussion

5.4.4.1 Response characteristics of the EPMEs

The responses of the electrodes were determined for both enantiomers L-PA and D-PA, at pH=2.5 (citrate buffer) using a potentiometric method. The responses obtained for D-PA were not linear and non-Nernstian. That proved that the electrodes couldn't be used for the assay of D-PA. The equations of calibration obtained for L-PA are as follows:

(I) E =503.0 - 53.42 p(L-PA) r = 0.9934

(II) E = 432.1 - 52.60 p(L-PA) r = 0.9906

(III) E = 614.8 - 52.19 p(L-PA) r = 0.9918

where E(mV) is the cell potential, p(L-PA) = -log[L-PA], and (I), (II) and (III) correspond to the EPMEs based on the maltodextrins (I), (II), and (III), respectively. The response characteristics for the EPMEs when used for the assay of L-PA are shown in Table 5.6. All electrodes showed very low detection limits, maltodextrin (I) and (II) based electrodes in the magnitude order of 10^{-9} mol/L and the maltodextrin (III) based electrode in the magnitude order of 10^{-12} mol/L. The electrodes responses showed good stability and reproducibility for all the performed tests for 6 months, when used daily for measurements (RSD<1.0%).

Table 5.6 Response characteristics of enantioselective, potentiometric membrane electrodes for L-pipecolic acid

Chiral selector	Slope (mV/pL-PA)	Intercept, E^o (mV)	Linear concentration range (mol/L)	Detection limit (mol/L)
Maltodextrin (I)	58.70	503.0	10^{-8}-10^{-3}	$1.00x10^{-9}$
Maltodextrin (II)	52.60	432.1	10^{-8}-10^{-5}	$6.00x10^{-9}$
Maltodexrin (III)	52.19	614.8	10^{-10}-10^{-6}	$1.66x10^{-12}$

All values are the average of ten determinations.

The response time was less than 1 minute for the concentration range between 10^{-8} and 10^{-5} mol/L, when maltodextrin (II) based electrode was used; for maltodextrin (I) based electrode the response time was higher than 1 min for concentrations between 10^{-8} and 10^{-6} mol/L, while for concentrations between 10^{-5} and 10^{-3} mol/L the response time was lower than 1min; for maltodextrin (III) based electrode, the response times was less than

1min for concentrations between 10^{-10} and 10^{-8} mol/L and higher than 1min for a concentration range between 10^{-7} and 10^{-6} mol/L.

5.4.4.2 The influence of pH on the responses of the electrodes

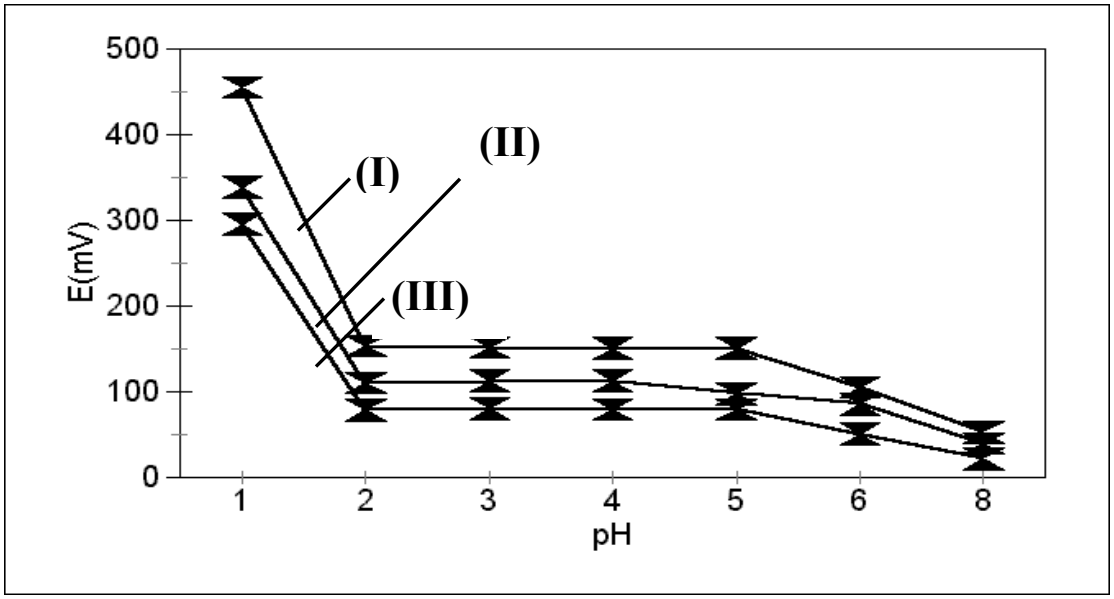

Figure 5.2 Effect of pH on the response of the enantioselective, potentiometric membrane electrodes for L-pipecolic acid (10^{-7} mol/L L-pipecolic acid solution). (I) – maltodextrin (I) based EPME; (II) – maltodextrin (II) based EPME; (III) – maltodextrin (III) based EPME.

The effect of pH on the responses of the electrodes was determined by recording the emf of the cell for a 10^{-7} mol/L solution of L-PA, at different pHs.

The E(mV) vs. pH plots presented in Figure 5.2 show the emf independency of pH in the following pH ranges of 2.0-6.0, 2.0-5.0, and 2.0-4.0 for maltodextrin (I), (II) and (III) based EPMEs, respectively.

5.4.4.3 Selectivity of the EPMEs

The selectivity of all EPMEs was checked using the mixed solutions method. The concentrations of the supposed interfering ion and L-PA were 10^{-6} and 10^{-7} mol/L,

respectively. The enantioselectivity was investigated against D-PA. The potentiometric selectivity coefficients vs D-PA for the maltodextrin (I), (II), and (III) based EPMEs: 3.2 x 10^{-3}, 3.0 x 10^{-3}, and 4.9 x 10^{-3}, respectively, proved that D-PA does not interfere in the determination of L-PA, demonstrating the enantioselectivity property of the electrodes.

Inorganic ions such as Na^{+}, K^{+} and Ca^{2+} do not interfere with the analysis of L-PA, because the potentiometric selectivity coefficients determined using mixed solutios method were less than 10^{-4}. The interference of creatine and creatinine was also checked. Potentiometric selectivity coefficients of $1x10^{-4}$, $1.2x10^{-4}$, $1.1x10^{-4}$ for creatine when the maltodextrin (I), (II), and (III) based EPMEs were used and $1.2x10^{-3}$, $1.5x10^{-3}$, $1.1x10^{-3}$ for creatinine when the maltodextrin (I), (II), and (III) based EPMEs were used, proved that creatine and creatinine do not interfere in the determination of L-PA.

5.4.4.4 Analytical applications

Table 5.7 Determination of L-pipecolic acid in the presence of D-pipecolic acid

Chiral Selector	Average recovery*, (%)				
	L:D (mol:mol)				
	2:1	1:1	1:2	1:4	1:9
Maltodextrin (I)	99.75±0.08	99.25±0.05	99.88±0.05	99.80±0.06	99.81±0.05
Maltodextrin (II)	99.68±0.04	99.20±0.05	99.75±0.05	99.75±0.04	99.73±0.05
Maltodextrin (III)	99.75±0.04	99.80±0.03	100.71±0.05	100.80±0.04	100.78±0.05

* All values are the average of ten determinations.

In order to determine the suitability of the EPMEs for the enantioanalysis of L-pipecolic acid, solutions containing both enantiomers in different ratios were prepared, and

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recovery test for L-PA ($C_{L\text{-}PA}=10^{-7}$mol/L) were performed. The results obtained (Table 5.7) demonstrated the suitability of the electrodes for the enantioanalysis of L-pipecolic acid due to the good recovery and RSD (%) values. No significant differences in the recovery values were recorded for the ratios between L:D enantiomers varying from 1:9 to 1:99.99.

The results obtained for the assay of L–pipecolic acid in serum samples are shown in Table 5.8. Samples (1-3) were collected from different patients suspected of peroxisomal disorders and buffered with citrate buffer (0.1mol/L, pH=2.5, buffer:sample=1:1(v/v)).

Table 5.8 Recovery of L-pipecolic acid in serum samples (nmol/L)

Sample no.	Standard method[14]	Maltodextrin (I)	Maltodextrin (II)	Maltodextrin (III)
1	13.00	12.91±0.02	12.96±0.03	12.92±0.04
2	10.00	9.94±0.01	9.97±0.03	9.91±0.03
3	27.00	26.95±0.02	26.91±0.02	26.98±0.04

All values are the average of 10 determinations.

The results obtained using the proposed EPMEs are in good concordance with those obtained using the standard method (spectrometric method) [41].

5.5 Amperometric biosensors for the enantioselective analysis of L- and D-pipecolic acids in biological fluids

5.5.1 New amperometric biosensors based immobilization of L- and D-amino acid oxidases on diamond paste for the determination of L- and D-pipecolic acids in serum samples

5.5.1.1 Apparatus

A 663 VA Stand (Metrohm, Herisau, Switzerland) in combination with the Autolab 20 and software (Ecochemie version 4.9) were used for all chronoamperometric measurements. A Pt electrode and an Ag/AgCl electrode served as the counter and reference electrodes in the cell.

5.5.1.2 Amperometric biosensors design

The enzyme solutions used for the design of the biosensors were prepared in a 0.1 mol/L phosphate buffer pH=7.0. Two plastic tips were filled with plane diamond paste leaving an empty space of 3-4 mm in the top part filled with the modified diamond paste containing the mixture of L- and D-AAOD as shown below. The diameters of the biosensors were 3 mm. Electric contacts were obtained by inserting silver wires into the diamond paste. The biosensors tips were gently rubbed on fine abrasive paper to produce a flat surface. The surface of the biosensors were wetted with de-ionized water and then polished with an alumina paper (polished strips 30144-001, Orion) before use. The biosensors were stored dry at 4^0C, when not in use.

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5.5.1.3 Recommended procedure: Direct DPV assay

The technique used for the direct voltammetric assay was differential pulse voltammetry (DPV) with the applied potential pulse amplitude of 25 mV vs. Ag/AgCl. The diamond paste electrode together with the reference and auxiliary electrodes were dipped into a cell containing phosphate buffer (pH=7.0) and the selected electrolyte (sodium pyrophosphate or NaCl) solution 0.1 mol/L, in a ratio of 3.5:1, as well as the synthetic mixture between L- and D-pipecolic acids. All solutions were deoxygenated for 5 min before measurements with N_2. The peak height measured at 700 mV and 200 mV vs Ag/AgCl for L- and D-pipecolic acid, respectively, was plotted versus their concentrations. The unknown concentrations of L- and D-pipecolic acids were determined from the corresponding calibration graphs.

5.5.1.4 Preparation of the modified diamond paste

Equal volumes of solutions of L-AAOD and D-AAOD (1mg enzyme/mL of phosphate buffer pH=7.0) were mixed. 30 µL paraffin oil and 400 mg natural monocrystalline diamond powder were mixed to form a diamond paste. 50 µL from the solution containing both enzymes was added to the diamond paste to form the modified diamond paste.

5.5.1.5 Results and discussion

5.5.1.5.1 Amperometric biosensors response

Two supporting electrolytes (sodium pyrophosphate (I) and NaCl (II)) that proved previously to give good results when diamond electrodes were used in the DPV technique

[35, 36], were tested for the determination of L- and D-pipecolic acids in order to determine the best working conditions. The biosensor response was determined using DPV technique. The response characteristics are shown in Table 5.9. The lowest limits of detection were found when sodium pyrophosphate was utilized as supporting electrolyte for both L- and D-pipecolic acid. The working concentration ranges are wide, with the exception of the determination of D-PA using NaCl as supporting electrolyte. Accordingly, the electrolyte of choice for the assay of L- and D-PA is sodium pyrophosphate.

Table 5.9 Response characteristics of the amperometric biosensor for L- and D-pipecolic acid assay.

Analyte	Electrolyte	Linear conc. range	Detection limit	Equation of calibration*	r
L-PA	I	0.0001-10 µmol/L	10 pmol/L	$^{1}I = 9.94 + 0.84C$	0.9984
	II	0.1 - 10000 nmol/L	0.01 nmol/L	$^{2}I = 0.19 + 0.031C$	0.9995
D-PA	I	0.0001 - 10 µmol/L	10 pmol/L	$^{1}I = 21.69 + 2.82C$	0.9956
	II	0.01 – 10 µmol/L	0.1 nmol/L	$^{1}I = 11.36 + 0.19C$	0.9995

*I is the intensity of the current in µA, and C is the concentration of L- and D-pipecolic acid, respectively, in [1] µmol/L and [2]nmol/L

5.5.1.5.2 Selectivity of the amperometric biosensors

The selectivity of the biosensor was checked by both the separate and mixed solution methods. Amperometric selectivity coefficients were determined following the method proposed by Wang [40]. In the evaluation, the concentration of the possible interferent, was ten times higher than that of the main analyte. The order of magnitude of all of the amperometric selectivity coefficients was 10^{-3} proving no interference in the

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determination of L- and D-PA (Table 5.10), and the proposed electrode can be used for the enantioanalysis of pipecolic acid. Inorganic ions such as Na^+, K^+ and Ca^{2+} did not interfere with the analysis of pipecolic acid.

Table 5.10 Enantioselectivity of the amperometric biosensor designed for L- and D-pipecolic acid

Analyte	Interfering enantiomer	Electrolyte	pK_{amp}
L-PA	D	I	2.11
	D	II	2.98
D-PA	L	I	3.00
	L	II	2.15

All values are the average of ten determinations.

5.5.1.5.3 Analytical Applications

The response characteristics and selectivity of the modified diamond paste based biosensor as well as the potential where the peaks are formed for the assay of L- and D-PA made the amperomeric biosensor suitable for the enantioanalysis of pipecolic acid.

Table 5.11 Determination of L-pipecolic acid in the presence of D-pipecolic acid

L: D	Recovery L-pipecolic acid, (%)	
	Electrolyte	
	I	II
2:1	99.76 ± 0.03	99.75 ±0.03
1:1	99.78 ± 0.02	99.76 ± 0.02
1:2	99.79 ± 0.02	99.73 ± 0.02
1:4	99.76± 0.03	99.77 ± 0.03
1:9	99.77 ± 0.02	99.78 ± 0.03

All values are the average of ten determinations

Table 5.12 Determination of D-pipecolic acid in the presence of L-pipecolic acid

D: L	Recovery D-pipecolic acid, (%)	
	Electrolyte	
	I	II
2:1	99.76 ± 0.03	99.74 ±0.04
1:1	99.89 ± 0.02	99.83 ± 0.06
1:2	99.85 ± 0.02	99.82 ± 0.05
1:4	99.89± 0.03	99.86 ± 0.05
1:9	99.87 ± 0.03	99.85 ± 0.04

All values are the average of ten determinations.

To prove that the amperometric biosensor can be used reliably in enantioanalysis, tests of recovery of L- in the presence of D-PA and of D- in the presence f L-PA, different ratios between the enantiomers were performed. The results shown in Tables 5.11 and 5.12 indicate that the new amperometric biosensor can be used reliably for such tests.

Table 5.13 Determination of L-and D-pipecolic acid in serum samples.

PA	Sample No.	Standard Method	Proposed Method	
			Sodium pyrophosphate	NaCl
		(: mol/L)	(: mol/L)	
L	1	1.10	1.09 ± 0.02	1.07 ± 0.04
	2	4.40	4.38 ± 0.02	4.37 ± 0.03
	3	6.00	5.97 ± 0.03	5.96 ± 0.03
		(nmol/L)	(nmol/L)	
D	4	13.90	13.87 ± 0.04	13.85 ± 0.04
	5	11.30	11.26 ± 0.03	11.24 ± 0.03
	6	30.00	29.96 ± 0.03	29.93 ± 0.03

All values are the average of ten determinations

Samples were collected from patients suspected of cerebrohepatorenal syndrome (1-3) and hepatitis B (4-6). These samples were analysed using the new amperometric biosensors and the results were compared with those obtained using a standard method

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[41] (Table 5.13). A good agreement was obtained between the results that show that the electrodes can be successfully used in the diagnosis of these diseases.

5.5.2 Carbon paste based amperometric biosensors for the enantioselective analysis of pipecolic acid

5.5.2.1 Apparatus

A663 VA Stand (Metrohm, Herisau, Switzerland) in combination with a μAutolab and software Ecochemie (version 4.9) were used for all chronoamperometric measurements. A Pt electrode and an Ag/AgCl electrode served as the counter and reference electrodes in the cell.

5.5.2.2 Amperometric biosensors design

Four plastic tips were filled with plane carbon paste leaving an empty space of 3-4 mm (which is the sensing part of the biosensors) to be filled with carbon paste containing the different enzymes. The diameters of all biosensors were 3 mm. Electric contacts were obtained by inserting silver wires into the carbon paste. The biosensors tips were gently rubbed on fine abrasive paper to produce a flat surface. The surface of the biosensors was wetted with de-ionized water and then polished with an alumina paper (polished strips 30144-011, Orion) before use. The biosensors were stored dry at 4^0C, when not in use. All the enzyme solutions used for the biosensors design were prepared in 0.1 mol/L phosphate buffer of pH=7.

5.5.2.2.1 Monoenzyme amperometric biosensors

Two biosensors, based on graphite paste, were designed as follows: paraffin oil and graphite powder were mixed in a ratio 1:4 (w/w) to form a carbon paste. 100 μL from the solution (1mg/enzyme/mL) of L-amino acid oxidase (L-AAOD) or D-amino acid oxidase (D-AAOD), respectively, were added to two separate portions of carbon paste.

5.5.2.2.2 Bienzyme amperometric biosensors

Two mixtures of enzymes were used for the design of amperometric biosensors: (1) 1mg of horseradish peroxidase (HRP) was dissolved in 50 μL of L-AAOD solution (0.25 mg/mL); (2) 1mg of HRP was dissolved in 50 μL of D-AAOD solution (0.25 mg/mL). Each mixture was incorporated in the carbon paste (100 mg graphite powder and 40 μL paraffin oil), to obtain two bienzyme electrodes.

5.5.2.3 Recommended procedures: Direct amperometry

The chronoamperometric technique was used for all the measurements. The electrodes were dipped into a cell containing 10 mL of phosphate buffer, pH =7.00 and different aliquots of L- or D-pipecolic acid solution. The intensity of current measured was plotted versus the concentration of L- or D-pipecolic acid. The unknown concentrations of L- and D-pipecolic acid were determined from the calibration graphs.

5.5.2.4 Determination of L- and D-pipecolic acid in serum samples

Six serum samples were collected from different patients suspected of peroxisomal disorders (samples 1-3) and liver cirrhosis (samples 4-6). Different aliquots of serum

samples were diluted with buffer solution (pH=7.00). Direct amperometry was involved to determine the content of L- and D-pipecolic acid in serum samples.

5.5.2.5 Results and discussion

5.5.2.5.1 Amperometric biosensors response

The response characteristics of the biosensors were measured at different potentials in order to determine the best working potential (higher sensitivity, lower limit of detection, shorter response time, etc.) for the assay of L- and D-pipecolic acid (Table 5.14).

Table 5.14 Response characteristics for the amperometric biosensors designed for L- and D-pipecolic Acid

Enzyme(s) used for the biosensor design	E (mV)	Linear conc. range	Detection limit	Response time, $t_{R90\%}$ (s)	Equations of calibration*	r
L-AAOD	650	0.02-0.1µmol/L	20pmol/L	60	^{1,a}I=-0.73+33.19C	0.9866
	50	2-600nmol/L	400pmol/L	30	^{2,b}I =168.9+18.23C	0.9993
	400	0.8-10pmol/L	1pmol/L	120	^{1,c}I =3.75+0.482C	0.9956
L-AAOD + HRP	400	4-200pmol/L	0.2pmol/L	30	^{2,c}I =-71.99+21.78C	0.9993
	650	20-200pmol/L	0.4pmol/L	120	^{1,c}I =0.24+9C	0.9980
D-AAOD	650	0.002-0.06 fmol/L	$1x10^{-4}$ fmol/L	30	^{1,d}I =0.02+67.01C	0.9999
	130	1-8nmol/L	20pmol/L	120	^{2,b}I =5.03+5.08C	0.9976
	550	20-800fmol/L	10fmol/L	120	^{1,d}I =9.75+0.035C	0.9988
D-AAOD+ HRP	650	2-10fmol/L	0.02fmol/L	120	^{2,d}I =404.3+46.45C	0.9935

* I is the intensity of the current in ^{1}µA, ^{2}nA, and C is the concentration of L-and D-pipecolic acid, respectively, in ^{a}µmol/L, ^{b}nmol/L, ^{c}pmol/L and ^{d}fmol/L.

The biosensor response was highly stable and reproducible over 35 days, when the biosensors were intensively used everyday for measurements and were kept into the fridge at 4^{0}C when not in use. The lower limits of detection for the analysis of L- and D-pipecolic acid were obtained at 400mV and at 650 mV when L-AAOD+HRP and D-AAOD, respectively, were used for the design of the biosensors. The higher sensitivity

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(defined by Otto as the slope of the equation of calibration [22]) was recorded at 650mV when L-AAOD+HRP and D-AAOD where used for the assay of L-PA and D-PA, respectively.

5.5.2.5.2 Enantioselectivity of the Amperometric Biosensors

The enantioselectivity of all biosensors was checked using both separate and mixed solution methods with respect to L- and D-pipecolic acid. Amperometric enantioselectivity coefficients (K_{amp}) were determined following the method proposed by Wang [40] for the same potentials used for the determination of the response characteristics of the amperometric biosensors. The ratios between the concentrations of the main enantiomer and the other enantiomer were 1:10. The values of the amperometric selectivity coefficients (pK_{amp}), obtained using the mixed solution method, for all the biosensors designed for L- and D-PA are higher than 2.00 (Table 5.15), showing that the proposed biosensors are enantioselective.

Table 5.15 Enantioselectivity of the amperometric biosensors designed for L- and D-pipecolic acid

Enzyme(s) used for the design of the biosensor	Interfering enantiomer	E(mV)	pK_{amp}
L-AAOD	D	650	3.00
	D	400	2.55
	D	50	2.82
L-AAOD + HRP	D	650	2.17
	D	400	3.82
D-AAOD	L	650	2.43
	L	550	2.48
	L	130	2.96
D-AAOD + HRP	L	650	2.47

All values are the average of ten determinations.

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The biosensors based on L-AAOD+HRP has got the best enantioselectivity for L-pipecolic acid analysis, when measurements are performed at 400mV while the biosensors based on D-AAOD enzyme has got the best enantioselectivity for D-pipecolic acid analysis, when measurements are performed at 130mV.

5.5.2.5.3 Analytical Applications

In order to determine the suitability of the proposed biosensors for the enantioanalysis of pipecolic acid, solutions containing both enantiomers in different ratios were prepared, and a recovery test for each enantiomer was performed.

Table 5.16 Determination of L-pipecolic acid in the presence of D-pipecolic acid

Enzyme(s) used for the design of the biosensors	E(mV)	Average recovery, L-PA (%)				
		L:D				
		2:1	1:1	1:2	1:4	1:9
L-AAOD	650	99.21±0.01	99.80±0.02	99.97±0.02	99.58±0.03	99.60±0.02
	400	99.37±0.02	99.89±0.03	99.86±0.02	99.40±0.01	99.70±0.02
	50	99.88±0.03	99.97±0.02	99.21±0.02	99.47±0.03	99.50±0.03
L-AAOD + HRP	650	99.42±0.03	99.52±0.03	99.52±0.01	99.53±0.02	99.53±0.02
	400	99.42±0.02	99.49±0.02	99.60±0.02	99.55±0.01	99.35±0.02

All values are the average of ten determinations.

Table 5.17 Determination of D-pipecolic acid in the presence of L-pipecolic acid

Enzyme(s) used for the design of the biosensors	E(mV)	Average recovery, D-PA (%)				
		D:L				
		2:1	1:1	1:2	1:4	1:9
D-AAOD	650	99.85±0.01	99.81±0.01	99.68±0.01	99.99±0.02	99.99±0.01
	400	99.61±0.01	99.46±0.02	99.99±0.01	99.95±0.02	99.98±0.01
	50	99.60±0.02	99.65±0.03	99.89±0.01	99.80±0.03	99.85±0.02
D-AAOD + HRP	650	99.25±0.01	99.89±0.03	99.85±0.02	99.99±0.02	99.92±0.02

All values are the average of ten determinations.

The results obtained (Tables 5.16 and 5.17) demonstrated the suitability of the proposed amperometric biosensors for the enantioanalysis of pipecolic acid due to the good recovery values obtained for the assay of one of the enantiomers in the presence of its antipode. No significant differences in the recovery values were recorded for the ratios between L:D or D:L enantiomers varying from 1:9 to 1:99.99.

The results obtained for the assay of L- and D-pipecolic acid in serum samples are shown in Tables 5.18 and 5.19. Samples 1-3 were used for the assay of L-pipecolic acid while samples 4-6 were used for the assay of D-pipecolic acid. The results obtained using the proposed biosensors at different working potentials is in concordance with those obtained using the standard method [41].

Table 5.18 Recovery of L-pipecolic acid in serum samples

Average recovery, L-PA (: mol/L)						
Sample No.	Standard method	L-AAOD			L-AAOD+HRP	
		650 mV	400mV	50mV	650mV	400mV
1	0.91	0.82±0.01	0.88±0.03	0.89±0.03	0.89±0.02	0.88±0.04
2	2.41	2.41±0.01	2.39±0.03	2.38±0.02	2.37±0.01	2.35±0.04
3	3.30	3.29±0.01	3.26±0.03	3.28±0.03	3.29±0.02	3.27±0.03

All values are the average of ten determinations.

Table 5.19 Recovery of D-pipecolic acid in serum samples

Average recovery, D-PA (nmol/L)					
Sample No.	Standard method	D-AAOD			D-AAOD+HRP
		650 mV	550mV	130mV	650mV
4	13.0	12.90±0.02	12.92±0.01	12.93±0.03	12.95±0.01
5	19.0	18.87±0.02	18.92±0.01	18.89±0.03	18.93±0.03
6	23.0	22.83±0.02	22.80±0.02	22.89±0.02	22.97±0.03

All values are the average of ten determinations.

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5.5.3 Diamond paste based amperometric biosensors based on L-AAOD and D-AAOD for the determination of L- and D-pipecolic acid

5.5.3.1 Apparatus

A 663 VA Stand (Metrohm, Herisau, Switzerland) in combination with a PGSTAT 20 and software Ecochemie (version 4.8) were used for all chronoamperometric measurements. A Pt electrode and an Ag/AgCl electrode served as counter and reference electrodes in the cell.

5.5.3.2 Amperometric Biosensors Design

Two plastic tips were filled with plane diamond paste (0.1 g of each diamond powder were mixed with 20 μL paraffin oil) leaving an empty space of 3-4 mm in the top part to be filled with modified diamond paste (containing the different enzymes). The diameters of all biosensors were 3 mm. Electric contacts were obtained by inserting silver wires into the diamond paste. The biosensors tips were gently rubbed on fine abrasive paper to produce a flat surface. The surface of the biosensors were wetted with de-ionized water and then polished with an alumina paper (polished strips 30144-011, Orion) before use. The biosensors were stored dry at 4^0C, when not in use. All the enzyme solutions used for the biosensors design were prepared in 0.1 mol/L phosphate buffer of pH=7.0.

Two modified diamond paste were prepared as follows: 30 μL paraffin oil and 400 mg natural monocrystalline diamond powder were mixed to form a diamond paste. 50 μL

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from the solution (1mg/enzyme/mL) of L-AAOD or D-AAOD, respectively, were added to two separate portions of diamond paste.

5.5.3.3 Recommended procedures: Direct amperometry

The chronoamperometric technique was used for the measurement of the intensity of current. The electrodes were dipped into a cell containing 10 mL of phosphate buffer, pH =7.00 and different aliquots of L- or D-pipecolic acid solution. The intensity of current measured was plotted versus the concentration of L- or D-pipecolic acid. The unknown concentrations of L- and D-pipecolic acid were determined from the calibration graphs.

5.5.3.4 Determination of L- and D-pipecolic acid in serum samples

Different aliquots of serum samples were diluted with buffer solution (pH=7.00). Direct amperometry was involved to determine the content of L- and D-pipecolic acid in serum samples.

5.5.3.5 Results and discussion

5.5.3.5.1 Response characteristics of the amperometric biosensors

The response characteristics of the biosensors were measured at different potentials in order to determine the best working potential (higher sensitivity, lower limit of detection, shorter response time, etc.) for the assay of L- and D-pipecolic acid (Table 5.20).

Table 5.20 Response characteristics for the amperometric biosensors designed for L- and D-pipecolic acid

Enzyme used for the design of the biosensor	E (mV)	Linear conc. Range	Detection limit	t_R (s)	Equation of calibration*	r
L-AAOD	650	$2x10^{-4}$- $1x10^{-3}$ fmol/L	$2x10^{-5}$ fmol/L	60	[1,a] $I = 1x10^{-3} + 58.6C$	0.9955
	400	20-100 pmol/L	0.2 pmol/L	120	[2,b] $I = 0.9 + 1.2C$	0.9937
D-AAOD	650	0.2 - 1.0 pmol/L	0.02 pmol/L	180	[2,b] $I = 0.7 + 8.1C$	0.9813
	300	20 – 100 fmol/L	0.6 fmol/L	120	[2,a] $I = 0.01 + 0.1C$	0.9987

*I is the intensity of the current in [1]mA and [2]μA, and C is the concentration of L- and D-pipecolic acid, respectively, in [a]fmol/L, [b]pmol/L

The biosensor response was highly stable and reproducible over one month, when the biosensors were intensively used everyday for measurements and were kept into the fridge at 4^0C when not in use. The best response characteristics (larger concentration range, lower limit of detection and highest sensitivity) for the assay of L-pipecolic acid and D-pipecolic acid were obtained at 650 mV and 300 mV, respectively. The low limits of detections and the high reliability of the biosensors is due to the type of the matrix used for the biosensors design (diamond paste), way adopted for the design of biosensors as well as to the selection of the working potential.

5.5.3.5.2 Enantioselectivity of the Amperometric Biosensors

The enantioselectivity of all biosensors was checked using both separate and mixed solution method with respect to L- and D-pipecolic acid. Amperometric enantioselectivity coefficients were determined following the method proposed by Wang [40], for the same potentials used for the determination of the response characteristics of the proposed amperometric biosensors. The ratio between the concentration of the main

enantiomer and the other enantiomer was 1:10. The values of the amperometric selectivity coefficients (pK_{amp}) (obtained using mixed solution method) for all the biosensors designed for L- and D-pipecolic acid are higher than 2.00, showing that the proposed biosensors are enantioselective. The biosensor based on L-AAOD enzyme has got the best enantioselectivity for L-pipecolic assay, when measurements are performed at 650 mV (pK_{amp}=2.82) while the biosensor based on D-AAOD has got the best enantioselectivity for D-pipecolic acid assay, when measurements are performed at 300 mV (pK_{amp}=2.77).

5.5.3.5.3 Analytical Applications

Table 5.21 Determination of D-pipecolic acid in the presence of L-pipecolic acid

E (mV)	Recovery L-pipecolic acid, (%)				
	L: D				
	2:1	1:1	1:2	1:4	1:9
650	99.59 ± 0.03	99.70 ± 0.03	99.69 ± 0.02	99.82± 0.03	99.80 ± 0.02
400	99.65 ±0.03	99.69 ± 0.02	99.27 ± 0.02	99.97 ± 0.03	99.67 ± 0.02

Table 5.22 Determination of L-pipecolic acid in the presence of D-pipecolic acid

E (mV)	Recovery D-pipecolic acid, (%)				
	D: L				
	2:1	1:1	1:2	1:4	1:9
650	99.90 ± 0.02	99.98 ± 0.03	99.96 ± 0.02	99.89 ± 0.01	99.94 ± 0.02
300	99.89 ± 0.01	99.96 ± 0.02	99.90 ± 0.02	99.88 ± 0.01	99.92 ± 0.03

The assay of L- and D-pipecolic acid was conducted by use of different ratios between L- and D-pipecolic acid. The results obtained (Table 5.21 and 5.22) demonstrated the suitability of the proposed amperometric biosensors for the enantioanalysis of pipecolic acid due to the good recovery values obtained for the assay of one of the enantiomers in

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the presence of its antipode. No significant differences in the recovery values were recorded for the ratios between L:D or D:L enantiomers varying from 1:9 to 1:99.9.

The results obtained for the assay of L- and D-pipecolic acid in serum samples are shown in Table 5.23. The serum samples were collected from different patients suspected of peroxisomal disorders (samples 1-3, for the assay of L-PA) and liver cirrhosis (samples 4-6, for the assay of D-PA). The results obtained using the proposed biosensors at different working potentials are in good concordance with those obtained using the standard method [41].

Table 5.23 Determination of L-and D-pipecolic acid in serum samples

PA	Sample No.	Standard Method (: mol/L)	400 mV	Proposed Method (: mol/L) 650 mV	300 mV
L	1	1.00	0.99 ± 0.02	1.01 ± 0.03	-
	2	4.00	3.91 ± 0.03	3.87 ± 0.01	-
	3	5.50	5.49 ± 0.02	5.42 ± 0.01	
		(nmol/L)		(nmol/L)	
D	4	14.00	-	13.92 ± 0.03	13.91 ± 0.01
	5	12.00	-	11.97 ± 0.02	11.98 ± 0.02
	6	30.00		29.98 ± 0.03	29.90 ±0.01

All values are the average of ten determinations.

5.6 Sequential injection analysis utilizing amperometric biosensors as detectors for the simultaneous determination of L- and D-pipecolic acid

5.6.1 Apparatus

A 663 VA stand (Metrohm, Herisau, Switzerland) in combination with a PGSTAT 20 and software (Eco chemie version 4.9) was used for all chronoamperometric

measurements. A platinum electrode and Ag/AgCl electrodes were used as counter and reference electrodes in the cell.

5.6.2 Biosensor's design

Carbon pastes based biosensors described in section (3.5.2) were used as detectors in the SIA system for the simultaneous assay of L- and D-pipecolic acids. Monoenzymatic biosensors are based on the physical immobilization of L-AAOD or D-AAOD in carbon paste and the bi-enzymatic biosensor is based on the physical immobilization of L-AAOD and HRP or D-AAOD and HRP in carbon paste.

5.6.3 Sequential injection system

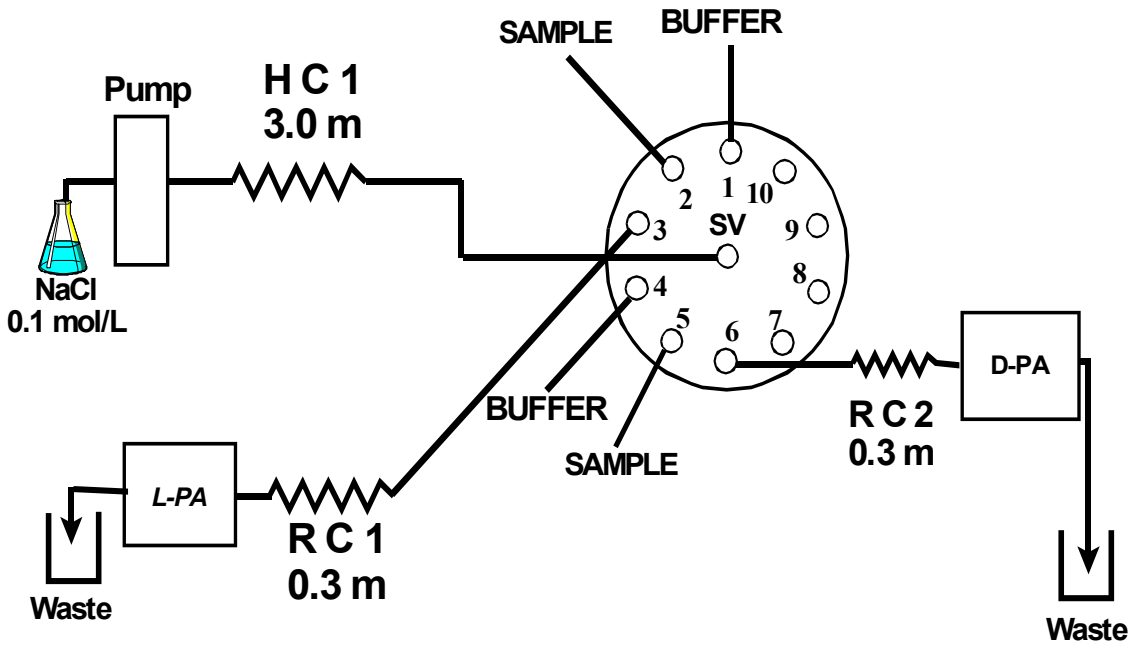

Figure 5.2 Schematic flow diagram of the sequential injection analysis/amperometric biosensors system used for the simultaneous determination of L- and D-pipecolic acid.

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Table 5.24 Device sequence for one cycle of the SIA system

Time(s)	Pump	Valve	Description
0	Off	Buffer	Pump stops, select buffer stream (valve position 1)
5	Reverse	Buffer	Draw up buffer solution
9.5	Off		Pump stops
10.5		Sample	Select sample stream (valve position 2)
11.5	Reverse	Sample	Draw up sample solution
16	Off		Pump stops
17		L-pipecolic cell	Select L-pipecolic cell line (valve position 3)
18	Forward		Pump stack of zones to L-pipecolic acid cell
48	Off		Pump stops
49		Buffer	Select buffer stream (valve position 4)
50	Reverse	Buffer	Draw up buffer solution
54.5	Off		Pump stops
55.5		Sample	Select sample stream (valve position 5)
56.5	Reverse	Sample	Draw up sample solution
61	Off		Pump stops
62		D-pipecolic acid cell	Select D-pipecolic acid cell line (valve position 6)
63	Forward		Pump stack of zones to D-pipecolic acid cell
93	Off	Home	Pump stops, return valve to the starting position (valve position 1)

The amperometric biosensors were incorporated into the conduits of a SIA system (Figure 5.2) constructed from a 10-port electrically actuated selection valve (Model ECSD10P, Valco Instruments, Houston, TX) and a Gilson Minipuls peristaltic pump. Tygon tubing (0.76 mm for both holding coils and 0.89 mm i.d. for both mixing coils) was used to construct the manifold; coils were wound round suitable lengths of glass tubing (15 mm o.d.); 0.1 mol/L NaCl was used as carrier. The capacity of the system is about 38 samples per hour.

The device operating sequence is shown in Table 5.24. The device control was achieved using a PC30-B interface board (Eagle electric, Cape Town, South Africa). The FlowTEK [42] software package (obtained from MINTEK) for computer-aided flow analysis was used through out the system. An optimum flow rate of 3.61 ml/min was used to propel the solutions. The sample and buffer consumption is only 270 µl of each per measurement of L- and D-enantiomers.

5.6.4 Results and discussions

5.6.4.1 The response characteristics of the biosensors in the SIA system

The response of all the biosensors was determined at different potentials using a chronoamperometric technique in order to obtain the best response characteristics (e.g., higher sensitivity, lower limit of detection, wide linear concentration range, etc.) for the simultaneous detection of L-and D-pipecolic acid (Table 5.25). The working concentration ranges as well as the limits of detection demonstrated the suitability of the

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proposed amperometric biosensors for the simultaneous on-line monitoring of both enantiomers.

The best response characteristics were obtained when the biosensors based on L-AAOD and HRP (at 400mV) and the biosensor based on D-AAOD (at 650mV) were used as detectors for the simultaneous assay of L- and D-PA, respectively in the SIA system. The response obtained for all the biosensors showed good stability and reproducibility for tests performed daily, for 3 weeks.

Table 5.25 Response characteristics of the amperometric biosensors designed for the simultaneous assay of L- and D-pipecolic acids in a SIA system. All values are the average of ten determinations

Enzyme used for the biosensor design	E (mV)	Linear conc. Range	Detection limit	Equations of calibration*	r
L-AAOD	650	10-100 nmol/L	2 nmol/L	$^{1,c}H$=-0.89+0.056C	0.9788
	50	10-400 nmol/L	8 nmol/L	$^{1,c}H$ =-0.83+0.11C	0.9948
	400	0.04-20 pmol/L	0.02 pmol/L	$^{1,b}H$ =2.13+0.31C	0.9998
L-AAOD + HRP	+400	2-100 pmol/L	0.08 pmol/L	$^{2,b}H$ =-260.78+80.0C	0.9982
	650	8-100 pmol/L	2 pmol/L	$^{3,b}H$ =3.23+0.1C	0.9974
D-AAOD	650	0.001- 0.8 fmol/L	$8x10^{-4}$ fmol/L	$^{1,a}H$ =0.31+0.41C	0.9953
	130	20-100 nmol/L	8 nmol/L	$^{2,c}H$ =24.99+0.14C	0.9980
	550	0.6-40 fmol/L	0.4 fmol/L	$^{1,a}H$ =0.11+0.27C	0.9992
D-AAOD+ HRP	650	0.002-0.4 fmol/L	0.001 fmol/L	$^{2,a}H$ =0.015+2.045C	0.9939

* H is the peak height in $^{1}\mu A$, ^{2}nA, and ^{3}mA and C is the concentration of L-and D-pipecolic acid, respectively, in $^{a}fmol/L$, $^{b}pmol/L$ and $^{c}nmol/L$.

5.6.4.2 Selectivity of the amperometric biosensors

The selectivity of all biosensors was checked using both the mixed solutions and separate solutions methods with respect to L- and D-pipecolic acid. Amperometric selectivity coefficients (K_{amp}) were determined at the same potentials used for the determination of the response characteristics of the amperometric biosensors following the method proposed by Wang [40]. The ratio between the concentration of the enantiomer of interest and the other enantiomer was 1:10.

Table 5.26 Enantioselectivity of the amperometric biosensors designed for the simultaneous assay L- and D-pipecolic acids in a SIA system. All values are the average of ten determinations

Enzyme(s) used for the design of the biosensor	Interferent	E(mV)	pK_{amp}
L-AAOD	D-PA	650 400 50	3.11 2.45 2.63
L-AAOD + HRP		650 400	3.01 3.14
D-AAOD	L-PA	650 550 130	3.33 2.97 2.73
L-AAOD + HRP		650	3.96

The values of the amperometric selectivity coefficients (pK_{amp}) (obtained using the mixed solution method) for all the biosensors designed for L- and D-PA are higher than 2.00, revealing that all the proposed biosensors are enantioselective when used as detectors in SIA system (Table 5.26). The best enantioselectivity for the assay of L-PA was achieved by the bienzymatic sensor is based on L-AAOD and HRP when measurements are performed at 400 mV, while the best enantioselectivity for the assay of D-PA was achieved by using the bienzymatic sensor based on D-AAOD and HRP when

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measurements are performed at 650 mV. Also the biosensor based on D-AAOD exhibit a good enantioselectivity for the assay of D-PA at 650 mV.

5.6.4.3 Analytical applications

The SIA system incorporated with the amperometric biosensors proved to be useful for the simultaneous assay of L- and D-pipecolic acid. The results (Tables 5.27 and 5.28) obtained by using different ratios between L- and D-pipecolic acid demonstrated the suitability of the proposed SIA/amperometric biosensors system for the enantioanalysis of pipecolic acid due to the good recovery values obtained for the assay of one of the enantiomers in the presence of its antipode.

Table 5.27 Determination of L-pipecolic acid in the presence of D-pipecolic acid

Enzyme(s) used for the design of the biosensors	E(mV)	Average recovery*, (%)				
		L:D (mol:mol)				
		2:1	1:1	1:2	1:4	1:9
L-AAOD	650	99.56±0.02	98.99±0.04	99.77±0.05	99.22±0.06	99.01±0.05
	400	99.55±0.03	99.17±0.04	99.63±0.03	99.98±0.02	99.20±0.03
	50	99.65±0.07	99.87±0.05	99.77±0.05	99.22±0.06	99.01±0.05
L-AAOD + HRP	650	99.72±0.03	99.53±0.04	99.37±0.04	99.96±0.04	99.90±0.03
	400	99.59±0.04	99.82±0.03	99.79±0.03	99.13±0.03	99.24±0.04

All values are the average of 10 determinations.

No significant differences in the recovery values were recorded for the ratios between L:D or D:L enantiomers varying from 1:9 to 1:99.99.

Table 5.28 Determination of D-pipecolic acid in the presence of L-pipecolic acid

Enzyme(s) used for the design of the biosensors	E (mV)	Average recovery L-PA*, (%) D:L (mol:mol)				
		2:1	1:1	1:2	1:4	1:9
D-AAOD	650	99.46±0.03	99.38±0.03	99.77±0.02	99.45±0.02	99.48±0.04
	550	99.84±0.04	99.80±0.03	99.26±0.03	99.18±0.03	99.47±0.02
	130	99.83±0.06	99.72±0.05	99.61±0.05	99.62±0.04	99.88±0.04
D-AAOD + HRP	650	99.20±0.04	99.15±0.05	99.69±0.03	99.39±0.04	99.42±0.05

All values are the average of 10 determinations.

The results obtained for the assay of L- and D-pipecolic acid in serum samples are shown in Tables 5.29 and 5.30. The samples were collected from different patients suspected of peroxisomal disorders and liver cirrhosis for the simultaneous assay of L- and D-pipecolic acid, respectively. The results using the proposed SIA/amperometric biosensors system at different working potentials are in good concordance with those obtained using the standard method [41].

Table 5.29 Recovery of L-pipecolic acid in serum samples (μmol/L)

Sample No.	Standard method	L-AAOD			L-AAOD+HRP	
		650 mV	400mV	50mV	650mV	400mV
1	1.10	1.09±0.01	1.08±0.03	1.09±0.01	1.07±0.02	1.10±0.01
2	2.30	2.28±0.02	2.29±0.02	2.28±0.03	2.30±0.02	2.27±0.02
3	0.80	0.79±0.02	0.80±0.02	0.79±0.03	0.8±0.01	0.78±0.03

All values are the average of 10 determinations.

Table 5.30 Recovery of D-pipecolic acid in serum samples (nmol/L)

Sample No.	Standard method	D-AAOD			D-AAOD + HRP
		650 mV	550mV	130mV	650mV
1	30.00	29.83±0.03	29.40±0.03	29.81±0.02	29.70±0.03
2	13.00	12.88±0.02	12.87±0.01	12.91±0.02	12.89±0.02
3	27.50	27.41±0.02	27.39±0.02	27.98±0.03	27.47±0.02

All values are the average of 10 determinations.

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5.7 Conclusion

Different types of electrochemical techniques have been applied for the enantioanalysis of L- and D-pipecolic acid in biological fluids using carbon or diamond paste based electrodes. The construction of the proposed electrodes is simple, fast and reproducible and it is also assuring reliable response characteristics for the proposed electrodes.

The proposed EPMEs proved to be successful for the enantioanalysis of L-pipecolic acid in serum samples, helping for a fast and reliable diagnosis of peroxisomal disorders. The serum samples did not need any pre-treatment before the analysis.

The proposed amperometric biosensors and the amperometric electrode based diamond paste have excellent features for enantioselective clinical analysis. The selection of the new type of matrix (diamond paste) as well as of the best working potential in the assay of the enantiomers of L- and D-pipecolic acid proved to have a high effect on the performances of the amperometric biosensors, in terms of sensitivity, limit of detection, linear concentration range, response time and enantioselectivity. Due to the high biocompatibility of the diamond, the biosensors will be able to be used, after miniaturization, for *in vivo* assay of L- and D-pipecolic acid.

34 serum samples may be analyzed in 1 hour, without the need of any pre-treatment by using SIA/amperometric biosensor system for the simultaneous assay of L- and D-pipecolic acids in serum samples. The main advantages of the system are high reliability of analytical information, rapidity, simplicity and low cost of analysis. The selection of

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the enzyme(s) and working potentials in the assay of the enantiomers of L- and D-pipecolic acid proved to have a high influence on the performance of amperometric biosensors in terms of sensitivity, limit of detection, linear concentration range, and enantioselectivity. The best amperometric biosensors that can be used as detectors in the proposed sequential injection analysis system proved to be the one based on L-AAOD and HRP and the one based on D-AAOD for the assay of L- and D-PA, when the measurements are performed at 400 and 650 mV, respectively.

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Chapter 6

Diamond paste-based electrodes for the determination of L- and D-fucose using differential pulse voltammetry

6.1 Introduction

Electrochemical techniques have been developed for the analysis of trace amounts of many organic molecules of biological significance. The selection of electrode material is very crucial for the selective assay of specific group of compounds. Different materials have been used, such as glass carbon, pyrolytic graphite, and carbon paste for the amperometric electrodes design. Glassy carbon and pyrolytic graphite electrodes have got practical problems, such as electrode surface fouling due to binding of reaction products and non-electrochemically active biochemical species [1]. Diamond exhibits several unique features, which make it superior to those conventional electrode materials [2-12]. Polycrystalline boron-dopped diamond thin film electrodes have been used in electrochemical studies due to their wide electrochemical potential window allowing a sensitive electroanalytical detection of chemical species that reacts at relatively high potentials [5], low background current, long-term stability due lack of adsorption of chemical species [8] on the inert electrode surface and finally their relative insensitivity to dissolved oxygen in both alkaline and acidic aqueous electrolytes [13,14].

The advances in single-crystal diamond have enabled the development of a wide range of monocrystalline diamond products. Electrical properties of the monocrystalline diamond,

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based on the mobilities of negative electrons and positive holes, the inherent structural defects and the presence of small amounts of impurities in its structure enhance its use for research in the design of electrochemical sensors [15, 16].

Differential pulse voltammetry has proved to be effective for the determination of several chiral compounds in biological, pharmaceutical and clinical matrices involving limited or no sample preparations such as the time-consuming extraction techniques. Chirality remains a vital diagnosis tool as biological marker for many clinical diseases and a key parameter in the development of pharmaceuticals and agrochemicals [17]. In many cases only one isomer in a chiral compound is responsible for the desired activity, while the other may exhibit no value or interference. Recent descriptions of diseases producing abnormal chiral metabolites have provided the need for individual enantiomer assay.

The fucose enantiomers are L- and D-6-deoxygalactoses monosaccharide (Figure 6.1). D-fucose exists in simple glycosides comprising only a few sugar units, limited to plant products, microbial and antibiotic substances [18]. The L-enantiomer occurs in many bacterial and plant glycosides and polysaccharides [18]. L-fucose is also found in oligosaccharides of human milk, and in many glycolipids and glycoproteins, including several families of blood group antigens [19]. Changes have been detected in the fucosylation pattern of these molecules in the tissues of cancers patients, due to fucosyltarnsferase activity, which is especially high in the serum of patients suffering from high malignant or metastatic tumors (such as colon carcinoma, breast and liver cancer) [20]. Serum and urinary levels of free L-fucose of cancer patients had

significantly higher levels than healthy persons [18-19]. Clinically, levels of free L-fucose in serum and urine, can be used as a marker for tumor malignancy.

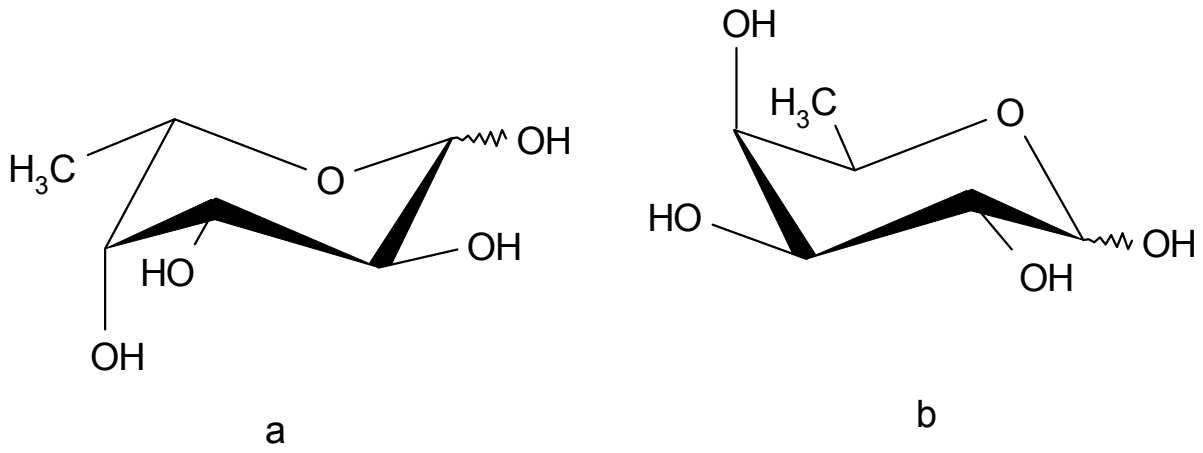

Figure 6.1. Chair structures of fucose (a) L-fucose and (b) D-fucose

Different analytical methods have been used for the assay of L- and D-fucose including enzymatic assay [21-22], gas chromatography [23], high performance liquid chromatography [24-25], anion exchange chromatography [26], and spectrophotometry [27-29].

The aim of this chapter is to develop a simple and reliable method based on utilization of diamond electrodes for the determination of L- and D-fucose in biological fluids (e.g., serum, urine). A diamond paste was preferred for the electrode design due to the high reliable construction [30]. A natural and two synthetic types of diamonds (1 and 2) were used for the design of the diamond paste.

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6.2 Experimental section

6.2.1 Apparatus

A 663 VA stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 100 and software (Eco Chemie version 4.9) were used for all cyclic and differential pulse voltammetric measurements. An Ag/AgCl (0.1 mol/l KCl) electrode and a Pt electrode were used as reference and counter electrodes in the cell.

6.2.2 Diamond paste electrodes design

Three diamond paste electrodes were prepared for L- and D-fucose using different diamond powder (natural diamond, synthetic 1 and synthetic 2). All diamond paste electrodes were prepared by mixing 100mg of each diamond powder with 20μL of paraffin oil. A portion of the diamond paste was then placed in a plastic pipette tip (3mm). The electrode diameter was approximately 2.3mm. Electrical conductivity was achieved by inserting a silver wire (0.5 mm in diameter) into the diamond paste. Before each use, the electrode surface was smoothed by polishing with alumina foils (polishing strips 30144-001, Orion). All sensors were stored at room temperature, when not in use.

6.2.3 Recommended procedures

6.2.3.1 Cyclic voltammetry

For the optimization step, all cyclic voltammetry scans between –1.0 and +1.0 V were carried out. The diamond paste-based sensors were used as working electrodes, an Ag/AgCl was used as reference electrode and a Pt wire as counter electrode. The scan

rate was adjusted to 20mV/s. The experiments were carried out under deaerated conditions.

6.2.3.2 Direct differential pulse voltammetry (DPV)

The technique used for the direct assay was differential pulse voltammetry with applied potential pulse amplitude of 25 V, scan rate of 20 mV/s, pulse width of 50 ms, and pulse time of 40 ms. All measurements were carried out at 25 ^{o}C. The diamond electrode together with the reference and auxiliary electrodes were dipped into the compartment cell containing 10^{-4} mol/L of L- or D-Fucose, phosphate buffer and supporting electrolyte (0.1mol/L). All conditions of different DPV scan measurements are summarized in Table 6.1. The peak heights of different electrodes, measured at the suitable potentials, were plotted versus the concentrations of L- or D-fucose. The unknown concentrations of L- and D-fucose were determined from the calibration graphs.

6.2.4 Reagents and materials

All solutions were prepared by using deionized water (A Modulab System, Continental Water Systems, San Antonio, TX). L- and D-fucose, monocrystalline natural diamond powder (1 μm) (99.9%) and monocrystalline synthetic diamond powder 1 μm (synthetic-1) (99.9%) and 50 μm (synthetic-2) (99.9%) powder were purchased from Aldrich (Milwaukee, WI). The paraffin oil was purchased from Fluka (Buchs, Switzerland). 250 mL of 10^{-2} mol/L of L- and D-fucose were prepared as stock solutions. All solutions of different concentrations were prepared from the stock solutions by serial dilutions. Phosphate buffer (pH 7) was prepared from KH_2PO_4 (Saarchem-Holpro Analytic)) and

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Na_2HPO_4 (Chemical Suppliers) using de-ionized water. Solutions of different pH values (1-10) were prepared by the addition of HCl or NaOH solutions (0.1mol/L) to the solutions containing L- or D-fucose. The pH value was monitored using pH-meter (Orion, Model 420A, Labotec).

6.2.5 L-fucose samples.

Three serum samples (1-3) and three urine samples (4-6) of suspected cancer patients were provided from local hospitals. The direct DPV method was used for the analysis of L-fucose concentrations in the serum and urine samples.

6.3 Results and discussion

6.3.1 Optimization of working conditions

Optimization of working conditions was carried out for the different types of diamond paste-based electrodes using L- and D-fucose as analytes. Cyclic voltammograms for different electrolyte solutions and pHs (1-10) were recorded before and after the addition of different aliquots of L- or D-fucose, separately. Four different supporting electrolytes (0.1mol/L NaCl, 0.1mol/L KCl, 0.1mol/L $NaNO_3$, and 0.1mol/L KNO_3) were tested. Figures 6.2 and 6.3 show the effect of pH and supporting electrolyte on the peak height for L-fucose and D-fucose, respectively. The shapes and heights of the peaks contribute to the selection of the optimum electrolyte and pH. The best peak shapes for the three L-fucose assay electrodes were recorded at: pH 3.0 using NaCl as electrolyte, at pH 10.0 using KNO_3 as electrolyte, and at pH 10.0 using KCl as electrolyte for natural diamond, synthetic 1 and synthetic 2 base electrodes.

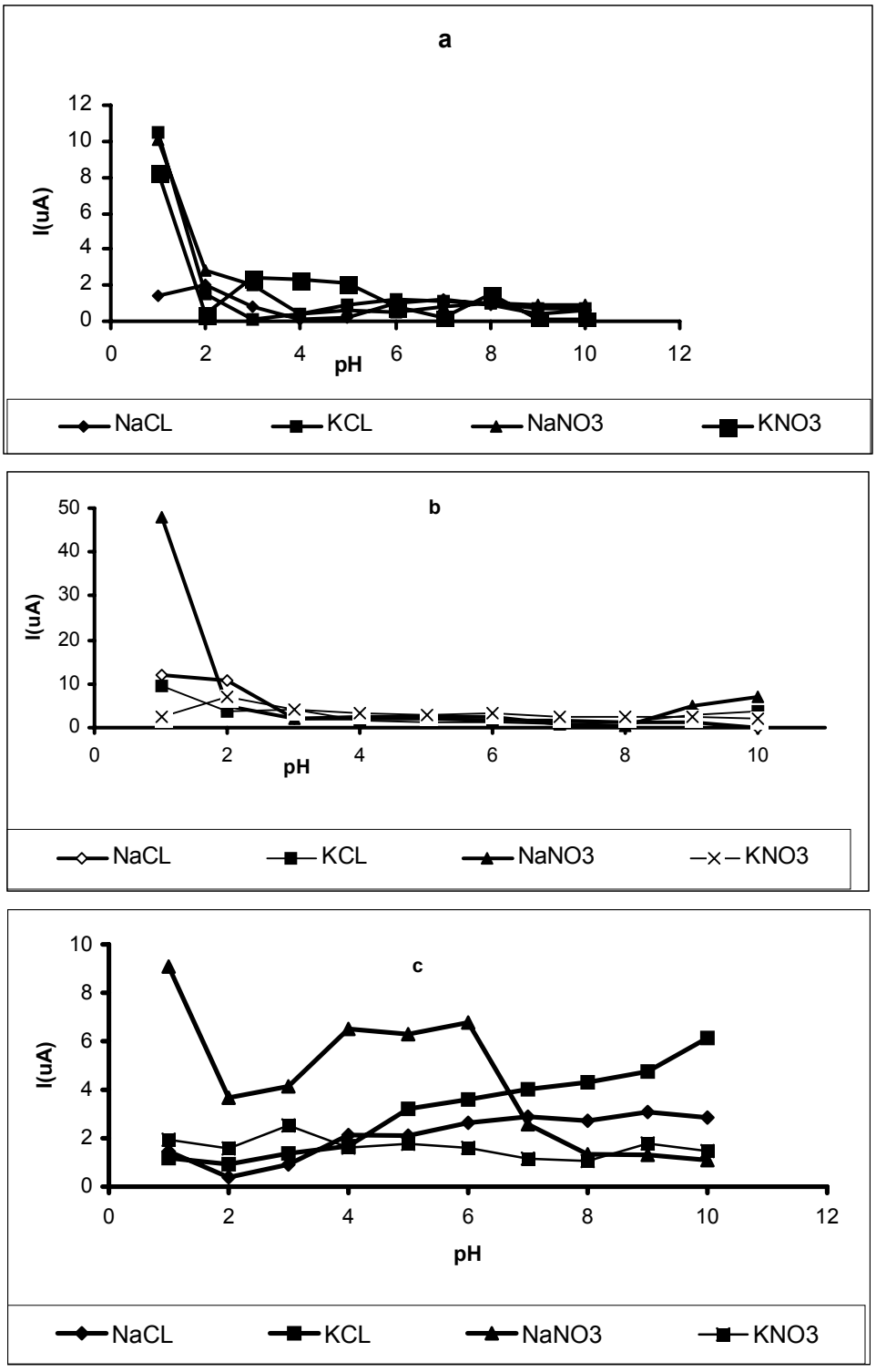

Figure 6.2 The effect of electrolyte and pH on the peak height (μA) for a concentration of L-Fucose solution of 10^{-4} mol/L when (a) natural diamond, (b) synthetic-1, and (c) synthetic-2 based electrodes are used.

The peaks measured a pH 1.0 in 0.1 mol/L of $NaNO_3$ were approximately higher than the peaks recorded for the rest of the electrolytes at different pHs. Despite of these highest peaks, they were broad causing poor resolution which enhances the effect of interferences in the determination of L-fucose in serum and urine samples. As well, the optimum conditions for D-fucose electrodes based on natural diamond, synthetic-1, and synthetic-2 diamonds were recorded at: pH 1.0 using KCl as electrolyte, at pH 1.0 using KNO_3 as electrolyte, and at pH 1.0 using $NaNO_3$ as electrolyte. Table 6.1 summarizes the optimum working conditions, pH and supporting electrolyte, for L- and D-fucose assay using the diamond paste based electrode.

Table 6.1 Optimum conditions for L- and D-fucose determination

Analyte	Optimization factor	**Electrodes Based on**		
		Natural diamond	Syntheic-1	Synthetic-2
L-fucose	Electrolyte (0.1mol/l)	NaCl	KNO_3	KCl
	pH	3.00	10.00	10.00
	Potential range (mV)	0.00-500	0.00-500	0.00-300
	Peak Position(mV)	240	130	90
	Background current (μA)	0.895	0.5967	0.068
D-fucose	Electrolyte (0.1mol/l)	KCl	KNO_3	$NaNO_3$
	pH	1.00	1.00	3.00
	Potential range (mV)	0.00-350	0.00-350	-50.00-350
	Peak Position(mV)	120	130	130
	Background current (μA)	17.43	3.722	0.232

All measurements were made at 25^{o}C; all values are the average of ten determinations.

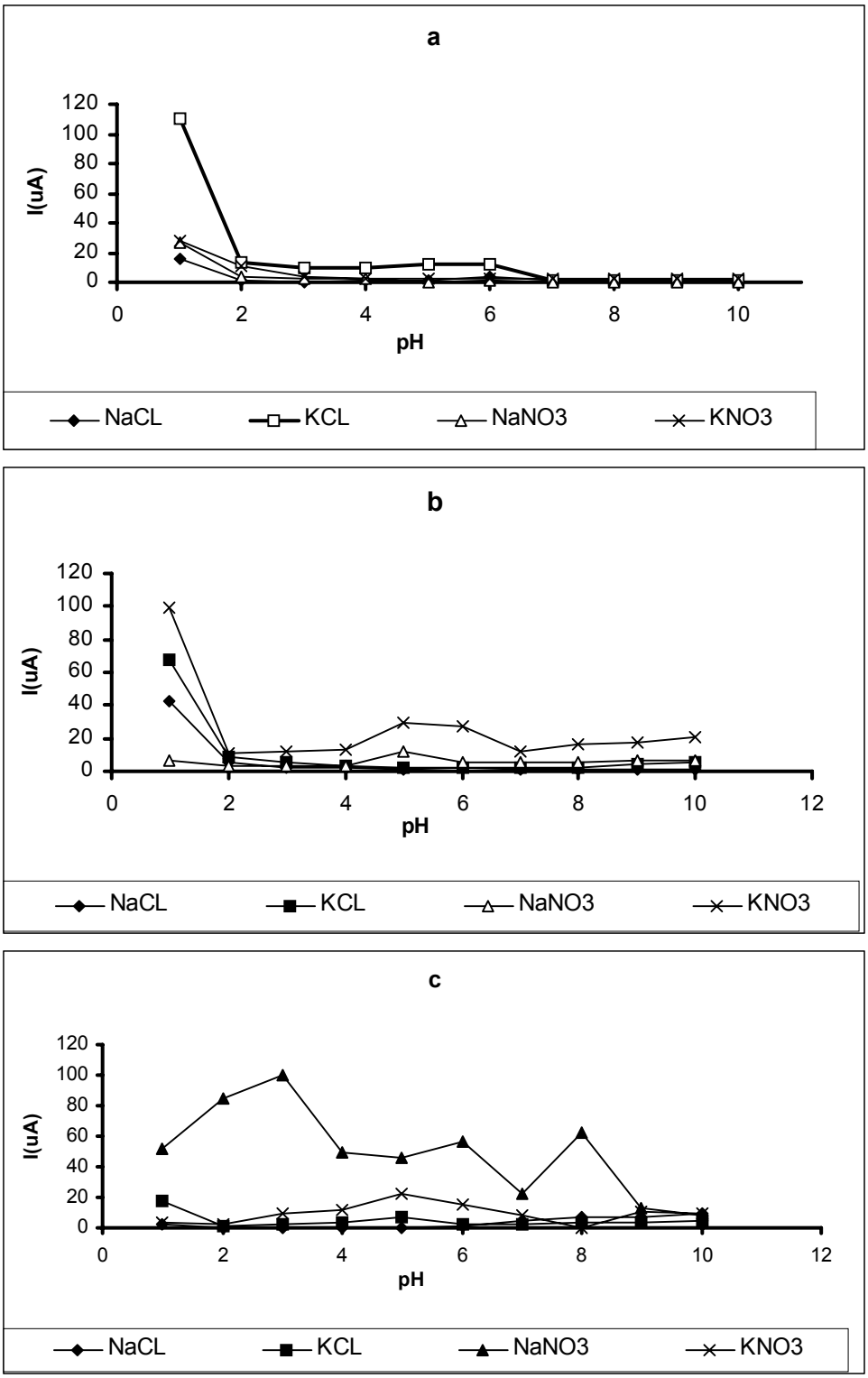

Figure 6.3 The effect of electrolyte and pH on the peak height (μA) for a concentration of D-Fucose solution of 10^{-4} mol/L when (a) natural diamond, (b) synthetic-1, and (c) synthetic-2 based electrodes are used..

6.3.2 Response of the diamond paste based electrodes

The response characteristics of the diamond paste-based electrodes for L- and D-fucose were determined using DPV. The responses of the electrodes were linear over wide concentration ranges and can be described by the calibration equations for L- and D-fucose (Table 6.2). The limits of detections were calculated using the following equation [31]:

$$DL = \frac{I_B + 3\sigma_S - a}{S} \qquad \textbf{(6.1)}$$

where I_B is the background current recorded, σ_S is the standard deviation for the measurement of the background current, a is the slope of the calibration equation.

Table 6.2 Response characteristics of the diamond paste-based amperometric electrodes for L- and D-fucose assay**.**

		Electrodes based on		
		Natural diamond	Synthetic-1	Synthetic-2
L-fucose	Equation of calibration	$H^a=1.38+1.99C^d$	$H^a=1.13+3.35C^e$	$H^b=10.67+32.04C^c$
	Linear concentration range (mol/L)	$10^{-13} – 10^{-9}$	$10^{-11} – 10^{-8}$	$10^{-6} – 10^{-3}$
	Limit of detection (mol/L)	10^{-14}	10^{-12}	10^{-8}
	Regression coefficient (r)	0.9994	0.9940	0.9931
D-fucose	Equation of calibration	$H^a=0.48+0.2C^c$	$H^a=1.41+0.08C^c$	$H^a=3.74+3.66C^c$
	Linear concentration range (mol/L)	$10^{-6} – 10^{-3}$	$10^{-5} – 10^{-3}$	$10^{-9} – 10^{-3}$
	Limit of detection (mol/L)	10^{-7}	10^{-7}	10^{-10}
	Regression coefficient (r)	0.9999	0.9994	0.9917

[a] All measurements were made at 25°C; all values are the average of ten determinations H is the peak height ([a] μA and [b] nA), C is the concentration of L- or D-fucose ([c] mmol/L, [d] nmol/L, and [e] pmol/L) and r is the regression coefficient.

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The peak heights for natural diamond, synthetic-1, and synthetic-2 diamonds based electrodes were proportional to the L- and D-fucose concentrations with low detection limits as shown in table 6.2. A previous comparison analysis using monocrystalline diamonds (natural, synthetic-1, and synthetic-2), glassy carbon (GC), and carbon paste (CP) electrodes was shown by our group [32]. The signal to noise ratio for the diamond paste-based electrodes was higher comparing to GC and CP [32]. The diamond paste-based electrodes proved a good reproducibility of peak current according to the obtained values of standard deviations (RSD < 1%).

6.3.3 Selectivity of the diamond paste electrodes

The selectivity of all the diamond paste-based electrodes was checked. Mixed and separate solutions method was used to calculate the amperometric selectivity coefficients [30]. The ratio between L- or D-fucose and the interfering species was 1:10 (mol/mol). The amperometric selectivity coefficients for diamond paste-based electrodes (Table 6.3) proved the enantioselectivity of the proposed electrodes. Accordingly, these results proved that L- and D-fucose can be analyzed one in the presence of the other, using the proposed electrodes.

Table 6.3 Amperometric Selectivity Coefficients[a]

Analyte(I)/ Interfering species (J)	K_{sel}^{amp}		
	Electrodes based on		
	Natural diamond	Synthetic-1	Synthetic-2
L-fucose/D-fucose	3.95×10^{-3}	7.05×10^{-3}	4.19×10^{-4}
D-fucose/L-fucose	4.06×10^{-3}	4.41×10^{-3}	1.21×10^{-3}

[a] All measurements were made at 25°C; all values are the average of ten determinations.

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6.3.4 Analytical applications

The optimum working conditions (Table 6.1) were used for all recovery tests. The suitability of the proposed diamond paste electrodes for the enantioanalysis of fucose was determined using solutions containing both enantiomers in different ratios. The recovery values for the assay of one enantiomer in the presence of its antipode proved no significant differences in these results for the ratios between L:D or D:L enantiomers varying from 2:1 to 1:9 (RSD <1%), (Tables 6.4 and 6.5).

Table 6.4 Recovery of L-fucose in the presence of D-fucose

	Average recovery, (%)[a]		
L:D (mol:mol)	Electrodes based on Natural diamond	Electrodes based on Synthetic-1	Electrodes based on Synthetic-2
2:1	99.92±0.03	99.97±0.03	99.95±0.04
1:1	99.90±0.02	99.99±0.04	99.93±0.03
1:2	99.89±0.02	99.96±0.04	99.94±0.03
1:4	99.96±0.03	99.97±0.04	99.95±0.04
1:9	99.92±0.03	99.95±0.04	99.96±0.03

[a] All measurements were made at 25°C; all values are the average of ten determinations.

Table 6.5 Recovery of D-fucose in the presence of L-fucose

	Average recovery, (%)[a]		
D:L (mol:mol)	Electrodes based on Natural diamond	Electrodes based on Synthetic-1	Electrodes based on Synthetic-2
2:1	99.48±0.03	99.62±0.02	99.56±0.03
1:1	99.67±0.03	99.76±0.03	99.63±0.03
1:2	99.99±0.02	99.86±0.03	99.76±0.02
1:4	99.98±0.03	99.85±0.02	99.70±0.02
1:9	99.99±0.02	99.87±0.02	99.69±0.03

[a] All measurements were made at 25°C; all values are the average of ten determinations.

The results obtained demonstrated the suitability of diamond paste electrodes for the enantioanalysis of fucose in biological fluids (serum and urine) (Table 6.6). The recovery tests performed for the assay of L-fucose in serum and urine samples using DPV were higher than 99.00% with RSD < 1% (n=10). Due to precision and accuracy of results, diamond electrodes can be reliably used for the enantioselective assay of L-fucose in serum and urine samples with a high average recovery and low RSD % values.

Table 6.6 Recovery of L-fucose from serum and urine samples using electrodes based on natural diamond, synthetic 1 and synthetic 2

Type of sample	L-fucose, Average recovery		
	Sample No.	Standard method [27]	Amperometric electrodes
Electrodes based on Natural diamond (pmol/L)			
Serum	1	60	59.73±0.024
	2	233	232.25±0.07
	3	333	331.83±0.1
Urine	4	430	428.67±0.129
	5	500	498.40±0.20
	6	910	906.18±.0.273
Electrodes based on Synthetic-1 (nmol/L)			
Serum	1	6.00	5.99±0.01
	2	23.3	23.24±.0.01
	3	33.3	33.23±0.01
Urine	4	43.0	42.90±0.02
	5	50.0	49.87±0.02
	6	91.0	90.76±0.02
Electrodes based on Synthetic-2 (μmol/L)			
Serum	1	18.0	17.92±0.01
	2	70.0	69.75±0.02
	3	100.0	99.69±0.04
Urine	4	280.0	279.10±0.11
	5	500.0	498.45±0.15
	6	910.0	907.82±0.36

[a] All measurements were made at 25°C; all values are the average of ten determinations

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6.3.5 Statistical comparison between diamond paste electrodes and the standard method for fucose analysis

Statistical comparison between the results obtained for L- and D-fucose using the diamond paste electrodes and the standard method were performed for all serum and urine samples using the paired t-test. These data were compared statistically with the standard method at the 99.00% confidence level. The two methods are compared by analyzing several different samples. The comparison refers to paired data, and t_{calc} is computed by the expression [33],

$$t_{calc} = \left| \bar{X}_A - \bar{X}_B \right| \sqrt{\frac{N(N-1)}{\sum (d_i - \bar{d})^2}} \qquad (6.2)$$

where $\bar{X}_A$ and $\bar{X}_B$ are the average recoveries of L-fucose using standard method and proposed diamond paste electrodes method, respectively. N is number of paired samples, which are analyzed by both methods, d_i is the individual difference between the two methods for each sample, and $\bar{d}$ is the mean of all d_i values. t_{calc} values were 2.96, 0.89, and 2.37 for natural, synthetic-1, and synthetic-2 diamond paste electrodes method versus the standard method. All t_{calc} values, at the 99.00% confidence level, are less than the tabulated theoretical t-value: 4.032. Since all experimental t_{calc} are less than theoretical t-value, there is no statistically significant difference between the results obtained by the standard method and diamond paste electrodes.

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6.4 Conclusion

Monocrystalline diamonds (natural, synthetic-1, and synthetic-2) were found to be excellent materials for the electrodes designs, and enantioanalysis of L- and D-fucose. The electrodes were enantioselective, precise, and accurate with low detection limits. The design is simple and the electrodes exhibit a long term stability. The low RSD (<1%) values of the recovery tests using DPV, proved the reliability of analytical results. This work is opening an excellent vision of miniaturization of the electrodes for application for *in vivo* analysis of L-fucose in patients with various types of cancer.

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Chapter 7

Enantioselective, potentiometric membrane electrodes for the determination of L- and D-glyceric acids

7.1 Introduction

Inborn errors of metabolism disorders are rare genetic diseases. They can be diagnosed by assay of organic acids (e.g., L(D)-glyceric acids) in human body fluids. Glyceric acid (2,3-dihydroxypropionic acid, Figure 7.1) exists in two configurations, L- and D-enantiomers in mammalian metabolism.

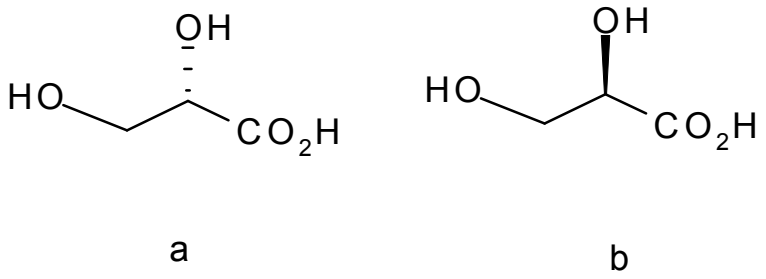

Figure 7.1 Glyceric acid enantiomers (a) L(-)-glyceric acid and (b) D-(+)-glyceric acid

The presence of one of these enantiomers in blood or urine in abnormal concentration caused a different type of illness. Enantiomers may originate from different metabolic pathway, due to the enzymes' deficiencies. D-glyceric dehydrogenase (D-GDH) catalyzes the interconversion of hydroxypyruvate to D-glycerate in the degradation pathway of serine metabolism [1]. D-GDH also has a glyoxylate-reductase (GR) activity, and it catalyzes the cytoplasmic reduction of glyoxylate to glycolate [2]. The genetic deficiency of D-GDH and GR causes a metabolic disorder named primary hyperoxaluria

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type 2 (PH II) or L-glyceric acidurias/acidemia [3,4] where hydroxypyruvate is converted to L-glycerate by L-lactate dehydrogenase (LDH) in the presence of NADH (Figure 7.2) [5].

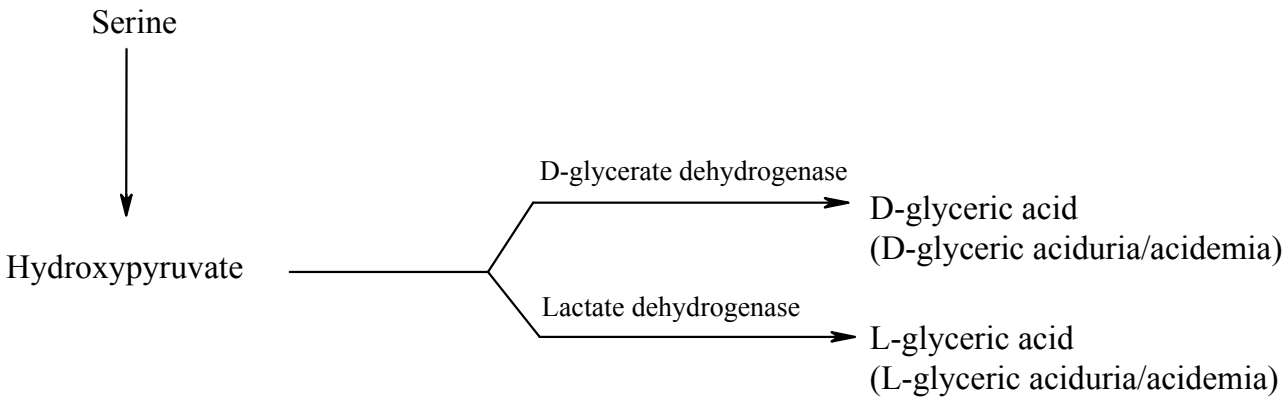

Figure 7.2 Metabolic pathways of L- and D-glyceric acid [5].

The metabolic disorder leads to excessive urinary oxalate and excretion of L-glycerate. PH II is characterized by urolithiasis or nephrocalcinosis with terminal renal failure [6]. Primary hyperoxaluria type 1 (PH I) is caused by deficiency of hepatic alanine: glyoxylate aminotransferase (AGT), which promotes the transamination of glyoxylate to oxalate. Deficiency of AGT caused oxidation of glyoxylate to oxalate and glycolate by glycolate oxidase and reductase, respectively [7,8]. PH I and PH II are described by marked increase of oxalate production associated with hyperglycolate or hyper-L-glycerate in blood and urine [9].

Another disease entity involving glyceric acid is D-glyceric acidemia/aciduira. This disease was first reported by Brandt, *et. al.* [10,11] showing ketotic hyperglycinemia with abnormal excretion of D-glyceric acid in urine and serum. D-glyceric acid is an

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intermediate in the pathways of serine degradation and fructose metabolism [11,12]. This increase of excretion was explained by deficiency of D-glycerate kinase that is involved in the conversion of D-glyceric acid to D-2-phosphoglycerate [13-17]. D-glyceric acidemia/acidurias is associated with delayed psychomotor growth, mental retardation and seizures [11, 12, 14, 18]. The different syndrome entity of increased excretion of L and D-glyceric acid in urine and serum is a hallmark of two totally different inborn diseases, hyperoxaluria II and D-glyceric acidamia/aciduira, respectively. Accordingly, enantioanalysis of L- and D-GA is important for the diagnosis of patients with PH II and D-glyceric acidamia/aciduira.

Several methods have been reported for the configurational analysis of L- and D-GA based on capillary electrophoresis [19], colorimetry [20], polarimetry [15] and chromatographic methods [9, 21-26].

Enantioanalysis of chiral substances of clinical importance is a vital subject for the biomedical applications and early discovery of illnesses. Enantioselective, potentiometric membrane electrodes (EPMEs) based on chiral selectors had been developed for enantiomeric assay. Cyclodextrins [27, 28], macrocyclic antibiotics [29], and maltodextrins [30] were used as chiral selectors in the design of EPMEs.

In this chapter, nine EPMEs based on different chiral selector have been designed for the enantioanalysis of L- and D-glyceric acids in serum and urine sample. EPMEs based on maltodextrin I, maltodextrin III, α-CD, γ-CD and vancomycin were used for the

determination of L-glyceric acid while EPMEs based on maltodextrin III and β-CD, 2-hydroxy-3-trimethylammoniopropyl-β-CD and teicoplanin were used for the analysis of D-glyceric acid. The proposed EPMEs were applied for the enantiomers analysis of L- and D-glyceric acids in biological fluid.

7.2 Reagents and materials

L- and D-glyceric acids, vancomycin and teicoplanin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Graphite powder (1-2μm, synthetic) and maltodextrins [DE 4.0-7.0 (I), 13.0-17.0 (II), and 16.5-19.5 (III)] were purchased from Aldrich (Milwaukee, WI, USA). Paraffin oil was purchased from Fluka (Buchs, Switzerland). α-, β-, γ-, and 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrins were supplied by Wacher-Chemie GmbH (Germany). Phosphate buffer (pH = 3.5) was supplied by Merck (Darmstadt, Germany).

De-ionized water from a Modulab system (Continental Water Systems, San Antonio, TX, USA) was used for all reagents and solutions preparation. 0.1mol/L stock solutions of L- and D-glyceric acids were buffered with phosphate buffer (pH 3.5). Solutions of L- and D-glyceric acid ($1x10^{-10}$ - $1x10^{-2}$ mol/L) were prepared by serial dilution from the stocks solutions of L- and D- glyceric acids and were buffered with phosphate buffer (pH 3.5).

10^{-3} mol/L solutions of each maltodextrin (I, II and III), and cyclodextrins (α-, β-, γ-, and 2-hydroxy-3-trimethylammoniopropyl-β-CD) were prepared. The solution of vancomycin ($2x10^{-3}$ mol/L) was prepared in phosphate buffer (pH 4.00). The solution of teicoplanin

($2x10^{-3}$ mol/L) was prepared using pH 6.00 phosphate buffer. The solution of teicoplanin ($2x10^{-3}$ mol/L) containing acetonitrile was prepared using pH 6.00 phosphate buffer containing 40% (v/v) of acetonitrile.

7.3 Enantioselective, potentiometric membrane electrodes based on maltodextrins

7.3.1 Apparatus

All chronopotentiometric (zero current) measurements were recorded using a Metrohm 663 VA stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 20 and a software version 4.9 (Eco Chemie, Utretch, The Netherlands). An Ag/AgCl (0.1 mol/L KCl) electrode was used as reference electrode in the cell.

7.3.2 Electrodes design

Plain carbon paste was prepared by thoroughly mixing 100 mg of graphite powder with 40 µL paraffin oil. Paraffin oil and graphite powder [1:4, (w/w)] were mixed well, followed by the addition of 10^{-3} mol/L aqueous solution of maltodextrin [DE 4.0-7.0 (I), 13.0-17.0 (II), or 16.5-19.5 (III)] (100 µL of each maltodextrin solution to 100 mg graphite powder). The plain carbon paste was filled into a plastic pipette peak leaving a space of 3-4 mm into the top to be filled with the modified carbon paste that contains the maltodextrin. The diameter of enantioselective, potentiometric membrane electrode was 3 mm. Electric contact was obtained by inserting a Ag/AgCl wire into the carbon paste. The internal electrolyte solution of EPMEs was 0.1 mol/L KCl. All the EPMEs tips were

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gently rubbed on fine abrasive paper to produce a flat surface. The surface of the electrodes was wetted with de-ionized water and then polished with an alumina paper (polished strips 30144-011, Orion) before use for the analysis. When not in use, L- and D-GA electrodes were immersed in 10^{-3} mol/L L- or D-glyceric acid solution, respectively.

7.3.3 Recommended procedure

Direct potentiometric method was used for potential determination of each standard solution (10^{-10}-10^{-2} mol/L), utilizing two electrodes system. The electrodes were placed in stirred standard solutions. Calibration graphs were obtained by plotting E (mV) versus p(L-GA) or p(D-GA), respectively. The unknown concentrations of L- and D-glyceric acids were determined in serum and urine samples by interpolation of the potential measured into the calibration graphs (E (mV) versus p(L-GA) or E (mV) versus p(D-GA)).

7. 3.4 Results and discussion

7. 3.4.1 EPMEs response characteristics

The response characteristics exhibited by the EPMEs impregnated with maltodextrins were determined for enantiomers, L-glyceric and D-glyceric acids at pH=3.5 (phosphate buffer). The response obtained for L-glyceric acid was near-Nernstian only for maltodextrins I and III based EPMEs, while the response obtained for D-glyceric acid was near-Nernstian only for EPME based on maltodextrin II. The linear concentration ranges obtained are the following: for L-glyceric: 10^{-8}-10^{-6}mol/L and 10^{-6}-10^{-3}mol/L for

EPMEs based on maltodextrin I and III, respectively, and for D-glyceric acid 10^{-5}-10^{-3} mol/L. The equations of calibration and the corresponding correlation coefficients (r) are as follows:

EPME based on maltodextrin I:	E(mV) = 58.00 p(L-GA) + 517.67	r = 0.9681
EPME based on maltodextrin III:	E(mV) = 59.07 p(L-GA) - 198.00	r = 0.9994
EPME based on maltodextrin II:	E(mV) = 59.00 p(D-GA) - 156.67	r = 0.9999

where E (mV) is the cell potential, p(L-GA) = -log [L-GA] and p(D-GA) = -log [D-GA]. The limits of detection obtained for the assay of L-glyceric acid are 1.19 x 10^{-9} and 1.00 x 10^{-7} mol/L when maltodextrin I and III based EPME, respectively, are used while for the assay of D-glyceric acid the limit of detection is 1.00x10^{-6}mol/L when the EPME based on maltodextrin II is used. The electrode based on maltodextrin I exhibited the lowest limit of detection. The electrodes responses have a good stability and reproducibility for the tests performed for 1 month, when used daily for measurements (RSD<0.1%). The best stability was recorded for EPME based on maltodextrin I, because maltodextrin I has got a more rigid conformation compared to maltodextrin II and III [31].

The best response time was recorded for the maltodextrin II, where the electrode potential was recorded in 1min for the concentration range 10^{-5}-10^{-3}mol/L. The response time recorded for the assay of L-glyceric acid is 2min for maltodextrin I based EPME in the concentration range 10^{-8}-10^{-6}mol/L and 90s for maltodextrin III based EPME in the concentration range 10^{-6}-10^{-3}mol/L.

7. 3.4.2 Effect of pH on the responses of the electrodes

Influence of pH on the response of the electrodes was studied by recording the emf of the cell containing solutions of L- or D-glyceric acid at different pH values (pH range 1-10). For L-glyceric, measurements were performed for a concentration of 10^{-6} mol/L, whereas for D-glyceric measurements were investigated for a concentration of 10^{-4}mol/L. These solutions were prepared by adding very small volumes of HCl or NaOH solution (10^{-1} mol/L or 1 mol/L of each) to L- or D-glyceric acid solutions, respectively.

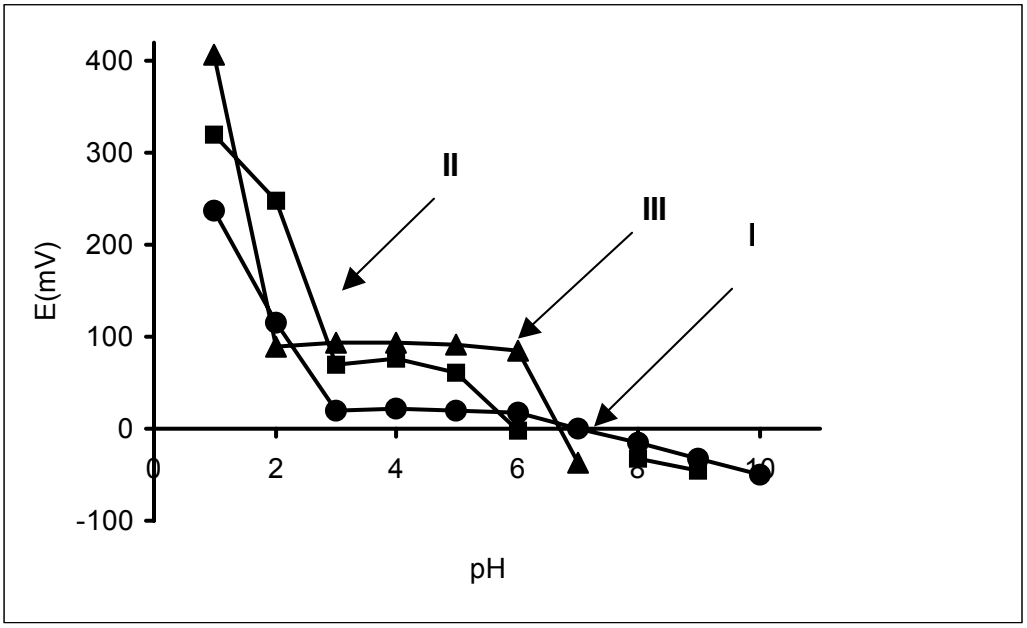

Figure 7.3 Influence of pH variation on the response of EPMEs based on maltodextrins (I) and (III) to solutions of L-glyceric acid (10^{-6} mol/L L-GA) and of EPME based on maltodextrin (II) to solutions of D-glyceric acid (10^{-4}mol/L).

The plots of E(mV) versus pH show the influence of pH variation on the responses of EPMEs (Figure 7.3). For the assay of L-glyceric acid, the responses of EPMEs based on maltodextrins I and III are pH-independent in the pH ranges 3.0-6.0 and 2.0-6.0 while for

the assay of D-glyceric acid the response of EPME based on maltodextrins II is pH-independent in the pH range 3.0-5.0.

7. 3.4.3 Selectivity of the electrodes

The selectivity of the electrodes was investigated using the mixed solutions method [32]. The selectivity of the proposed electrodes was checked over L- or D-GA, creatine and creatinine. A ratio of 1:10 was used between the concentrations (mol/L) of analyte and possible interferent. The potentiometric selectivity coefficients, K_{sel}^{pot}, for maltodextrin (I) and (III) based EPMEs proved that D-GA, creatine and creainine do not interfere in the determination of L-GA; as well, K_{sel}^{pot} values of maltodextrin (II) based EPME proved that L-GA, creatine and creatinine do not interference in the assay of D-GA (Table 7.1). The interference of inorganic cations such a Na^{+}, K^{+}, and Ca^{2+} was verified for all proposed EPMEs; the values of K_{sel}^{pot} (all lower than 10^{-3}) shown that these ions do not interfere in the analysis of L- and D-GA.

Table 7.1 Selectivity coefficients of enantioselective, potentiometric membrane electrodes.

Interfering species (J)	K_{sel}^{pot}		
	EPME based on		
	Maltodextrin I	Maltodextrin III	Maltodextrin II
L-glyceric acid	-	-	3.98×10^{-3}
D-glyceric acid	3.89×10^{-3}	3.98×10^{-3}	-
Creatine	4.05×10^{-3}	7.50×10^{-3}	7.51×10^{-3}
Creatinine	8.26×10^{-3}	3.82×10^{-3}	3.83×10^{-3}

All measurements were made at room temperature; all values are the average of ten determinations.

7. 3.4.4 Analytical applications

The suitability of the proposed EPMEs for the enantioanalysis of GA was investigated by recovery of each enantiomer in the presence of its antipode. Therefore, solutions containing different ratios between L:D or D:L (2:1 to 1:99.99) enantiomers of glyceric acid were prepared. The recovery tests demonstrated the suitability of the enantioselective, potentiometric membrane electrodes for the analysis of the enantiopurity of GA (Tables 7.2 and 7.3). No significant differences in the recovery values were recorded for the ratios between L:D or D:L enantiomers varying from 1:9 to 1:99.99.

Table 7.2 The results obtained for the determination of L-glyceric acid in the presence of D-glyceric acid.

L:D (mol/mol)	EPMEs based on % L-GA, Recovery	
	Maltodextrin I	Maltodextrin III
2:1	99.28±0.01	99.46±0.01
1:1	99.71±0.01	99.42±0.02
1:2	99.69±0.01	99.51±0.03
1:4	99.24±0.02	99.31±0.01
1:9	99.72±0.01	99.84±0.01

All measurements were made at room temperature; all values are the average of ten determinations.

Table 7.3 The results obtained for the determination of D-glyceric acid in the presence of L-glyceric acid.

D:L (mol/mol)	EPME based on maltodextrin II % D-GA, Recovery
2:1	99.87±0.03
1:1	99.80±0.02
1:2	99.48±0.01
1:4	99.27±0.01
1:9	99.63±0.03

All measurements were made at room temperature; all values are the average of ten determinations.

Healthy volunteers donated three serum and five urine human samples. These samples were stored at -20^{o}C before use. Serum and urine samples were spiked with L- or D-glyceric acid to make the final concentrations as shown in Tables 7.4 and 7.5. Serum (1-3) and urine (4-8) samples were spiked with different aliquots of L-GA. On the other hand, serum (9-11) and urine (12-16) samples were spiked with aliquots of D-GA. These samples were used for the recovery of L-GA and D-GA in the real human matrices. The results obtained for the analysis of L–GA and D-GA in serum and urine samples are shown in Tables 7.4 and 7.5, respectively. The results obtained by using the proposed EPMEs are in good concordance with the quantities of the acids added to the real samples, showing the suitability of the electrodes for the diagnosis of the associated diseases.

Table 7.4 Recovery of L-glyceric acid in serum and urine samples.

Type of sample	Sample no.	Spiked L-GA final concentrations (mg/L)	EPMEs based on % L-GA, Recovery	
			Maltodextrin I	Maltodextrin III
Serum	1	0.0212	99.59±0.02	99.89±0.03
	2	0.2120	98.89±0.05	99.90±0.02
	3	0.6360	99.40±0.07	99.27±0.02
Urine	4	0.0424	99.29±0.02	99.35±0.01
	5	0.0848	99.39±0.01	99.27±0.01
	6	0.1060	99.50±0.03	99.18±0.01
	7	0.4240	99.14±0.08	99.15±0.03
	8	0.7420	99.44±0.04	99.18±0.02

All measurements were made at room temperature; all values are the average of ten determinations.

Table 7.5 Recovery of D-glyceric acid in serum and urine samples.

Type of sample	Sample no.	Spiked D-GA final concentrations (mg/L)	EPME based on Maltodextrin II
			% D-GA, Recovery
Serum	9	2.120	99.30±0.02
	10	21.20	99.76±0.05
	11	63.60	99.76±0.03
Urine	12	4.240	99.64±0.08
	13	8.480	99.94±0.08
	14	10.60	99.39±0.02
	15	42.40	99.78±0.01
	16	74.20	99.82±0.01

All measurements were made at room temperature; all values are the average of ten determinations.

7.4 Enantioselective, potentiometric membrane electrodes based on cyclodextrins

7.4.1 Apparatus

The direct potentiometric measurements was recorded using a Metrohm 663 VA stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 20 and a software version 4.9 (Eco Chemie, Utretch, The Netherlands). An Ag/AgCl (0.1 mol/l KCl) electrode was used as reference electrode in the cell.

7.4.2 Electrodes design

Graphite powder and paraffin oil were mixed with in a ratio 1:4 (w/v) followed by the addition of solution containing $1x10^{-3}$mol L^{-1} of α-, β-, γ-, and 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrins (100μL chiral selector solution to 100 mg carbon paste). A plain carbon paste was also prepared by thoroughly mixing 100 mg of

graphite powder with 40µL paraffin oil. The plain carbon paste was filled into a plastic pipette peak leaving a space of 3-4 mm into the top to be filled with the carbon paste that contains the chiral selector. The diameter of enantioselective, potentiometric membrane sensor was 3 mm. Electric contact was obtained by inserting Ag/AgCl wire into the carbon paste. The internal electrolyte solution of EPMEs was 0.1mol L^{-1} KCl. All the EPMEs tips were gently rubbed on fine abrasive paper to produce a flat surface. The surface of the electrodes was wetted with de-ionized water and then polished with an alumina paper (polished strips 30144-011, Orion) before use for the analysis. When not in use, L- and D-GA electrodes were immersed in a 10^{-3} mol/L solution of L- or D-glyceric acid, respectively.

7.4.3 Recommended procedure

The direct potentiometric method was used for the potential determination of each standard solution (10^{-10}-10^{-2} mol/L, pH 3.50) of L- and D-glyceric acids. Calibration graphs were obtained by plotting E (mV) versus pL-GA and pD-GA respectively. The unknown concentrations of L- and D-glyceric acid were determined in serum and urine samples by interpolation of the potential measured, into the calibration plots.

7. 4.4 Results and discussion

7. 4.4.1 EPMEs response characteristics

The truncated cone shape of cyclodextrins and its relative hydrophobic cavity able to host analytes by means of inclusion-complexation is the reason of interaction beween enantiomer and chiral selectors. Weak bonds between substituent groups on the

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asymmetric center of analytes and secondary and/or primary hydroxyl groups of the CD are mainly responsible for chiral recognition [33]. From the proposed EPMEs, only those based on α-CD and γ-CD worked for the assay of L-glyceric acid, while β-CD and 2-hydroxy-3-trimethylammoniopropyl-β-CD based EPME worked for the asay of D-glyceric acid. The calibration equations obtained for the L- and D-glyceric acid are:

$$E(mV) = -59.00\ pL\text{-}GA + 639.00 \qquad (\alpha\text{-CD based EPME})$$

$$E(mV) = 52.80\ pL\text{-}GA - 64.30 \qquad (\gamma\text{-CD based EPME})$$

$$E(mV) = 58.00\ pD\text{-}GA + 100.33 \qquad (\beta\text{-CD based EPME})$$

$$E(mV) = 59.00\ pD\text{-}GA - 160.50 \qquad (\beta\text{-derivative-CD based EPME})$$

where E (mV) is the cell potential and pL-GA = -log [L-GA] and pD-GA = -log [D-GA]. All the response characteristics of the electrodes are shown in Table 7.6. EPMEs displayed a good stability and reproducibility over the tests performed for 2 months, when they were used daily for measurements.

The response times were lower than 1 min and 1min for the EPMEs designed for the assay of L-glyceric acid, respectively based on α- and γ-CD in the concentration ranges (10^{-9}-10^{-7} and 10^{-5}-10^{-2} mol/L). The response times for the determination of D-glyceric acid was higher than 1min when EPME based on β-CD was used in the range 10^{-5}-10^{-3} mol/L and lower than 1min when EPME based on β-derivative-CD was ued in the concentration range 10^{-6}-10^{-3} mol/L.

Table 7.6 Response characteristics of EPMEs for the determinations of D- and L-glyceric acids.

Analyte	Chiral selector	EPMEs characteristics			
		Slope [mV/p(C)]	Intercept, E^o [mV]	Linear range [mol/L]	Detection limit [mol/L]
L-GA	α-CD	59.00	139.00	10^{-9}-10^{-7}	$1.48x10^{-11}$
	γ-CD	52.80	-64.30	10^{-5}-10^{-2}	$1.00x10^{-6}$
D-GA	β-CD	58.00	-100.33	10^{-5}-10^{-3}	$1.00x10^{-6}$
	β-derivative-CD	59.00	-160.50	10^{-6}-10^{-3}	$1.00x10^{-7}$

[a]All measurements were made at room temperature; all values are the average of ten determinations.

7.4.2.2 Effect of pH on the responses of the electrodes

The influence of the pH on the response of the proposed EPMEs was checked by measuring the potential of the potentiometric cells at pHs between 1 and 10. Solutions of pHs between 1 and 10 of L- and D-glyceric acid were prepared by adding different volumes of HCL (0.1 mol/L) or NaOH solutions (0.1 mol/L) to their standard solutions. Plots showing the variation of E (mV) with pH values are shown in Figure 7.4a and 7.4b. For L-glyceric acid, the responses of EPMEs are pH-independent in the pH ranges 2.0-5.0 (α-CD based EPME) and 2.0-6.0 (γ-CD based EPME), while for D-glyceric acid the repones of EPMEs are not depending on pH in the ranges 3.0-5.0 (β-CD based EPME) and 4.0-7.0 (β-derivative-CD based EPME).

7. 4.2.3 Selectivity of the electrodes

The selectivity of electrodes has been investigated using the mixed solutions method proposed by Ren [32] and it was checked against L- and D-glyceric acid, creatine, creatinine, Na^+, K^+ and Ca^{+2}. The ratio between the concentration of the main analyte and interfering ion was 1:10. The potentiometric selectivity coefficients, K_{sel}^{pot} Table 7.7, proved that L (D)-glyceric acid, creatine and creatinine do not interfere in the

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determination of L- and D-glyceric acid, and that the proposed EPMEs are enantioselective. Also, inorganic cations such a Na^{+}, K^{+}, and Ca^{2+} do not interfere in the analysis of L- and D-GA, as the values of K_{sel}^{pot} obtained were lower than 10^{-3}.

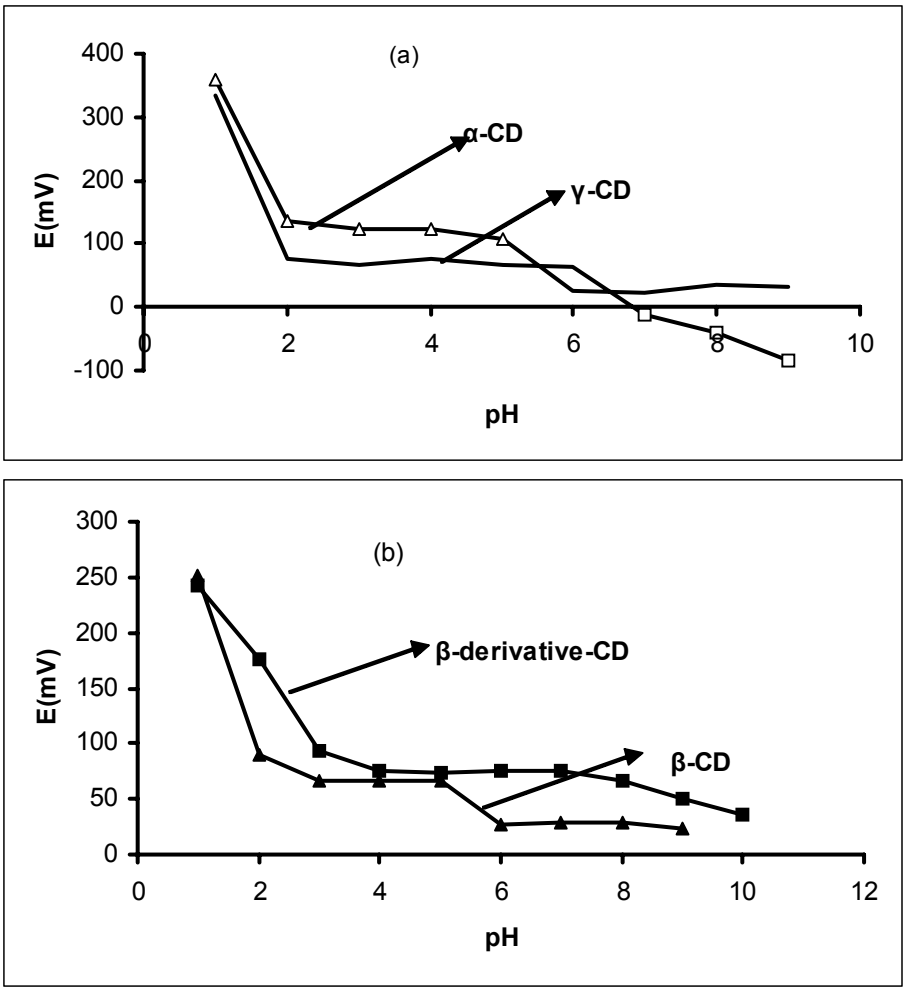

Figure 7.4 (a) Influence of pH variation on the response of EPMEs based on α- and γ-cyclodextrins, respectively, for the assay of L-glyceric acid, (b) Influence of pH variation on the response of EPMEs based on β-cyclodextrin and 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrin (as chloride salt) (β-CD-derivative) for the assay of D-glyceric acid.

Table 7.7 Selectivity coefficients for the response of EPMEs for the determination of L- and D-glyceric acid.[a]

Interfering species (j)	K_{sel}^{pot} of EPME based on			
	α-CD	γ-CD	β-CD	B-derivative-CD
L-glyceric acid	-	-	3.89×10^{-3}	3.98×10^{-3}
D-glyceric acid	3.98×10^{-3}	4.46×10^{-3}	-	-
Creatine	3.83×10^{-3}	4.27×10^{-3}	4.05×10^{-3}	7.51×10^{-3}
Creatinine	7.51×10^{-3}	8.35×10^{-3}	7.63×10^{-3}	3.83×10^{-3}

[a]All measurements were made at room temperature; all values are the average of ten determinations.

7. 4.2.4 Analytical applications

Table 7.8 The recovery results obtained for the analysis of L-glyceric acid in the presence of D-glyceric acid[a]

L:D (mol/mol)	% L-GA, Recovery	
	EPMEs based on	
	α-CD	γ-CD
2:1	99.88±0.01	99.38±0.01
1:1	99.60±0.03	99.69±0.01
1:2	99.71±0.02	99.94±0.02
1:4	99.65±0.01	99.68±0.02
1:9	99.73±0.01	99.52±0.01
D:L (mol/mol)	% D-GA, Recovery	
	EPMEs based on	
	β-CD	β-derivative-CD
2:1	99.17±0.04	99.99±0.01
1:1	99.52±0.01	99.97±0.01
1:2	99.78±0.01	99.98±0.01
1:4	99.17±0.02	99.98±0.02
1:9	99.99±0.03	99.96±0.01

[a] All measurements were made at room temperature; all values are the average of ten determinations.

The suitability of EPMEs was investigated for the recovery of L- and D-GA in the solutions containing the antipode of the enantiomer assayed. Solutions containing L:D or

D:L of glyceric acid were prepared in different ratios (2:1 to 1:99.99) to check the recovery for L- and D-GA, respectively.

The recovery tests demonstrated the suitability of the enantioselective, potentiometric membrane electrode for the enantioanalysis of L- and D-GA (Table 7.8). No significant differences in the recovery values were recorded for the ratios between L:D or D:L enantiomers varying from 1:9 to 1:99.99.

Table 7.9 Recovery of L-glyceric acid in serum and urine samples[a].

Type of sample	Sample no.	% L-GA, recovery		
		Chromatographic method [19]	EPMEs based on	
			α-CD	γ-CD
Serum	1	99.36	99.44±0.01	99.09±0.04
	2	99.68	98.65±0.02	99.72±0.02
	3	99.39	98.37±0.03	99.03±0.03
Urine	4	99.75	99.76±0.02	99.73±0.03
	5	99.57	99.54±0.02	99.59±0.01
	6	99.60	99.52±0.01	99.69±0.01
	7	66.57	99.60±0.03	99.50±0.02
	8	99.80	99.81±0.01	99.72±0.01
	9	99.63	99.62±0.01	99.65±0.04

[a]All measurements were made at room temperature; all values are the average of ten determinations.

Healthy volunteers donated serum and urine human samples. These samples were stored at -20^{o}C. Serum and urine samples were spiked with L- and D-glyceric acid These samples were used for the recovery of L-GA and D-GA in the real matrices and to show the suitability of the EPMEs for the enantioanalysis of L- and D-GA in serum and urine samples. The results obtained for the analysis of L–glyceric and D-glyceric acid in serum and urine samples (Table 7.9 and 7.10) were compared with those obtained using a

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chromatographic method of analysis [19]. The results obtained using the proposed EPMEs are in good concordance with those obtained using the chromatographic method [19] showing the suitability of the proposed EPMEs for diagnosis of hyperoxaluria type 2 (PH II) and D-glyceric acidemia/aciduria.

Table 7.10 Recovery of D-glyceric acid in serum and urine samples[a]

Type of sample	Sample no.	% D-GA, Recovery		
		Chromatographic method [19]	EPME based on	
			β-CD	β-derivative-CD
Serum	1	99.44	99.08±0.02	99.55±0.01
	2	99.79	99.27±0.01	99.98±0.02
	3	99.51	99.36±0.01	99.87±0.02
Urine	4	99.47	99.57±0.02	99.33±0.02
	5	99.68	99.75±0.01	99.63±0.01
	6	99.87	99.99±0.01	99.52±0.02
	7	99.76	99.93±0.02	99.66±0.01
	8	99.78	99.91±0.02	99.59±0.02
	9	99.72	99.89±0.01	99.67±0.01

[a] All measurements were made at room temperature; all values are the average of ten determinations.

7.5 Enantioselective, potentiometric membrane electrodes based on macrocyclic antibiotics

7.5.1 Apparatus

A 663 VA Stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 100 and software (Eco Chemie version 4.9) was used for all chronopotentiometric (zero current) measurements. An Ag/AgCl (0.1 mol/l KCl) electrode was used as reference electrode in the cell.

7.5.2 Electrodes design

Paraffin oil and graphite powder were mixed in a ratio 1:4 (w/w) followed by the addition of the aqueous solutions of vancomycin (pH=4) and teicoplanin (pH=6) (10^{-3} mol/l) (100 µl chiral selector solution to 100 mg carbon paste to form the modified carbon paste. A plastic pipette peak was filled with carbon paste, a space of 3-4 mm was left into its top to be filled with the modified carbon paste. The diameter of enantioselective, potentiometric membrane electrodes was 3 mm. Electric contact was obtained by inserting Ag/AgCl wire into the carbon paste. The internal solution used for EPME was 0.1 mol/L KCl. All the sensors tips were gently rubbed on fine abrasive paper to produce a flat surface. The surface of the sensors was wetted with de-ionized water and then polished with an alumina paper (polished strips 30144-011, Orion) before use for the analysis. When not in use, the electrodes were immersed in a 10^{-3} mol/l L- or D-glyceric acid solution, respectively.

7.5.3 Recommended procedure

Direct potentiometric method was used for potential determination of each solution (10^{-10}-10^{-2} mol/l). Calibration graphs were obtained by plotting E(mV) versus p(L-GA) or p(D-GA), respectively. The unknown concentrations were determined from the calibration graphs.

7.5.4 Results and discussion

7.5.4.1 Response characteristics of EPMEs

The response characteristics of EPMEs were determined for both enantiomers, L-glyceric (L-GA) and D-glyceric acid (D-GA) at pH=3.5 (phosphate buffer). The response obtained for L-GA was linear and near-Nernstian only when vancomycin was used as chiral selector for the design of EPME, while response obtained for D-GA was linear and near-Nernstian only when teicoplanin based EPME was used. The equations of calibration obtained for L-GA and D-GA when vancomycin and teicoplanin based EPME were used, respectively, are as follows:

L-glyceric acid: $E = 574.6\text{-}58.6\ p$ (L-GA) $\quad r = 0.9957$

D-glyceric acid: $E = 206 - 50\ p$ (D-GA) $\quad r = 0.9988$

where E (mV) is the cell potential, p (L-GA) = -log [L-GA] and p (D-GA) = -log [D-GA]. The response characteristics of the two EPMEs are shown in Table 7.11. The limits of detection are very low of 10^{-10} and 10^{-5} mol/L orders of magnitude for L-glyceric acid and D-glyceric acid, respectively. The electrodes responses displayed a good stability and reproducibility for the tests performed for 3 months, when daily used for measurements (RSD<1.0%).

The response time recorded for the assay of L-glyceric acid is higher than 2 min in the 10^{-9} – 10^{-7} mol/L concentration range, while for D-glyceric acid is lower than 2 min in the 10^{-4}-10^{-2} mol/L concentration range.

Table7.11 Response characteristics of enantioselective, potentiometric membrane electrodes for L-and D-glyceric acid [a]

Analyte	Parameters			
	Slope (mV/decade)	Intercept, E^o (mV)	Linear range (mol/L)	Detection limit (mol/L)
L-glyceric acid	58.6(mV/*p*L-GA)	574.6	10^{-9}-10^{-7}	$1.56x10^{-10}$
D-glyceric acid	50.00(mV/*p*L-GA)	206	10^{-4}-10^{-2}	$7.60x10^{-5}$

[a] All measurements were made at room temperature; all values are the average of ten determinations.

7.5.4.2 The influence of pH on the responses of the EPMEs

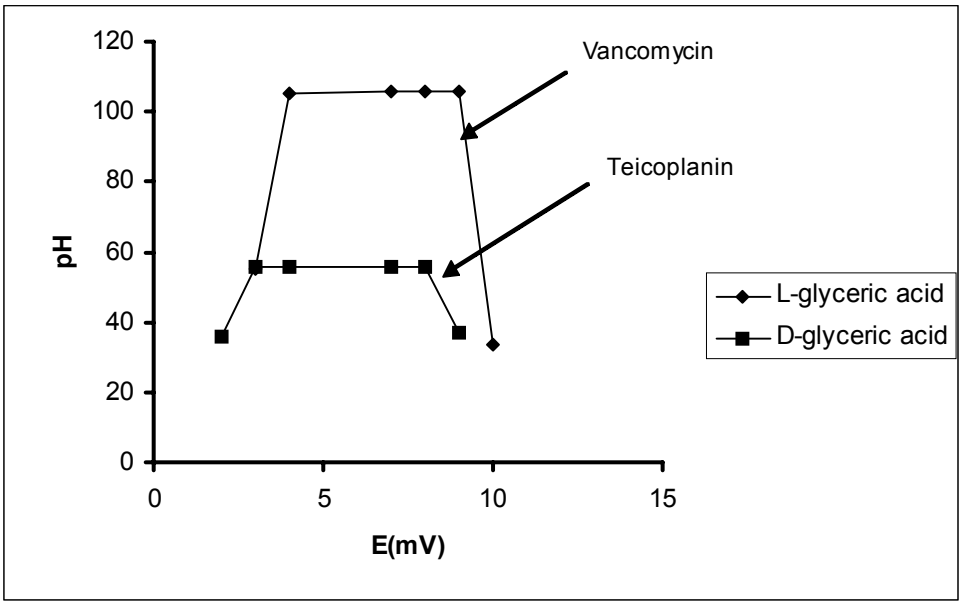

Figure 7.5 Effect of pH on the response of the EPMEs to L-glyceric acid (10^{-8} mol/l L-GA), and D-glyceric acid (10^{-3}mol/l) solutions. (I) Vancomycin based EPME; (II) Teicoplanin based EPME

The effect of pH on the response of the electrodes was checked by recording the emf of the cell. Solutions of L-(10^{-8} mol/L) and D-(10^{-3}mol/L) glyceric acids having different pHs were prepared by adding small volumes of HCl (0.1 mol/L) or NaOH solution (0.1 mol/L). Plots of E (mV) vs. pH (Figure 7.5) show that the emf does not depend on pH in the ranges of 4.0-9.0 for vancomycin and 3.0-8.0 teicoplanin based EPMEs, respectively.

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7.5.4.3 Selectivity of the EPMEs

The selectivity of both electrodes was checked using the mixed solutions method proposed by Ren [32], over L-(or D)-glyceric acid, creatine, creatinine and some inorganic ions. The ratio between the concentrations of interfering ion and enantiomer was 10:1. The potentiometric selectivity coefficients (Table 7.12) for vancomycin and teicoplanin based EPMEs proved that L(D)-GA, creatine and creatinine do not interfere in the determination of L- and D-GA demonstrating the enantioselectivity property of the EPMEs. Inorganic cations such a Na^+, K^+, and Ca^{2+} do not interfere in the analysis of L- and D-GA.

Table 7.12 Selectivity coefficients for the response of the enantioselective membrane electrodes used for L- and D-glyceric acid assay.[a]

Interference species (J)	pK^{pot}_{sel}	
	Vancomycin based EPME	Teicoplanin based EPME
L-glyceric acid	-	2.39
D-glyceric acid	2.415	-
Creatine	2.086	2.08
Creatinine	2.41	2.39

[a]All measurements were made at room temperature; all values are the average of ten determinations.

7.5.4.4 Analytical applications

Solutions containing L- and D-GA in different ratios were prepared to test the recovery for each enantiomer and the suitability of the EPMEs for the enantioanalysis of L- and D-GA in serum and urine samples. The results of recovery for enantioanalysis of each enantiomer in the presence if it antipode (Tables 7.13 and 7.14) proved the suitability of the electrodes. No significant differences in the recovery values were recorded for the ratios between L:D or D:L enantiomers varying from 1:9 to 1:99.99.

Table 7.13 The results obtained for the determination of L-glyceric acid in the presence of D-glyceric acid[a]

L:D (mol/mol)	Recovery, %
2:1	99.25±0.01
1:1	99.75±0.02
1:2	99.26±0.06
1:4	99.30±0.04
1:9	99.67±0.06

[a]All measurements were made at room temperature; all values are the average of ten determinations.

Table 7.14 The results obtained for the determination of D-glyceric acid in the presence of L-glyceric acid[a]

D:L (mol/mol)	Recovery, %
2:1	99.96±0.04
1:1	99.57±0.03
1:2	99.99±0.03
1:4	99.95±0.02
1:9	99.93±0.03

[a]All measurements were made at room temperature; all values are the average of ten determinations.

Table 7.15 Recovery of L-glyceric acid in serum and urine samples, (%)[a]

Type of sample	Sample no.	% Recovery, L-GA	
		Standard method [24]	EPMEs
Serum Samples	1	98.47	98.52±0.04
	2	98.15	98.08±0.08
	3	98.02	98.00±0.06
Urine samples	4	99.30	99.25±0.02
	5	99.50	99.49±0.03
	6	99.45	99.50±0.03
	7	99.86	99.87±0.02
	8	99.12	99.13±0.01
	9	99.89	99.99±0.02

[a]All measurements were made at room temperature; all values are the average of ten determinations.

The results obtained for the analysis of L–glyceric and D-glyceric acid in serum and urine samples are shown in Tables 7.15 and 7.16, respectively. Serum and urine samples were

collected from patients suspected of L-glyceric academia (1-3) or aciduria (4-9) and D-glyceric acidemia (10-12) or aciduria (13-18). The results obtained using the proposed EPMEs is in good concordance with those obtained using the standard method [34]

Table 7.16 Recovery of D-glyceric acid in serum and urine samples, (%)[a]

Type of sample	Sample no.	% Recovery, D-GA	
		Standard method [24]	EPMEs
Serum Samples	10	97.7.0	97.23±0.02
	11	96.70	96.65±0.03
	12	97.70	97.21±0.08
Urine samples	13	99.20	99.18±0.02
	14	99.50	99.48±0.01
	15	99.93	100.00±0.02
	16	99.43	99.40±0.03
	17	99.15	99.12±0.02
	18	99.11	99.13±0.02

[a]All measurements were made at room temperature; all values are the average of ten determinations.

7.6 Conclusion

The proposed enantioselective, potentiometric membranes electrodes have excellent features in the real time enantioselective analyses of glyceric acid in biological fluids, e.g., serum and urine samples. The construction of the electrodes is simple, fast and reproducible. One of the main advantages of the proposed method is that the serum and urine samples need only a minimum of pre-treatment before the assay of any of the enantiomers that makes the method simple, fast and economical.

EPMEs based on maltodextrin I, maltodextrin III, α-CD, γ-CD and vancomycin were applicable for the determination of L-glyceric acid while EPMEs based on maltodextrin

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II, β-CD, 2-hydroxy-3-trimethylammoniopropyl-β-CD and teicoplanin were used for the analysis of D-glyceric acid. These EPMEs describe a reliable direct method for the analysis of L- and D-glyceric acids in real human fluids (serum and urine). EPMEs exhibited a good enantioselectivity over D- or L-glyceric acid, creatine , creatinine and inorganic ions.

For L-glyceric acid determination, α-CD based EPME has the best slope and the lower limit of detection, while 2-hydyroxy-3-trimethylammoniopropyl-β-CD has the best slope and the lower limit of detection. All the proposed EPMEs can be used successfully for fast and reliable diagnosis of L- or D-glyceric acidemia/aciduria. Miniaturization of the electrodes will make possible *in vivo* diagnosis of hyperoxaluria type 2 (PH II) and D-glyceric acidemia/acidurias

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Chapter 8

Diagnosis of L- and D-2-hydroxyglutarias using enantioselective, potentiometric membrane electrodes

8.1 Introduction

Organic acidurias is a group of inherited metabolic disorders characterized by increased excretion of organic acids. 2-Hydroxyglutaric acid (2-HGA) occurs in human urine of healthy individuals in minimal concentrations, and exists in the L- and D- configurations (Figure 8.1). Excess excretion of L- or D-2-hydroxyglutaric acid in urine is a clinical marker for L- or D-2-hydroxyglutaric acidurias. D-2-hydroxyglutaric aciduria is a rare neurometabolic disorder. The severe cases are characterized by encephalopathy of early infantile onset, seizures, hypotonia, cortical blindness and marked developmental delay [1-2]. L-2-hydroxyglutaric aciduria is a rare inborn error of metabolism [3]. It is an autosomal recessively inherited neurodegenerative disorder characterized by psychomotor retardation and progressive ataxia combined with sub-cortical leukoencephalopathy and cerebellar atrophy [4]. D-2-hydroxyglutaric aciduria is a severe neurological syndrome with neonatal onset, while L-2-hydroxyglutaric aciduria is usually associated with slowly progressive encephalopathy presenting in childhood [2]. The differentiation between these diseases requires the enantioanalysis of 2-hydroxyglutaric acid.

L-2-Hydroxyglutaric acid (L-2-HGA) may be found in abnormally higher concentrations in urine as a result of genetic errors or metabolic disorders [5,6] and it is a marker for L-

2-hydroxyglutaric aciduria (a rare neurometabolic disorder) [7-9]. L-2-hydroxyglutaric aciduria was first described by Duran [6]. Its effects are: mental retardation, progressive ataxia combined with subcortical leukoencephalopathy, cerebral atrophy, seizures, pyramidal and extra pyramidal symptoms and severe cerebral dysfunction [8, 10-12].

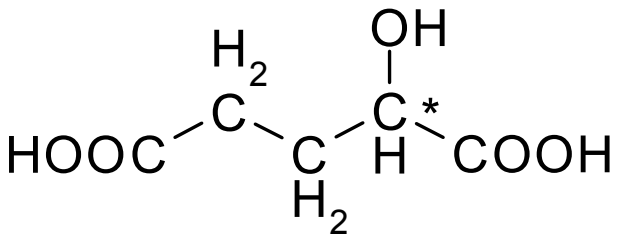

Figure 8.1 2-hydroxyglutaric acid

D-2-hydroxyglytaric aciduria is a rare genetic neurometabolic disorder biochemically characterized by high urinary excretion of D-2-hydroxygluaric acid. It was first described in 1980 by Chalmers et.al. [13]. This disorder is clinically characterized by two wide spectrums of phenotypes. The first one is a severe form with early-infantile-onset encephalopathy, seizures, hypotonia, crotical blindness and marked developmental delay. The mildest case usually has psychomotor retardation, macrocephaly and hypotonia [14]. D-2-hydroxyglutaric acid is a metabolite intermediate in different pathways in mankind [1,15]. It could be produced in mammalian species form 2-keoglutarate and 5-aminolevulinic acid, as shown in Figure 8.2 [2]. The conversion of D-2-hydroxyglutarate to 2-ketoglutarate is catalyzed by the enzyme D-2-hydroxyglutarate dehydrogenase and transhydrogenase that convert succinic semialdehyde into 4-hydroxybutyrate [16,17]. The production of D-2-hydroxyglutarate from 5-aminolevulinic acid is a minor pathway, as 5-aminolevulinic acid is predominately involved in the synthesis of porphyrins [15]. High

excretion of D-2-hydroxyglutaric acid also occurs in multiple acyl-CoA dehydrogenase deficiency that is due to a defect of the electron transfer flavoprotein (EP) or of the mitochondrial enzyme ETF-ubiquinone oxidoreductase [18]. D-2-hydroxyglutaric acid (D-2-HGA) dehydrogenase deficiency was considered as potential cause of this disease, even though the activity of the enzyme was normal or increased in the liver of patients. This suggests that their metabolism may result from a secondary pathway [19,20]. The existence of high metabolism of D-2-HGA in D-2-hydroxyglutaric aciduria patients suggests the vital need of an accurate method for quantitative analysis of D-2-HGA in human fluids.

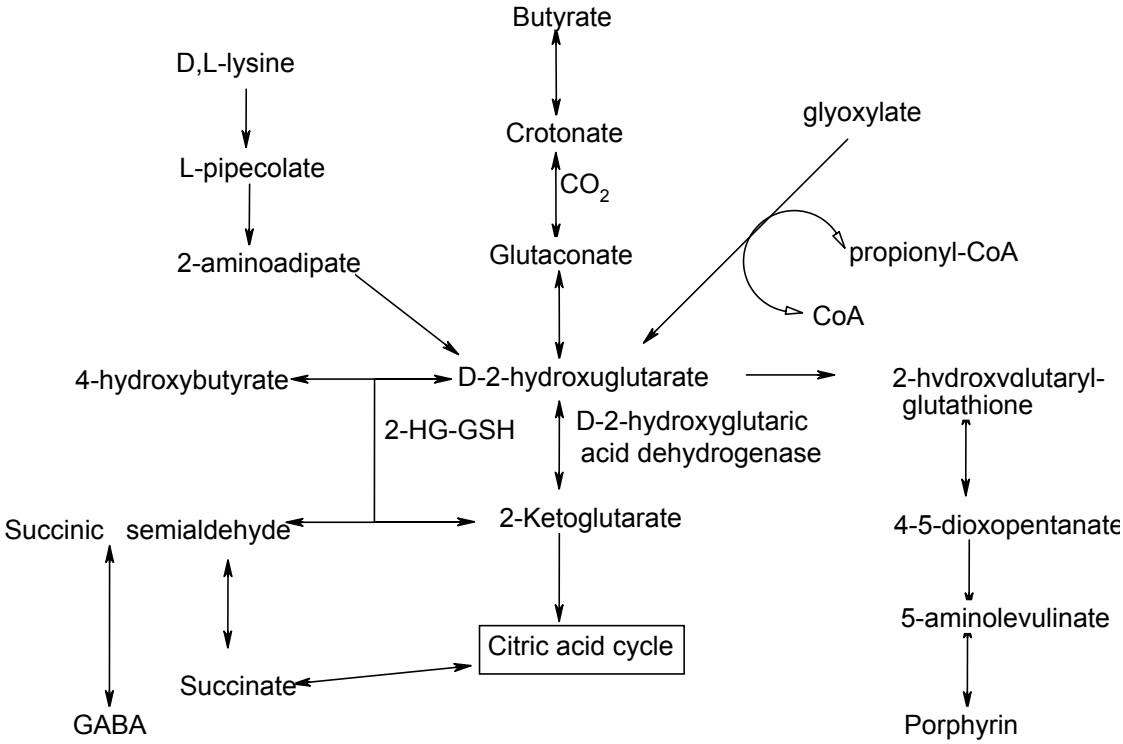

Figure 8.2 Metabolic pathways involving D-2-hydroxyglutaric acid in mammalian and bacterial species (2-HG-GSH = 2-hydroxyglutaryl-gluathione) [2].

Enantioanalysis of 2-hydroxyglutaric acid (HGA) is very important to differentiate between the two inherited metabolic diseases L- and D-2-hydroxyglutaric acidurias.

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Configurational analyses of L- and D-2-hydroxyglutaric acids were reported using ^{1}H and ^{13}C NMR and MRI [12, 21-25]. Capillary gas chromatography [20, 26-29] and liquid chromatography [30] have been used for the determination of 2-hydroxyglutaric acid in urine.

In this chapter, five EPMEs based on maltodextrins I, II and III with different dextrose equivalent values (DE: 4.0-7.0 (I), 13.0-17.0 (II), 16.5-19.5 (III)) and cyclodextins (β- and 2-hydroxy-3-trimethylammoniopropyl-β-derivative-CD) are proposed for the enantioanalysis of L-2-hydroxyglutaric acid in urine samples. Also, four EPMEs based on γ-cyclodextrin and macrocyclic antibiotics (vancomycin, teicoplanin and teicoplanin modified with acetonitrile) were proposed for the assay of L-2-hydroxyglutaric acid. Modified carbon paste was proved to be reliable for the construction of EPMEs [31].

8.2 Reagents and materials

Graphite powder (1-2μm) and maltodextrins (DE 4.0-7.0 (I), 13.0-17.0 (II), 16.5-19.5 (III)) were purchased from Aldrich (Milwaukee, WI, USA), paraffin oil was purchased from Fluka (Buchs, Switzerland). L- and D-2-hydroxyglutaric acids creatine and creatinine were purchased from Sigma-Aldrich (Milwaukee, WI, USA). α-, β-, γ-, and 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrins were supplied by Wacher-Chemie GmbH (Germany). Phosphate buffer (pH = 3) was purchased from Merck (Darmstadt, Germany).

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10^{-3} mol/L solutions of each maltodextrin (I, II and III), and each cyclodextrin (α-, β-, γ-, and 2-hydroxy-3-trimethylammoniopropyl-β-CD) were prepared. The solution of vancomycin ($2x10^{-3}$ mol/L) was prepared in phosphate buffer (pH 4.00). The solution of teicoplanin ($2x10^{-3}$ mol/L) was prepared using pH 6.00 phosphate buffer. The solution of teicoplanin ($2x10^{-3}$ mol/L) containing acetonitrile was prepared using pH 6.00 phosphate buffer containing 40% (v/v) of acetonitrile.

De-ionized water from a Modulab System (Continental Water Systems, San Antonio, TX, USA) was used for all solution preparations. The L- and D-2-hydroxyglutaric acid solutions were prepared by serial dilutions from standard L- and D-2-HGA solution (10^{-1} mol/L). All diluted and standard solutions of L- and D-2-HGA were buffered at pH=3.00 using phosphate buffer.

Urine samples were donated from healthy persons. Different aliquots of urine samples were spiked with L- and D-2-HGA. All spiked urine samples were buffered at pH=3 using phosphate buffer.

8.3 Enantioselective, potentiometric membrane electrodes based on maltodextrins

8.3.1 Equipments and apparatus

A 663 VA stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 100 computer-controlled potentiostat (Eco Chemie, Ultrech, The Netherlands) and software

version 4.9 was used for all potentiometric measurements. An Ag/AgCl (0.1mol/L KCl) electrode was used as reference electrode in the cell.

8.3.2 Electrodes design

Paraffin oil and graphite powder were mixed in a ratio 1:4 (w/w) to form the plain carbon paste. The modified carbon pastes were prepared by impregnating 100µL of 10^{-3} mol/L of each maltodextrin in 100mg of the plain carbon paste. A quantity of carbon paste, free of maltodextrins, was filled in a plastic pipette tip, leaving 3-4mm in the upper part to be filled with the modified carbon paste containing the maltodextrin. The diameter of all EPMEs was 3 mm. Electric contact was obtained by inserting silver wires into the carbon paste. The internal solution was 0.1mol/L KCl. The entire electrode surface was gently rubbed on fine abrasive paper to produce a flat surface. The surface of the electrode was wetted with de-ionized water, refreshed with modified carbon paste and then polished with an alumina paper (polished strips 30144-011, Orion) before use for the analysis. When not in use, each sensor was immersed in 10^{-3}mol/L of L-2-HGA solution.

8.3.3 Recommended procedure

Direct potentiometric method was employed for potential measurements, E (mV), of each standard L-2-HGA (10^{-10}-10^{-2} mol/L) solution and urine sample. The electrodes were placed in the standard solutions. Calibration graphs were obtained by plotting E (mV) versus pL-2-HGA. Unknown concentrations of L-2-HGA in urine samples were determined from the calibration graphs.

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8.3.4 Results and discussion

8.3.4.1 EPMEs response

The response characteristics exhibited by the EPMEs based on different types of maltodextrins for the analysis of L-2-hydroxyglutaic acid are summarized in Table 8.1. All the proposed membrane electrodes exhibited linear and near-Nernestian responses (57-60 mV per decade of concentration) for EPMEs based on maltodextrins I, II and III, respectively, for the determination of L-2-HGA. The same EPMEs were shown non-Nernestian responses when used for the assay of D-2-hydroxyglutaric acid. The detection limits recorded for L-2-HGA are low, as shown in Table 8.1. Enantioselectivity using maltodextrins is based on the formation of inclusion complexes [33-35]. The stability of the complexes formed between the chiral selector and analytes increases with the value of DE, because increasing the DE value will result in an increase of the diameter of the helix. The response obtained for all three electrodes show good stability and reproducibility for tests performed for more than 2 months (RSD<1%).

Table 8.1 Response characteristics of enantioselective, potentiometric membrane electrodes for L-2-HGA[a]

EPME based on	Parameters			
	Slope (mV/decade of conc.)	Intercept E^o (mV)	Linear conc. range (mol/L)	Detection limit (mol/L)
Maltodextrin I	57.3	546.86	10^{-9}-10^{-5}	2.86×10^{-10}
Maltodextrin II	59.7	392.4	10^{-6}-10^{-3}	2.67×10^{-7}
Maltodextrin III	59.3	536.7	10^{-8}-10^{-5}	8.90×10^{-10}

[a]All measurements were made at room temperature; all values are the average of ten determinations.

The response times of EPMEs based on maltodextrins I and II are higher than 1 min for concentration of L-2-HGA between 10^{-9} and 10^{-5}, and 10^{-6} and 10^{-3} mol/L, respectively.

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For EPME based on maltodextrins III, the response time is lower than 1 min for the concentration of L-2-HGA between 10^{-8} and 10^{-5} mol/L.

8.3.4.2 The pH influence on the response of the EPMEs

The effect of the pH variation on the response of the EPMEs based on maltodextrins I, II, and III has been tested by recording the emf of the cell, using direct potentiometric method.

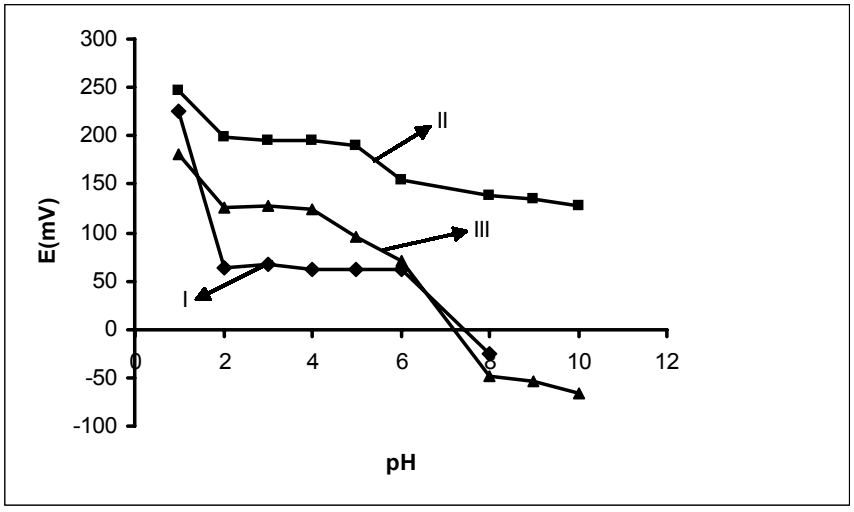

Figure 8.3 Effect of pH on the response of the enantioselective, potentiometric membrane electrodes based on maltodextrin I (I), II (II) and III (III), respectively, for the determination of L-2-HGA ($C_{L\text{-}2\text{-}HGA} = 10^{-6}$ mol/L).

All measurements were performed for a concentration of L-2-HGA of 10^{-6} mol/L, at different pH values selected between 1 and 10. These solutions were prepared by adding small volumes of HCl (0.1 mol/L) and/or NaOH solution (0.1 mol/L) to a solution of L-2-HGA. The E (mV) vs. pH plots presented in Figure 8.3 shows that the response of the EPMEs are pH-independent in the pH ranges of 2.0-6.0 (maltodextrins I based EPME), 2.0-5.0 (maltodextrin II based EPME) and 2.0-4.0 (maltodextrin III based EPME).

8.3.4.3 Selectivity of the electrodes

The selectivity of all EPMEs was checked using mixed solutions method proposed. The ratio between the concentrations of interfering ion and L-2-HGA was 10:1. The selectivity was investigated against D-2-hydroxglutaric acid (D-2-HGA), creatine, creatinine, Na^+, K^+ and Ca^{2+}. The selectivity coefficients for the enantioselective, potentiometric membrane electrodes, K_{sel}^{pot}, obtained are summarized in Table 8.2. The values obtained for D-2-HGA, creatine, creatinine, and the inorganic cations (Na^+, K^+ and Ca^{2+}, $K_{sel}^{pot} < 10^{-4}$) demonstrated the enantioselectivity and selectivity properties of the proposed EPMEs for the assay of L-2-HGA.

Table 8.2 Selectivity coefficients for the enantioselective, potentiometric membrane electrodes used for the assay of L-2-HGA[a]

Interference species (J)	pK_{sel}^{pot}		
	EPMEs based on		
	Maltodextrin I	Maltodextrin II	Maltodextrin III
D-2-HGA	2.40	2.42	2.42
Creatine	2.40	2.41	2.40
Creatinine	2.39	2.42	2.40

[a]All measurements were made at room temperature; all values are the average of ten determinations.

8.3.4.4 Analytical applications

The high selectivity and enantioselectvity of proposed EPMEs based on maltodextrins made them suitable for the enantioanalysis of L-2-HGA in urine in order to diagnose 2-hydroxyglutaric aciduria. The analysis of L-2-hydroxyglutaric acid was investigated in the presence of D-2-hydroxyglutaric acid by using different ratios between L- and D-2-HGAs. The results obtained (Table 8.3) proved again the suitability of the proposed potentiometric membrane electrodes for the enantioanalysis of L-2-HGA. No significant

differences in the recovery values were recorded for the different ratios between the enantiomers.

Table 8.3 The results obtained for the analysis of L-2-HGA in the presence of D-2-HGA[a]

L:D (mol/mol)	% L-2-HGA, Recovery		
	EPMEs based on		
	Maltodextrin I	Maltodextrin II	Maltodextrin III
2:1	99.60±0.03	99.60±0.01	99.78±0.01
1:1	99.91±0.01	99.46±0.02	99.75±0.01
1:2	99.35±0.01	99.54±0.01	99.04±0.01
1:4	99.82±0.01	99.92±0.03	99.80±0.02
1:9	99.76±0.03	99.71±0.02	99.43±0.01

[a] All measurements were made at room temperature; all values are the average of ten determinations.

Table 8.4 Recovery of L-2-HGA in urine samples[a]

Sample no.	LC/MS method [30]	% L-2-HGA, Recovery		
		EPMEs based on		
		Maltodextrin I	Maltodextrin II	Maltodextrin III
1	99.84	99.16±0.05	99.92±0.03	99.93±0.02
2	99.67	99.37±0.01	99.93±0.01	98.52±0.01
3	99.43	99.08±0.05	99.27±0.02	99.95±0.01
4	99.65	99.30±0.02	99.66±0.01	99.76±0.04
5	99.76	99.72±0.01	99.80±0.01	99.67±0.02
6	99.80	99.71±0.02	99.68±0.03	99.82±0.03

[a]All measurements were made at room temperature; all values are the average of ten determinations.

Urine samples (1-6) were donated from healthy persons and spiked with different amounts of L-2-HGA. All urine samples were buffered at pH=3 using phosphate buffer. The results recorded for the assay of L-2-HGA in urine samples are shown in Table 8.4 and they are in good agreement with those obtained using the method proposed by Rashed et al [30].

8.4 Enantioselective, potentiometric membrane electrodes based on cyclodextrins for the determination of L and D-2-hydroxyglutaric acid in urine samples

8.4.1 Equipments and apparatus

All potentiometric measurements were performed using a 663 VA Stand (Metrohm, Herisau, Switzerland) connected to an Autolab PGSTAT 100 (Eco Chemie, Netherlands) and software version 4.9. An Ag/AgCl (0.1 mol/l KCl) electrode and was used as reference electrode in the cell.

8.4.2 Electrodes design

The paraffin oil and graphite powder were thoroughly mixed in a ratio of 1:4 (w/w) followed by the addition of the aqueous solutions of β- cyclodextrin, β- cyclodextrin-derivative (2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrin) and γ-cyclodextrin (10^{-3} mol/L 100 µL chiral solution to 100mg carbon paste was added). Plain carbon paste was prepared by mixing 100mg of graphite powder with 40 µl paraffin oil. The plain carbon paste was filled into a plastic pipette peak leaving a space of 3-4 mm into the top to be filled with the modified carbon paste. The diameter of enantioselective, potentiometric membrane electrodes was 3 mm. Electric contact was obtained by inserting Ag/AgCl wire into the carbon paste. The internal solution was 0.1 mol/L KCl. All the EPMEs tips were gently rubbed on fine abrasive sand paper to produce a flat surface. The surface of the EPMEs was wetted with de-ionized water and then polished with an alumina paper (polished strips 30144-001, Orion) before use in each experiment.

When not in use, the electrodes were immersed in 10^{-3} mol/l L- or D-2-hydroxyglutaric acid solution.

8.4.3 Recommended procedure

The direct potentiometric method was used for the measurement of the potentials (E) of each standard solution (10^{-10} – 10^{-2} mol/L). Calibration graphs were obtained by plotting E(mV) versus p(L-HGA) or p(D-HGA). The unknown concentrations were determined from calibration graphs.

Ten urine samples were collected from patients suffering of L- or/and D-2-hydroxyglutaric acidurias. Different aliquots of the urine samples were buffered using phosphate buffer solution (pH=3). All samples were kept in refrigerator during experimental period, at 4^{o}C. Direct potentiometry was used for the enantioanalysis of L- and D-2-hydroxyglutaric acid in the urine samples.

8.4.4 Results and discussion

8.4.4.1 EPMEs response

The three cyclodextrin-carbon paste electrodes were tested for their response characteristics towards L- and D-2-hydroxyglutaric acid at pH=3.0 (phosphate buffer). Enantioanalyses was based on the formation of inclusion complexes between the cyclodextrin (host) and L- or D-2HGA (guest). The response obtained for L-HGA was linear and near-Nernstian only using β-CD and 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrins based EPMEs, while the response obtained for D-HGA was linear and

near-Nernstian only when γ-CD based EPME was used. The equations of calibration and correlation coefficients (r) for L-HGA and D-HGA are as follows:

L-2-HGA: $E = 57.5\ p\ (L\text{-}HGA) - 315.5;\ r = 0.9999$

L-2-HGA: $E = 58.6\ p\ (L\text{-}HGA) + 465.2;\ r = 0.9990$

D-2-HGA: $E = 50.0\ p\ (D\text{-}HGA) + 460;\ r = 0.9946$

where E is the cell potential in mV and p(L-HGA) = -log [L-HGA] and p (D-HGA) = -log [D-HGA]. The response characteristics of the EPMEs are summarized in Table 8.5. The limits of detection are very low 1.0×10^{-9} and 1.47×10^{-8} mol/L for L-2-hydroxyglutaric acid when EPMEs based on β- and 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrins are used and 6.30×10^{-7} mol/L for D-2-hydroxyglutaric acid when γ-CD based is used. The EPMEs responses exhibited a good stability and reproducibility for the tests performed for 4 months, when daily used for measurements (RSD<1.0%).

The response times were: 1 min for L-HGA assay using β-CD based EPME in the concentration range 10^{-8} – 10^{-6} mol/L, 2 min for L-HGA assay using 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrin based EPME in the concentration range 10^{-7}-10^{-5} mol/L and for D-HGA using γ-CD based EPME in the concentration range 10^{-6}-10^{-4} mol/L

Table 8.5 Response characteristics of enantioselective, potentiometric membrane electrodes for L-and D-2-hydroxyglutaric acid [a]

Analyte	Chiral selector	Parameters			
		Slope (mV/decade of conc.)	Intercept, E^o (mV)	Linear conc. range (mol/L)	Detection limit (mol/L)
L-2-HGA	β-Cyclodextrin	57.5	-315.5	10^{-8}-10^{-6}	1.00×10^{-9}
L-2-HGA	β-Cyclodextrin derivative	59.4	465.2	10^{-7}-10^{-5}	1.47×10^{-8}
D-2-HGA	γ-Cyclodextrin	50.0	460.0	10^{-6}-10^{-4}	6.30×10^{-7}

[a] All measurements were made at room temperature; all values are the average of ten determinations.

8.4.4.2 The pH influence on the response of the EPMEs

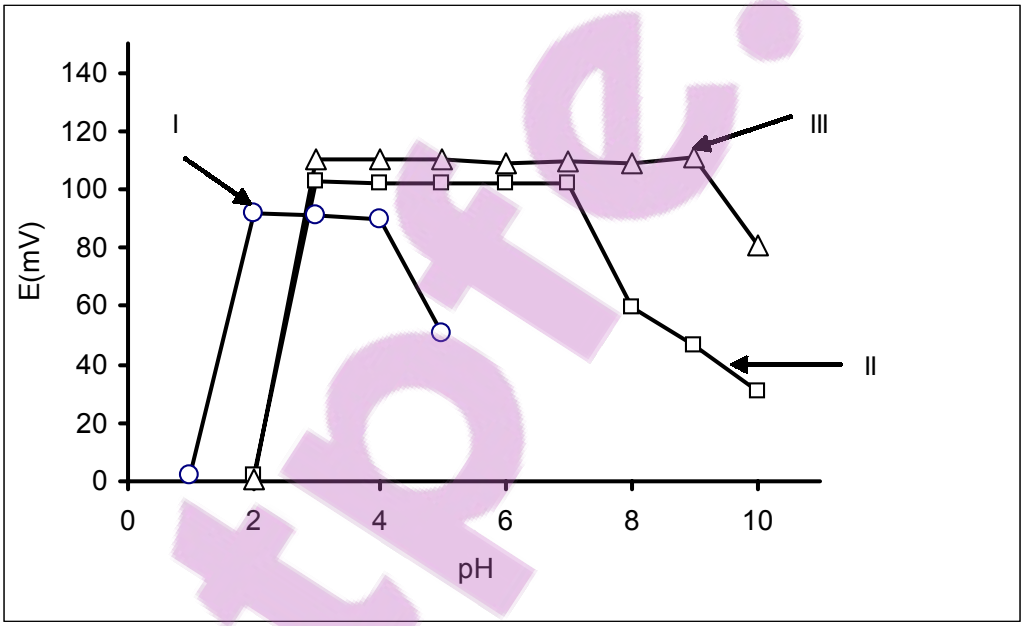

Figure 8.4. Effect of pH on the response of the enantioselective potentiometric membrane electrodes for L- and D-2-hydroxyglutaric acid ($C_{L\text{-}HGA} = 10^{-7}$ and $C_{D\text{-}HGA} = 10^{-5}$ mol/L). I &II for L-2-hydroxyglutaric acid using β and β-derivative cyclodextrin, respectively, and III for L-2-hydroxyglutaric acid using γ cyclodextrin.

Different solutions of L-HGA and D-HGA in the pH from 1 to 10, were prepared by adding small volumes of HCl (0.1 mol/l) or NaOH (0.1 mol/l) solutions to stock solutions

of L-HGA and D-HGA, respectively. The E (mV) vs. pH plots presented in Figure 8.4 show that the emf is not dependant on the pH in the following ranges 2.0-4.0 for β-CD based EPME, 3.0-10.0 for 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrin based EPME and 3-7 for γ-CD based EPME.

8.4.4.3 Selectivity of the electrodes

The selectivity of the electrodes was checked using the mixed solutions method proposed by Renk [33], over L- or D-HGA, creatine, creatinine and some inorganic cations. The ratio between the concentration of interfering ion and enantiomer was 10:1. The potentiometric selectivity coefficients, K_{sel}^{pot}, (Table 8.6) proved the high enantioselectivity and selectivity of the proposed EPMEs. Inorganic cations such a Na^+, K^+, and Ca^{2+} do not interfere in the analysis of L- and D-HGA.

Table 8.6 Potentiometric selectivity coefficients for the enantioselective, potentiometric membrane electrodes.[a]

Interference species (J)	K^{pot}_{sel}		
	EPME based on		
	β-Cyclodextrin	2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrin)	γ-Cyclodextrin
L-2-HGA	-	-	3.80×10^{-3}
D-2-HGA	4.09×10^{-3}	3.80×10^{-3}	-
Creatine	3.93×10^{-3}	3.95×10^{-3}	9.65×10^{-3}
Creatinine	8.34×10^{-3}	8.06×10^{-3}	4.71×10^{-3}

[a] All measurements were made at room temperature; all values are the average of ten determinations.

8.4.4.4 Analytical applications

The electrodes can be applied for the enantioanlaysis of L- and D-2-hydroxyglyutaric acid in urine matrices using direct potentiometric method. The recovery tests

demonstrated the suitability of these EPMEs for the assay of enantiopurity assay of L- and D-2-hydroxyglyutaric acid (Tables 8.7 and 8.8). The assay of one enantiomer in presence of its antipode was conducted by using different ratios between enantiomers. No significant differences in the recovery values were recorded for the ratios between L:D or D:L enantiomers varying from 1:9 to 1:99.99.

Table 8.7 The results obtained for the analysis of L-2-Hydroxyglutaric acid in the presence of D-2-Hydroxyglutaric acid [a]

L:D (moL/moL)	Recovery, % L-2-HGA	
	EMPE based on	
	β-Cyclodextrin	β-Cyclodextrin derivative
2:1	99.87±0.01	99.85±0.04
1:1	99.60±0.03	99.84±0.06
1:2	99.73±0.02	99.63±0.06
1:4	99.46±0.01	100.0±0.05
1:9	99.88±0.06	99.88±0.07

[a] All measurements were made at room temperature; all values are the average of ten determinations.

Table 8.8 The results obtained for the analysis of D-2-Hydroxyglutaric acid in the presence of L-2-Hydroxyglutaric acid [a]

D:L (mol/mol)	Recovery, % D-2-HGA
	γ-cyclodextrin based EPME
2:1	99.70±0.02
1:1	99.76±0.04
1:2	99.75±0.05
1:4	99.67±0.03
1:9	99.75±0.04

[a] All measurements were made at room temperature; all values are the average of ten determinations.

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Table 8.9 Recovery of L-2-Hydroxyglutaric acid in urine samples, (%) [a]

Sample no.	Standard method [32] (ng/L)	Recovery %, L-2-HGA	
		Chiral selector	
		β-Cyclodextrin	β-Cyclodextrin derivative
1	29.60	98.04±0.09	99.98±0.09
2	74.00	99.97±0.12	98.78±0.07
3	118.40	99.43±0.04	99.69±0.03
4	148.00	99.42±0.02	99.30±0.01
5	370.00	99.54±0.09	99.96±0.05

[a] All measurements were made at room temperature; all values are the average of ten determinations.

Table 8.10 Recovery of D-2-Hydroxyglutaric acid in urine samples, (%) [a]

Sample no.	Standard method [32] (mg/L)	Chiral selector
		γ-cyclodextrin
		Recovery % D-2-HGA
6	59.20	99.18±0.02
7	118.40	99.48±0.01
8	148.00	100.00±0.02
9	592.00	99.40±0.03
10	1036.00	99.12±0.02

[a] All measurements were made at room temperature; all values are the average of ten determinations.

The results obtained for the analysis of L- and D-2-hydroxyglutaric acid in urine samples are shown in Table 8.9 and 8.10, respectively. The results obtained by using the proposed EPMEs are in good concordance with those obtained using the standard method [32]. The advantage of the proposed method is the simplicity and high precision

8.5 Determination of D-2-hydroxyglutaric acid in urine samples using enantioselective, potentiometric membrane electrodes based on antibiotics

8.5.1 Apparatus

A 663 VA stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 100 computer-controlled potentiostat (Eco Chemie, Ultrech, The Netherlands) and software version 4.9 was used for all potentiometric measurements. An Ag/AgCl (0.1 mol l^{-1} KCl) electrode was used as reference electrode in the cell.

8.5.2 Electrodes design

Paraffin oil and graphite powder were mixed in a ratio 1:4 (w/w) to form the plain carbon paste. The modified carbon pastes were prepared by impregnating 100 µl of 10^{-3} mol/L solution of antibiotic (vancomycin, teicoplanin, or teicoplanin modified with acetonitrile), in 100 mg of the plain carbon paste. A certain quantity of carbon paste, free of antibiotics, was filled in a plastic pipette peak, leaving 3-4 mm in the top to be filled with the modified carbon paste. The diameter of the EPMEs was 3 mm. Electric contact was obtained by inserting silver wires into the carbon paste. The internal solution was 0.1 mol/L KCl.

The entire electrode surface was gently rubbed on fine abrasive paper to produce a flat surface. The surface of the electrode was wetted with de-ionized water, refreshed with modified carbon paste and then polished with an alumina paper (polished strips 30144-

011, Orion) before use for the analysis. When not in use, each sensor was immersed in 10^{-3} mol l^{-1} of D-2-hydroxyglutaric acid solution.

8.5.3 Recommended procedure

Direct potentiometric method was employed for all potential measurements, E (mV), of each solution of D-2-hydroxyglutaric acid (10^{-10} -10^{-2} mol L^{-1}) and of urine samples. Calibration graphs were obtained by plotting E (mV) versus p(D-2-HGA). Unknown concentrations of D-2-HGA in urine samples were determined by interpolating of the potential value in the calibration graph.

8.5.4 Results and discussion

8.5.4.1 EPMEs response characteristics

The calibration equations obtained for the D-enantiomer are:

(I) E = 192.0 + 59.3 p (D-HGA), r = 0.9994 (vancomycin)

(II) E = 167.01 + 57.74 p (D-HGA), r = 0.9766 (teicoplanin)

(III) E = 85.6 + 54.0 p (D-HGA), r = 0.9993 (teicoplanin modified with acetonitrile)

The response characteristics exhibited by the EPMEs based on different types of antibiotics (vancomycin, teicoplanin and teicoplanin modified with acetonitrile) for the determination of D-2-hydroxyglutaric acid are summarized in Table 8.11. All the proposed membrane electrodes exhibited near-Nernstian responses (54-59.30 mV per decade of concentration) for the determination of D-2-hydroxyglutaric acid. Non-

Nernestian responses were recorded for L-2-hydroxyglutaric acid. Detection limits were low.

The response of the EPME based on vancomycin and teicoplanin modified with acetonitrile are lower than 1 min for concentration of D-2-hydroxyglutaric acid between 10^{-7} and 10^{-3} and between 10^{-6}-10^{-2} mol/L, respectively. For EPME based on teicoplanin, the response time is higher than 1 min for the concentration range between 10^{-7} and 10^{-2} mol/L of D-2-hydroxyglutaric acid. The response obtained for all three electrodes show good stability and reproducibility for tests performed for more than 1 months (RSD <0.1%). All electrodes were stored at 4^{o}C when not in use.

Table 8.11 Response characteristics of enantioselective, potentiometric membrane electrodes for D-2-hydroxyglutaric acid [a]

EPME based on	Parameters			
	Slope (mV/decade of concentration)	Intercept, E^{o} (mV)	Linear concentration range (mol/L)	Detection limit (mol/L)
Vancomycin	59.30	192.0	10^{-7}-10^{-3}	$1.00x10^{-8}$
Teicoplanin	57.74	167.0	10^{-7}-10^{-2}	$1.00x10^{-8}$
Teicoplanin & acetonitrile	54.00	85.60	10^{-6}-10^{-2}	$1.00x10^{-7}$

[a] All measurements were made at room temperature; all values are the average of ten determinations.

8.5.4.2 Effect of pH on the EPMEs response

The effect of pH variations on the response of the constructed electrodes, based on vancomycin, teicoplanin and teicoplanin modified with acetonitrile, was investigated for the assay of D-2-hydroxyglutaric acid by recording the emf of the cell, containing its

solutions at different pHs (1-10) the concentration of D-2-hydroxyglutaric acid was $1x10^{-6}$ mol/L for all measurements. These solutions were prepared by adding small volumes of HCl (0.1 mol/L) and/or NaOH solution (0.1 mol/L) to D-2-hydroxyglutaric acid solutions. In Figure 8.5, the plot investigates the relation between the cell potential, E (mV) and the pH variations in the solutions of D-2-hydroxyglutaric acid. EPME based on teicoplanin has the widest pH independency range from 2.0 to 10.0, while the one based on vancomycin is independent in the 2.0 to 5.0 pH range. EPME based on teicoplanin mixed with acetonitrile is pH independent in the range 2.0-6.0.

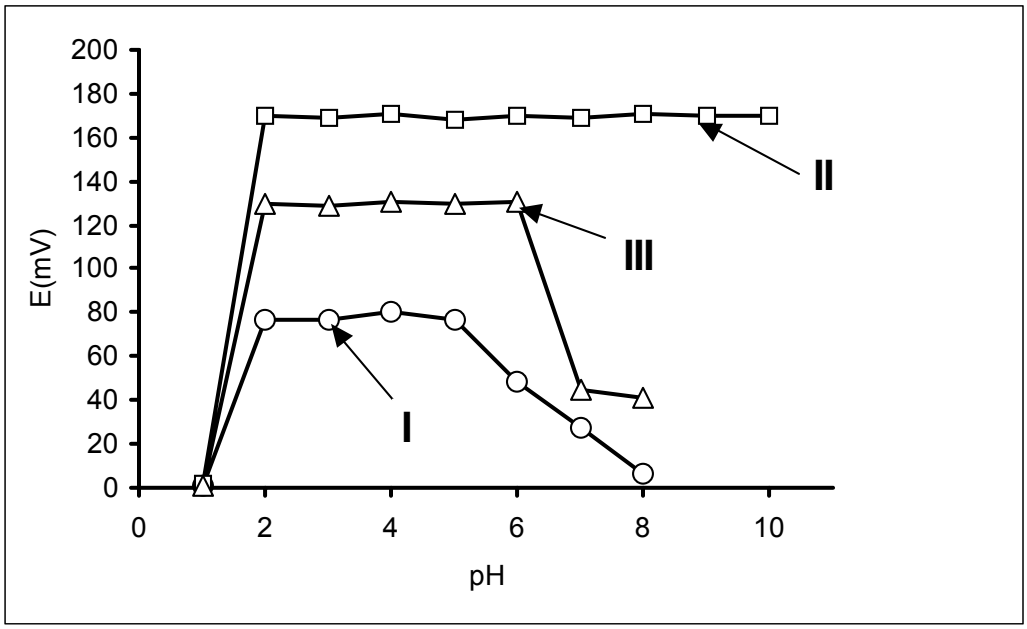

Figure 8.5 Effect of pH on the response of the enantioselective potentiometric membrane electrodes based on vancomycin (I), teicoplanin (II) and teicoplanin modified with acetonitrile (III).

8.5.4.3 Selectivity of the electrodes

The selectivity of all EPMEs was investigated using the mixed solution method proposed by Ren [42]. The concentrations of D-2-hydroxglutaric acid and interfering ion were 10^{-5} and 10^{-4} mol/L, respectively. The selectivity was investigated against L-2-hydroxglutaric

acid, creatine and creatinine. The selectivity coefficients for the enantioselective, potentiometric membrane electrodes, K_{sel}^{pot}, obtained are summarized in Table 8.12. The K_{sel}^{pot} values prove that the constructed potentiometric membrane electrodes are selective over creatine and creatinine. Inorganic cations such a Na^+, K^+, and Ca^{2+} do not interfere in the analysis of D-HGA.

Table 8.12 Selectivity coefficients of the enantioselective, potentiometric membrane electrodes based on macrocyclic antibiotics.[a]

Interference species (J)	pK_{sel}^{pot}		
	EPME based on		
	Vancomycin	Teicoplanin	Teicoplanin modified with acetonitrile
L-2-Hydroxyglutaric	3.81×10^{-3}	4.07×10^{-3}	4.17×10^{-3}
Creatine	3.81×10^{-3}	7.67×10^{-3}	4.17×10^{-3}
Creatinine	3.96×10^{-3}	3.91×10^{-3}	4.36×10^{-3}

[a] All measurements were made at room temperature; all values are the average of ten determinations.

8.5.4.4 Analytical applications

The electrodes proved to be useful for the determination of the enantiopurity of D-HGA raw material by direct potentiometric techniques and for its assay in urine samples. The analysis of D-2-hydroxyglutaric acid was investigated in the presence of L-2-hydroxyglutaric acid by using different ratios between D- and L-enantiomers.

The results obtained (Table 8.13) proved the suitability of the proposed potentiometric membrane electrodes for the enantioanalysis of D-2-hydroxyglutaric acid in the presence of its antipode. No significant differences in the recovery values were recorded for the ratios between 1:9 and 1:99.9 (L:D).

Table 8.13 The results obtained for the analysis of D-2-Hydroxyglutaric acid in the presence of L-2-Hydroxyglutaric acid [a]

Chiral selector	Recovery, % D-2-HGA		
	EPME based on		
D:L (moL:moL)	Vancomycin	Teicoplanin	Teicoplanin modified with acetonitrile
2:1	100.0±0.01	100.00±0.02	99.63±0.01
1:1	99.92±0.02	99.97±0.01	99.99±0.02
1:2	99.76±0.01	100.00±0.01	100.0±0.03
1:4	99.93±0.03	100.01±0.02	99.80±0.03
1:9	99.92±0.02	99.92±0.01	99.11±0.01

[a] All measurements were made at room temperature; all values are the average of ten determinations.

Table 8.14 Recovery of D-2-Hydroxyglutaric acid in urine samples, (%) [a]

Sample no.	Standard method [32] (µg/L)	Recovery, % D-2-HGA		
		EPME based on		
		Vancomycin	Teicoplanin	Teicoplanin with acetonitrile
1	44.40	99.56±0.02	99.30±0.05	99.51±0.02
2	88.80	99.18±0.02	99.27±0.02	98.59±0.03
3	133.20	99.59±0.01	99.29±0.03	99.60±0.01
4	177.60	99.83±0.01	99.52±0.02	99.54±0.02
5	370.0	99.35±0.03	99.87±0.01	99.50±0.03
6	1480.0	99.38±0.02	99.90±0.02	99.62±0.02

[a] All measurements were made at room temperature; all values are the average of ten determinations.

Urine samples (1-6) were donated from healthy persons and spiked with different aliquots of D-2-hydroxyglutaric acid. All spiked urine samples were buffered at pH=3 using phosphate buffer. The results recorded for the assay of D-2-hydroxyglutaric acid in urine samples are shown in Table 8.14. These results are in good concordance with those

obtained using the standard method [41]. The results obtained for samples (1-6) using the proposed EPMEs based on the different types of antibiotics (vancomycin, teicoplanin, and teicoplanin with acetonitrile) show the suitability of EPMEs for enantioanalysis of D-HGA and diagnosis of D-2-hydroxyglutaric acidurias.

8.6 Conclusions

The EPMEs based on vancomycin, teicoplanin, and teicoplanin modified with acetonitrile proved to be suitable for the enantioanalysis of D-2-hydroxyglutaric acid in solutions and urine samples by direct potentiometric technique. These electrodes can be reliable used for the diagnosis of D-2-hydroxyglutaric acidurias patients.

The EPMEs based on maltodextrins proved to be useful for the enantioanalysis of L-2-HGA in urine samples using direct potentiometric method. Therefore, these electrodes can be reliable used for the diagnosis of L-2-hydroxyglutaric aciduria.

β- and 2-Hydroxy-3-trimethylammoniopropyl-β-cyclodextrins based EPMEs can be reliable applied for the determination of L-2-hydroxyglutaric acid in urine samples and γ-cyclodextrin based EPME for the analysis of D-2-hydroxygluaric acid. The construction of the electrodes is simple, fast and reproducible. These electrodes have high precision, rapid response and the cost of construction and analysis is low. They can be successfully used for fast and reliable diagnosis of L- and D-2-hydroxyglutaric acidurias. EPME also have some advantages over the proposed chromatographic technique, such as high

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precision, high enantioselectiviy, rapidity, low cost of analysis and no need for special sample pre-treatment before analysis.

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Chapter 9

Enantioanalysis of L-vesamicol in serum samples using enantioselective, potentiometric membrane electrodes

9.1 Introduction

Human metabolism is involved in the production of both or only one of the enantiomers due to enzymatic deficiencies. Therefore, it is important to develop efficient methods for the enantioanalysis of chiral compounds of biological importance [1,2]. Biological activity differences of enantiomers arise due to differences between protein binding and transport.

L-vesamicol is a tertiary amine [L-(trans)-(-)-2-(4-phenyl-1-piperidinyl-cyclohexanol, Figure 9.1] and it is a potent, non-competitive inhibitor of acetylcholine uptake into the vesicles of cholinergic neurons [3-6]. It binds to an allosteric site on the specific vesicular acetylcholine transporter complex [7]. The transporter protein, which is responsible for the transfer of acetylcholine into synaptic vesicles, is located in cholinergic neurons. L-Vesamicol is potentially suitable for the study of presynaptic cholinergic mechanisms. In Alzheimer's disease, the degeneration of cholinergic neurons in brain is a significant neuropathological feature. The binding selectivity of L-vesamicol to the vesicular acetylcholine transporter could be used as a hallmark for diagnosis and follow up of degenerative disease [8]. L-Vesamicol was reported to exhibit high affinity, especially to σ-1 and σ-2 receptors, that are found in different parts of the central nervous system [9,10].

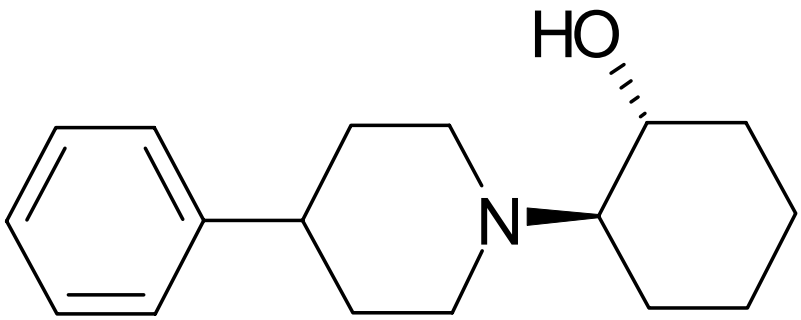

Figure 9.1 L-(-)-vesamicol

Acetylcholine, a neurotransmitter, stored by nerve terminals inside of synaptic vesicles is found in the cytoplasmic compartment. Stimulation of the nerve terminals effects excocytotic release of synaptic vesicle content into the synaptic gap and results in quantal transynaptic communication [11]. Cholinergic reduction in cerebral cortex and limbic brain may contribute to the cognitive impairments of patients with Alzheimer's disease and a disturbance in cholinergic neurotransmission [12,13], Parkinson's disease with dementia [14], Down's Syndrome [15] and olivopontocerebellar atrophy [16]. Since the vesicular storage of neurotransmitter is required for evoked release, factors that affect this storage are important to neurotransmission.

Very few methods have been used for the assay of L-vesamicol. Glidersleeve *et. al*. described direct resolution of vesamicol and its analogues by high performance liquid chromatography [17]. Zhou and Stewart determined vesamicol enantiomers in human serum by capillary electrophoresis using sulfated-β-cyclodextrin [18]. Hefnawy and Aboul-Enein described a validated liquid chromatographic method for the determination of vesamicol enantiomers in human plasma using vancomycin as chiral stationary phase [19].

This chapter reports the applications of ten enantioselective, potentiometric membrane electrodes based on cyclodextrins (α-, β-, γ-, and 2-hydroxy-3-trimethylammoniopropyl-β-derivative-CD), maltodextrins I, II and III with different DE values [I (4.0-7.0), II (13.0-17.0) and III (16.5-19.5)], and macrocyclic antibiotics (vancomycin and teicoplanin) as chiral selectors for the determination of L-vesamicol in serum samples. The proposed EPMEs were applied for the assay of L-vesamicol in serum samples.

9.2 Reagents and materials

L-vesamicol, vancomycin and teicoplanin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Graphite powder (1-2μm, synthetic) and maltodextrins [DE 4.0-7.0 (I), 13.0-17.0 (II), and 16.5-19.5 (III)] were purchased from Aldrich (Milwaukee, WI, USA). Paraffin oil was purchased from Fluka (Buchs, Switzerland). α-, β-, γ-, and 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrins were supplied by Wacher-Chemie GmbH (Germany). Phosphate buffer (pH = 5) was supplied by Merck (Darmstadt, Germany).

De-ionized water from a Modulab system (Continental Water Systems, San Antonio, TX, USA) was used for all reagents and solutions preparations. A 0.1mol/L stock solution of L- vesamicol was buffered with phosphate buffer (pH 5). Solutions of L-vesamicol ($1x10^{-10}$ - $1x10^{-2}$ mol/L) were prepared by serial dilutions from the stocks solution of L- vesamicol and were buffered with phosphate buffer (pH 5).

10^{-3} mol/L solutions of each maltodextrin (I, II and III), and each cyclodextrin (α-, β-, γ-, and 2-hydroxy-3-trimethylammoniopropyl-β-CD) were prepared. The solution of

vancomycin ($2x10^{-3}$ mol/L) was prepared in phosphate buffer (pH 4.00). The solution of teicoplanin ($2x10^{-3}$ mol/L) was prepared using pH 6.00 phosphate buffer. The solution of teicoplanin ($2x10^{-3}$ mol/L) containing acetonitrile was prepared using pH 6.00 phosphate buffer containing 40% (v/v) of acetonitrile.

9.3 Enantioselective, potentiometric membrane electrodes based on maltodextrins

9.3.1 Equipments and apparatus

All direct potentiometric measurements were recoded using a Metrohm 663 VA stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 100 computer-controlled potentiostat (Eco Chemie, Ultrech, The Netherlands) and software version 4.9. The electrochemical cell is a two-electrode compartment, consists of enantioselective, potentiometric membrane electrode (carbon paste electrode modified with the maltodextrins) and reference electrode (Ag/AgCl electrode).

9.3.1 Electrodes design

20 µL paraffin oil and 100 mg graphite powder were mixed followed by the addition of 100 µL of aqueous solution of maltodextrin {DE 4.0-7.0 (I), 13.0-17.0 (II), and 16.5-19.5 (III)}, (100 µL of chiral selector/100 mg carbon paste). Plain carbon paste was prepared by mixing graphite powder and paraffin oil in a ratio 4:1 (w/w). A certain quantity of carbon paste, free of chiral selector, was filled in a plastic pipette peak, leaving 3-4 mm in the top to be filled with the modified carbon paste. The diameter of all the EPMEs was 3 mm. Electric contact was obtained by inserting

silver wires into the carbon paste. The internal solution was 0.1 mol/L KCl. The entire electrode surface was gently rubbed on fine abrasive paper to produce a flat surface. The surface of the electrode was wetted with de-ionized water, refreshed with modified carbon paste and then polished with an alumina paper (polished strips 30144-011, Orion) before use for the analysis. When not in use, EPMEs were immersed in 10^{-3} mol/L of L-vesamicol solution.

9.3.3 Recommended procedure

Direct potentiometric method was employed for the measurement of potential variation, E (mV), over different standard solution concentrations (10^{-10} -10^{-2} mol/L) of L-vesamicol. The electrodes were immersed in the stirred standard solutions for assay. Calibration graphs were drawn by plotting E (mV) versus p(L-vesamicol). The same technique is used to record the produced potential for the spiked serum samples. Unknown concentrations of L-vesamicol in serum samples were determined from the calibration graphs.

9.3.4 Results and discussion

9.3.4.1 EPMEs response characteristics

The equations of calibration and correlation coefficients (r) obtained for L-vesamicol using maltodextrin (I), maltodextrin (II) and maltodextrin (III) based electrodes, are as follows:

Maltodextrin I:	E(mV) = 206.8 -55.00 p (L-vesamicol)	r = 0.9834
Maltodextrin II:	E(mV) = 294.7 -55.60 p (L-vesamicol)	r = 0.9913
Maltodextrin III:	E(mV) = 483.33 -51.00 p (L-vesamicol)	r = 0.9999

The response characteristics exhibited by the three EPMEs impregnated with maltodextrins were determined for L-vesamicol at pH 5.0 (phosphate buffer). All the response characteristics are summarized in Table 9.1. All EPMEs exhibited near-Nernestian slope (in the range 51 to 55.6 mV/decade of concentration of L-vesamicol). EPME based on maltodextrin (III) exhibited the lowest detection limit of 10^{-10} mol/L magnitude order.

Table 9.1 Response characteristics of EPMEs for the assay of L-vesamicol [a]

Chiral selector	Parameters			
	Slope (mV/decade)	Intercept, E^o (mV)	Linear concentration range (mol/L)	Detection limit (mol/L)
Maltodextrin I	-55.00 mV/p(L-ves)	206.80	10^{-4}-10^{-2}	1.34×10^{-5}
Maltodextrin II	-55.60 mV/p(L-ves)	294.70	10^{-5}-10^{-3}	5.00×10^{-6}
Maltodextrin III	-51.00 mV p(L-ves)	483.33	10^{-9}-10^{-7}	3.33×10^{-10}

[a] All measurements were made at room temperature; all values are the average of ten determinations.

The best response time was recorded for the maltodextrin I and II based EPMEs: less than 1 min for the concentration range 10^{-5}-10^{-2} mol/L and higher than 1 minute for the concentration range 10^{-10}-10^{-6} mol/L. The response time for maltodextrin III was higher than 2 min in the concentration range 10^{-9}-10^{-7} mol/L and 2 min in the concentration range 10^{-6}-10^{-2} mol/L.

9.3.4.2 Effect of pH on the responses of the electrodes

L-vesamicol solutions of different pH values were prepared by adding small aliquots of HCl (0.1 mol/L) or NaOH solution (0.1 mol/L) to a L-vesamicol solution. The effect of pH variation on the recorded potentials of EPMEs is shown in the Figure 9.2. The responses of EPMEs of L-vesamicol are pH-independent in the pH ranges 3.0-6.0 for maltodextrin I-based electrode, 6.0-10.0 for maltodextrin II-based electrode and 3.0-5.0 for maltodextrin III-based electrode.

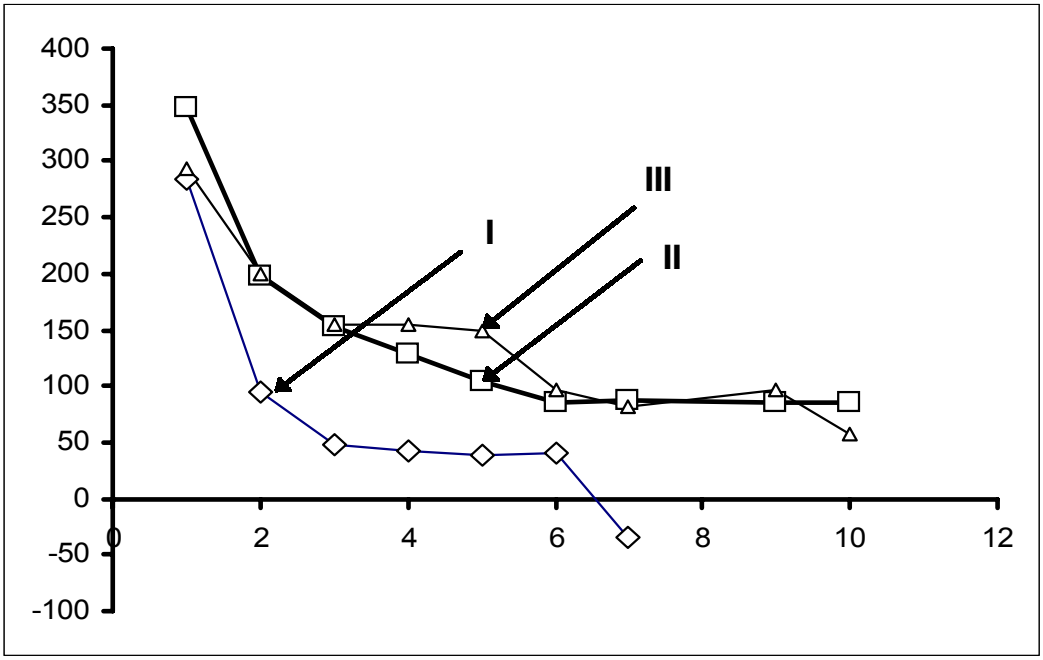

Figure 9.2 Influence of pH variation on the response of: I, EPME based on maltodextrin (I) (10^{-4} mol/L L-vesamicol); II, EPME based on maltodextrins (II) (10^{-4} mol/L L-vesamicol); and III, EPME based on maltodextrins (III) (10^{-8} mol/L L-vesamicol).

9.3.4.3 Selectivity of the electrode

The selectivity of the three electrodes have investigated using the mixed solutions method proposed by Ren [20]. The selectivity of L-vesamicol assay was tested over creatine, creatinine and some inorganic ions. The ratio between the concentrations of L-vesamicol and the imposed interfering ions was 1:10. The values for potentiometric selectivity coefficients, K_{sel}^{pot}, of maltodextrins I-, II- and III-based electrodes proved that creatine and creatinine do not interfere in the determination of L-vesamicol (Table 9.2). Inorganic cations such a Na^+, K^+, and Ca^{2+} do not interfere in the analysis of L- vesamicol.

Table 9.2 Potentiometric selectivity coefficients of the enantioselective, potentiometric membrane electrodes [a]

Chiral selector	pK_{sel}^{pot}	
	Interference species (J)	
	Creatine	Creatinine
Maltodextrin I	2.37	2.06
Maltodextrin II	2.10	2.347
Maltodextrin III	2.34	2.025

[a] All measurements were made at room temperature; all values are the average of ten determinations.

9.3.4.4 Analytical applications

Ten serum samples were donated form healthy volunteers and stored at -20°C. Samples are spiked with L-vesamicol. The results obtained for the analysis of L–vesamicol in serum sample using EPMEs based on maltodextrins I, II, and III are summarized in Table 9.3. The results obtained using the proposed EPMEs are in good concordance with the spiked values of L-vesamicol in serum samples.

Table 9.3 Recovery of L-vesamicol in serum samples using EPMEs based on maltodextrin I, II and III [a]

Sample no.	Concentration of L-vesamicol (ng/L)	% Recovery, L-vesamicol	Concentration of L-vesamicol (µg/L)	% Recovery, L-vesamicol	
		Maltodextrin III		Maltodextrin I	Maltodextrin II
1	15.54	99.23±0.01	1.554	99.22±0.02	99.72±0.01
2	20.72	98.50±0.01	2.072	99.13±0.03	99.22±0.04
3	25.90	99.83±0.01	2.590	99.44±0.05	99.82±0.01
4	31.08	99.67±0.01	3.108	99.54±0.01	99.44±0.01
5	36.26	99.27±0.02	3.626	99.50±0.03	99.28±0.01

[a] All measurements were made at room temperature; all values are the average of ten determinations.

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9.4 Cyclodextrins based enantioselective, potentiometric membrane electrodes

9.4.1 Apparatus

All measurements were carried out using a Metrohm 663 VA Stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 100 computer-controlled potentiostat (Eco Chemie, Ultrech, The Netherlands) and software version 4.9. The electrochemical cell consisted of enantioselective, potentiometric membrane electrode (carbon paste electrode modified with the cyclodextrin) and a reference electrode (Ag/AgCl electrode).

9.4.2 Electrodes Design

Paraffin oil and graphite powder were mixed thoroughly in the ratio 1:4, followed by the addition of aqueous solution of α-, β-, γ- or 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrins, (100 µl of chiral selector/100 mg carbon paste). Plain carbon paste was prepared by mixing 100mg of graphite powder with 40 µl paraffin oil. The plain carbon paste was filled into a plastic pipette peak leaving a space of 3-4 mm into the top of the peak to be filled with the modified carbon paste that contains the cyclodextrin. The diameter of the enantioselective, potentiometric membrane electrodes was 3 mm. Electric contact was obtained by inserting a silver/silver chloride wire into the carbon paste. The internal electrolyte solution was 0.1 mol/L KCl. The surface electrode was gently rubbed on fine abrasive paper to produce a flat surface. The surface of the electrode was also wetted with deionized water, refreshed with modified carbon paste and then polished with an alumina paper

(polished strips 30144-011, Orion) before each use. When not in use, EPMEs were immersed in 10^{-3} mol/L of L-vesamicol solution.

9.4.3 Recommended procedure

Direct potentiometry was employed for the measurement of potentials variation for different standard solution of L-vesamicol (10^{-10} -10^{-2} mol/L , pH 5.0) and samples. Calibration graphs were obtained by plotting E (mV) versus p[L-vesamicol]. The potentiometric technique was used for the analysis of L-vesamicol in the serum samples. The unknown concentrations of L-vesamicol in serum samples were calculated from the calibration graphs.

9.4.4 Results and discussion

9.4.4.1 EPMEs response characteristics

The response characteristics exhibited by the EPMEs impregnated with cyclodextrins were determined for L-vesamicol at pH = 5.0. The response obtained for L-vesamicol was near-Nernstian and linear for α-, β-, 2-hydroxy-3-trimethylammoniopropyl-β- and γ-cyclodextrins based EPMEs with correlation coefficients (r) 0.9694, 0.9990, 0.9844 and 0.9985, respectively. The linear concentration ranges obtained are 10^{-9}-10^{-7} mol/L for EPMEs based on α-, β- and 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrins and 10^{-5}-10^{-2} mol/L for γ-cyclodextrin based EPME. The equations of calibration are as follows:

EPME based on α-cyclodextrin: E(mV) = 529.33 -57.00 p (L-vesamicol)

EPME based on β-cyclodextrin: E(mV) = 562.50 -58.50 p (L-vesamicol)

EPME based on γ-cyclodextrin: E(mV) = 268.00 -52.50 p (L-vesamicol)

EPME based on 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrins -cyclodextrin:

$$E(mV) = 512.67 - 55.00\ p\ (L\text{-vesamicol})$$

where E (mV) is the cell potential, p(L-vesamicol) = -log[L-vesamicol]. The limits of detection obtained for the analysis of L-vesamicol are 5.70×10^{-10}, 2.42×10^{-10}, 4.77×10^{-10} and 7.86×10^{-6} mol/L when α-, β-, 2-hydroxy-3-trimethylammoniopropyl-β- and γ-cyclodextrins based EPMEs were used. The electrode based on β-cyclodextrin exhibited the lowest limit of detection, while electrode based on γ-cyclodextrin has the highest limit of detection. This may be explained by the excellent recognition power of β-cyclodextrin for chiral molecules containing a substituted aromatic ring [21]. The electrodes responses displayed a good stability and reproducibility for the tests performed for 3 weeks, when daily used for measurements (RSD<0.1%).

The best response time was recorded for β-cyclodextrin based EPME where the electrode potential was recorded in 26 seconds for the concentration range 10^{-9}-10^{-7} mol/L, while the response time recorded for the assay of L-vesamicol, EPME based on β-derivative-cyclodextrin, was one minute for the same concentration range. The response time for α- and γ-cyclodextrins based electrodes was 2 min in the concentration range 10^{-9}-10^{-7} mol/L and 10^{-5}-10^{-2} mol/L, respectively.

9.4.4.2 Influence of pH on the responses of the electrodes

Influence of pH on the response of the electrodes was tested by recording the emf of the cells, containing solutions of L-vesamicol at different pH values varied for 1 to 10. These solutions were prepared by adding small volumes of HCl or NaOH solution (10^{-1} mol/L or 1 mol/L of each) to a L-vesamicol solutions. The influence of pH

variation on the responses of EPMEs is shown in Figure 9.3. The responses of the electrode over L-vesamicol are pH-independent in the pH ranges 4.0-9.0, 5.0-8.0, 3.0-6.0 and 3.0-10.0 for electrodes based on α-, γ-, β-, and 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrins, respectively.

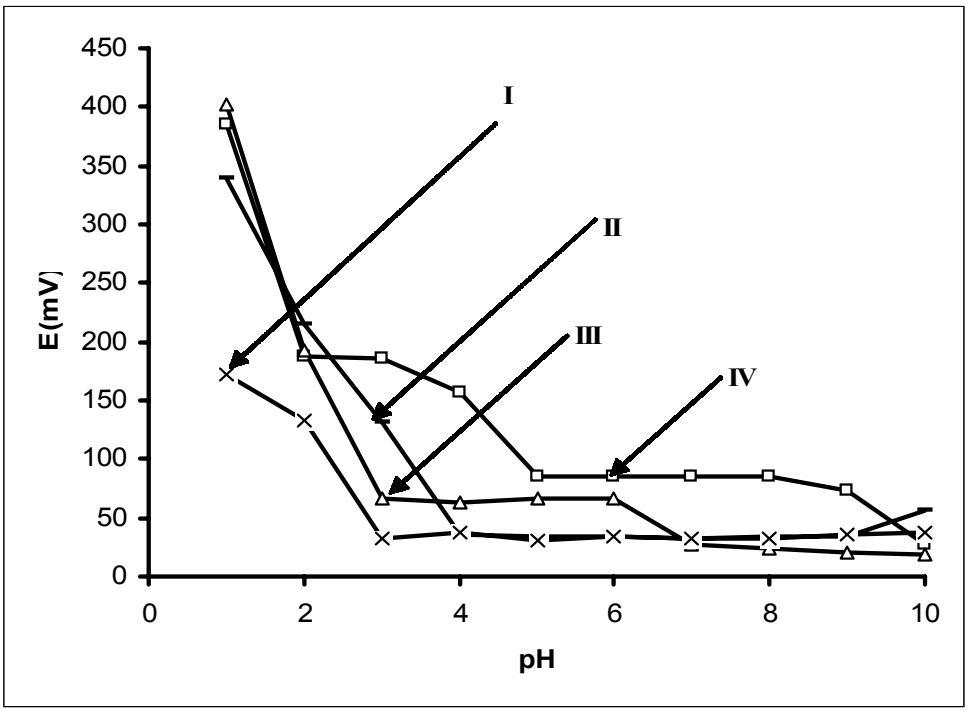

Figure 9.3 The influence of pH on functioning of the electrodes based on (I) 2-hydroxy-3-trimethylammoniopropyl-β- (II) α-cyclodextrin, (III) β-cyclodextrin ($C_{L\text{-vesamicol}} = 10^{-8}$ mol/) and (IV) γ-cyclodextrin ($C_{L\text{-vesamicol}} = 10^{-4}$ mol/l).

9.4.4.3 Selectivity of the electrode

The selectivity of the four electrodes was investigated using the mixed solutions method [32]. The selectivity of the proposed EPMEs was checked over creatine, creatinine, and some inorganic ions. The ratio between concentrations of L-vesamicol and interfering species was 1:10. The values of potentiometric selectivity coefficients, K_{sel}^{pot}, (Table 9.4) determined for electrodes based on α-, γ-, β-, and 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrins proved that creatine and creatinine do not interfere in the determination of L-vesamicol. The interference of inorganic cations

was checked for all proposed EPMEs, all K_{sel}^{pot} values are lower than 10^{-3} shown that these ions do not interfere in the analysis of L- vesamicol.

Table 9.4 Selectivity coefficients K_{sel}^{pot}, of the enantioselective, potentiometric membrane electrodes.[a]

EPME based on	K_{sel}^{pot}	
	Interfering species (j)	
	Creatine	Creatinine
α-cyclodextrin	4.28×10^{-3}	4.10×10^{-3}
β-cyclodextrin	4.02×10^{-3}	3.86×10^{-3}
2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrins	3.96×10^{-3}	4.12×10^{-3}
γ cyclodextrin	4.48×10^{-3}	4.29×10^{-3}

[a] All measurements were made at room temperature; all values are the average of ten determinations.

9.4.4.5 Analytical applications

Table 9.5 Determination of L-vesamicol in serum samples using EPMEs based on α-, β- 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrin [a]

Sample no.	Concentration of L-vesamicol (ng/L)	% Recovery, L-vesamicol		
		α-cyclodextrin	β- cyclodextrin	2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrin
1	8.88	99.50±0.02	99.86±0.02	99.22±0.02
2	11.84	99.41±0.01	99.93±0.01	99.13±0.01
3	14.80	99.36±0.01	99.90±0.01	99.44±0.04
4	17.76	99.74±0.01	99.72±0.02	99.54±0.02
5	20.72	99.22±0.01	99.89±0.01	99.50±0.02
Sample no.	Concentration of L-vesamicol (mg/L)	% Recovery, L-vesamicol		
		γ-cyclodextrin		
6	8.88	99.72±0.03		
7	11.84	98.56±0.02		
8	14.80	99.42±0.02		
9	17.76	99.57±0.02		
10	20.72	99.51±0.02		

[a] All measurements were made at room temperature; all values are the average of ten determinations.

Ten human serum samples were donated form different healthy volunteers and stored at -20^{o}C. The serum samples were spiked with L- vesamicol. The results obtained for the analysis of L–vesamicol in serum samples using EPMEs based on α-, β-, and 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrins, and γ-cyclodextrin are summarized in Table 9.5. The concentration of L-vesamicol calculated from results obtained by using the proposed EPMEs are in good concordance with the quantities of L-vesamicol in real samples, and proved the suitability of the electrodes for the proposed electrodes for the determination of L-vesamicol in serum samples.

9.5 Enantioselective, potentiometric membrane electrodes based antibiotics

9.5.1 Apparatus

A 663 VA stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 100 computer-controlled potentiostat (Eco Chemie, Ultrech, The Netherlands) and software 4.9 was used for all measurements. An Ag/AgCl electrode was used as reference electrode in the cell.

9.5.2 EPMEs Design

Plain carbon paste was prepared by mixing graphite powder and paraffin oil in a ratio 4:1 (w/w). Modified carbon pastes were prepared by the addition of 100 μl of 10^{-3} mol/L solution of each antibiotic (vancomycin or teicoplanin or teicoplanin with acetonitrile), to 100 mg of the plain carbon paste. A certain quantity of carbon paste, free of chiral selector, was filled in a plastic pipette peak, leaving 3-4 mm in the top to be filled with the modified carbon paste containing the chiral selector. The diameter

of all the EPMEs was 3 mm. Electric contact was obtained by inserting silver wires into the carbon paste. The internal solution was 0.1 mol/L KCl.

The electrode surface was gently rubbed on fine abrasive paper to produce a flat surface. The surface of the electrode was wetted with de-ionized water and polished with an alumina paper (polished strips 30144-011, Orion) before use for the analysis. When not in use, EPMEs were immersed in a 10^{-3} mol/L of L-vesamicol solution.

9.5.3 Recommended procedure

Direct potentiometric method was employed for the measurement of potential (E (mV)) of each L-vesamicol standard solution (10^{-10} -10^{-2} mol/L) and serum sample. Calibration graphs were obtained by plotting E (mV) versus p(L-vesamicol). Unknown concentrations of L-vesamicol in serum samples were determined from the calibration graphs.

9.5.4 Results and discussion

9.5.4.1 The response characteristics of EPMEs

The presence of several functional groups in the glycopeptides antibiotics contribute to enantiorecognition through hydrogen bonding, charge-charge, hydrophobic and steric interactions [22]. The calibration equations obtained for L-vesamicol are:

$$E(mV) = 386.83 - 55.50\ p(\text{L-vesamicol}), \quad r = 0.9929 \qquad (A)$$

$$E(mV) = 402.00 - 57.30\ p(\text{L-vesamicol}), \quad r = 0.9881 \qquad (B)$$

$$E(mV) = 399.58 - 53.66\ p(\text{L-vesamicol}), \quad r = 0.9870 \qquad (C)$$

where E(mV) is the potential, p(L-vesamicol) = -log[L-vesamicol], r is the correlation coefficient, and A, B, and C are indicating the EPMEs based on vancomycin, teicoplanin and teicoplanin modified with acetonitrile. The response characteristics exhibited by the EPMEs based on different types of antibiotics (vancomycin, teicoplanin and teicoplanin & acetonitrile) for the determination of L-vesamicol are summarized in Table 9.6. Proposed EPMEs exhibited linear and near-Nernstian responses between 53.0-58.0 mV per decade of L-vesamicol concentration with correlation coefficients of 0.9929, 0.9881 and 0.9870 for vancomycin, teicoplanin and teicoplanin modified with acetonitrile, respectively. The EPME based on teicoplanin modified with acetonitrile exhibited the wider linear concentration range and the lowest detection limit. These results may explained as organic modifiers are altering and/or inhibiting the aggregation of the teicoplanin monomers making more teicoplanin molecules available to interact with the enantiomer (L-vesamicol) [22].

Table 9.6 Response characteristics of EPMEs based on antibiotics for L-vesamicol [a]

EPME based on	Parameters			
	Slope (mV/decade of conc.)	Intercept, E^o (mV)	Linear conc. range (mol/L)	Detection limit (mol/L)
Vancomycin	-55.50	386.83	10^{-6}-10^{-4}	1.07×10^{-7}
Teicoplanin	-57.30	402.0	10^{-6}-10^{-3}	9.60×10^{-8}
Teicoplanin modified with acetonitrile	-53.66	399.58	10^{-7}-10^{-2}	3.60×10^{-8}

[a] All measurements were made at room temperature; all values are the average of ten determinations.

For all the proposed EPMEs, the response time was less than 1 minute for the concentration range 10^{-6}-10^{-2} mol/L and more than 1 minute for 10^{-10}-10^{-7} mol/L**.** The response show good stability and reproducibility for tests performed for more than 1 month, when used daily (RSD < 1%).

9.5.4.2 Effect of the pH on the response of EPMEs

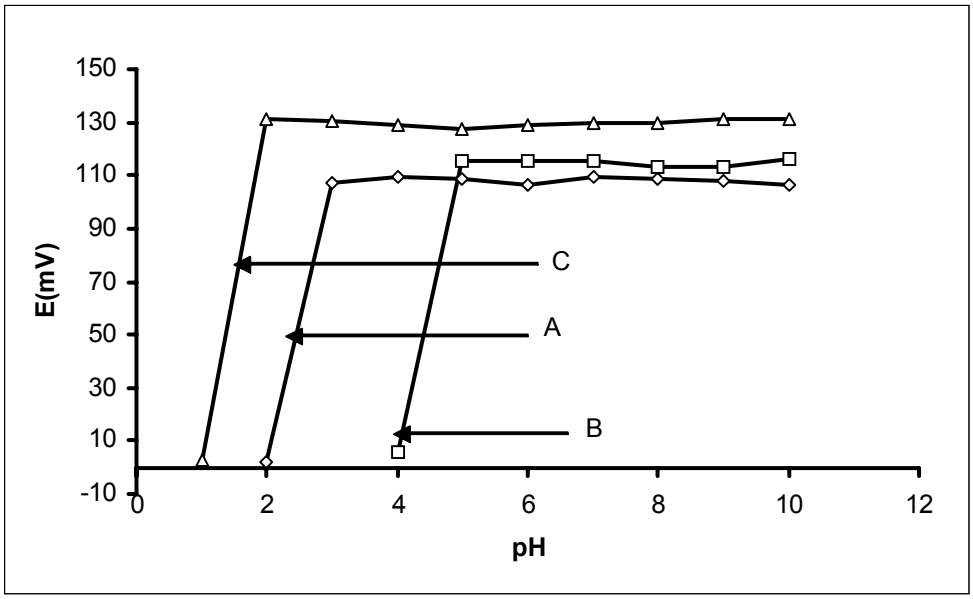

Figure 9.4 The influence of pH on the response of EPMEs for the determination of L-vesamicol (10^{-5} mol/L). (A) EPME based on vancomycin, (B) EPME based on teicoplanin and (C) EPME based on teicoplanin modified with acetonitrile.

The effect of pH variations on the response of the propped electrodes was checked for the assay of L-vesamicol in a pH range 1-10. The L-vesamicol concentration was 10^{-5} mol/L. In figure 9.4, the plot shows the variation of E(mV) with the pH. The response of EPME based on teicoplanin modified with acetonitrile does not depend on the pH value on the pH range 2.0-10.0, while the response of EPMEs based on teicoplanin and vancomycin are independent on pH on the pH ranges 5.0-10.0 and 3.0-10.0.

9.5.4.3 Selectivity of the Electrodes

The selectivity of all EPMEs was investigated using the mixed solution method. The concentrations of L-vesamicol and interfering ions were 10^{-5} and 10^{-4} mol/L, respectively. The selectivity was investigated over creatine, creatinine and inorganic cations such as Na^{+}, K^{+} and Ca^{2+}. The selectivity coefficients for the enantioselective, potentiometric membrane electrodes, K_{sel}^{pot}, obtained are summarized at Table 9.7. The

K^{pot}_{sel} values proved that the constructed EPMEs are selective over creatine and creatinine. D-vesamicol is also not interfering proving the enantioselectivity property of the electrodes (K^{pot}_{sel} $<10^{-4}$). For Na^+, K^+ and Ca^{2+}, the K^{pot}_{sel} is less than 10^{-4}, accordingly they do not interfere in the assay of L-vesamicol.

Table 9.7 Selectivity coefficients of EPMEs.[a]

Interference species (J)	PK^{pot}_{sel}		
	Vancomycin	Teicoplanin	Teicoplanin modified with acetonitrile
Creatine	4.06×10^{-3}	3.94×10^{-3}	4.38×10^{-3}
Creatinine	4.24×10^{-3}	4.10×10^{-3}	4.20×10^{-3}

[a]All measurements were made at room temperature; all values are the average of ten determinations.

9.5.4.4 Analytical Applications

The electrodes proved to be useful for the assay of L-vesamicol in serum samples. The results recorded for the assay of L-vesamicol in serum samples are shown in Table 9.8. These results are in good concordance with those obtained using the method proposed by Gildersleeve, *et al* [29], proving the suitability of these EPMEs for the enantioanalysis of L-vesamicol in serum sample. .

Table 9.8 Recovery of L-vesamicol in serum samples [a]

Sample no.	Electrophoresis method [29] (µg/L)	Recovery, % L-vesamicol		
		EPME based on		
		Vancomycin	Teicoplanin	Teicoplanin modified with acetonitrile
1	15.54	99.41±0.04	99.62±0.02	99.49±0.02
2	20.72	99.97±0.05	99.66±0.02	98.73±0.03
3	25.90	99.68±0.02	99.72±0.03	99.80±0.01
4	31.08	99.84±0.02	99.78±0.03	99.78±0.01
5	36.26	99.61±0.06	99.66±0.02	99.83±0.01

[a] All measurements were made at room temperature; all values are the average of ten determinations.

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9.6 Conclusions

Utilization of EPMEs have several advantages over chromatographic methods such as low cost, no or very simple sample preparation, short time of analysis and simplicity of analysis. The proposed enantioselective, potentiometric membrane electrodes based on maltodextrins (I, II and III) showed excellent features for enantioselective analysis of L-vesamicol. The maltodextrin (III)-based electrode exhibited the lowest detection limits (3.33×10^{-10} mol/L) compared to maltodextrin (I)- and (II)-based electrodes. The proposed electrodes showed good selectivity and proved to be reliable for the direct determination of L-vesamicol in serum samples, without any special pre-treatment before the analysis.

The proposed enantioselective, potentiometric membrane electrodes based on cyclodextrins were successfully applied for the determination of L-vesamicol in human serum samples. These electrodes proved to be reliable for the direct determination of L-vesamicol in real human serum samples.

The proposed enantioselective, potentiometric membrane electrodes based on antibiotics can be successfully used for the enantioselective analysis of L-vesamicol. EMPE also has some advantages over other techniques, such as high precision, rapidity, and low cost of analysis. The construction of these electrodes is simple, fast and reproducible. If miniaturized, these electrodes can be applied for *in vivo* diagnosis patients suffering of Alzheimer and Parkinson diseases.

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Chapter 10

Amperometric biosensor for the enantioanalysis of L-lysine in serum samples

10.1 Introduction

Advanced single-crystal diamond has enabled the development of a wide range of monocrystalline diamond products to meet the exacting requirements of for an amperometric transducer [1]. The reliability obtained for the electrical properties of single-crystal diamond is encouraging for research in the electrochemical sensors based on monocrystalline diamond, as well, it proves that the doping of monocrystalline diamond is not necessary which minimizes the time affected for electrode's construction and also simplified the steps adapted for the design of such electrochemical sensors [2]. Amperometric biosensors are a good alternative for chromatographic methods due to the fact that they can be used for the direct measurement of the enantiomers in solutions without any prior separation of the substance that has to be determined [3]. The biochemical reaction is very selective and sensitive [4]. The sensitivity of the enzymatic reaction must be correlated to the sensitivity of the transducer; the best electrochemical transducer for this biosensor is the amperometric one [5]. Design of amperometric biosensors based on monocrystalline diamond paste proved higher sensitivity and selectivity [5,6].

Lysine is one of the indispensable amino acids that can not be manufactured by the human body, but must be acquired from food sources [7]. L-lysine (Figure 10.1) is widely a available to the public as a non-prescription of oral supplement. L-lysine can be transported into brain [8]. In the metabolism of L-lysine, lysine is the precursor of

acetoacetyl-CoA, which is a very important enzyme in the biosynthesis of acetylcholine in the central nervous system [7].

Lysine is also the precursor in the biosynthesis of L-carnitine, which is involved in the transport of acyl groups into mitochondria for beta-oxidation [9]. L-carnitine is an essential cofactor in the transfer of long-chain fatty acids across the inner mitochondrial membrane [10] and significantly decrease the infarct volume compared with saline [11].

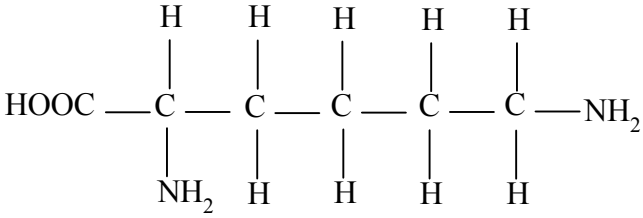

Figure 10.1 L-lysine

The determination of lysine was carried out by liquid chromatography [12,13], ion exchange chromatography [14] and capillary electrophoresis [15]. Unfortunately, these methods may be unsuitable for the analysis of large sets of samples since the separation step is often tedious and time consuming and need expensive equipments. For this reason, there is a great interest in developing faster, less expensive and simpler methods for L-lysine analysis. Biosensors offer the possibility of fast and inexpensive analysis. Four different enzymes have been used as biological components in the construction of these biosensors [16-19]. L-lysine oxidase is the widely used in enzymatic reaction of L-lysine. This enzyme was isolated from *Trichoderma Viride* Y244-2 (EC 1.4.3.14) [20]. The reaction of the enzymatic conversion of L-lysine is shown in Figure 10.2. The enzyme has been reported to immobilize by cross-linking with glutaraldehyde onto a polymer film directly on the

working electrode [26], or covalently on silanized silica gel [23], on nylon [27], or on immobilion membranes [24].

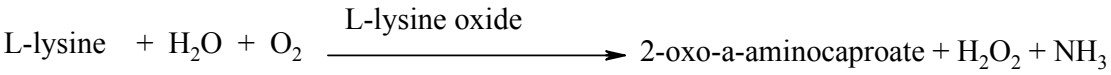

Figure 10.2 Enzymatic conversion of L-lysine

In this chapter, a diamond paste based amperometric biosensor is proposed for the analysis of L-lysine. The proposed amperometric biosensor based on physical immobilization of L-lysine oxidase in a monocrystalline natural diamond paste is designed for the enantioselective analysis of L-lysine in serum samples.

10.2 Reagents and Materials

Natural diamond powder with particle size 1μm (99.9%), β-alanine, creatine and creatinine were purchased from Aldrich (Milwaukee, USA); paraffin oil and L-lysine were purchased from Fluka (Buchs, Switzerland); L-glutamate, D-lysine and L-lysine oxidase (L-lysine oxygen oxidoreductase [deaminating]; (EC 1.4.3.14)) were purchased form Sigma (USA); asparagine and phosphate buffer (pH=7.0) were purchased from Merck (Darmstadt, Germany). D-lysine was purchased from Sigma (Germany). L-(+)-serin was purchased from Reidel-Haen (Germany) L-cystine from Hopkin & Williams LTD (England) L-leucine from Aldrich (Germany); histamine dihydrochloride from BDH (Poole, England); glycine from Analar, BDH (Poole, England).

De-ionized water from a Modulab System (Continental Water Systems, San Antonio, TX, USA) was used for all solution preparations. Stock solution of L-lysine was used (10^{-4} mol/L) for standard solutions preparation (10^{-16}-10^{-4} mol/L) by serial dilution.

10.3 Diamond Paste Based Amperometric Biosensor

Plain diamond was prepared by mixing 0.1 g of diamond powder with 20 µL paraffin oil, and filled in a plastic pipette peak leaving an empty space of 3-4 mm in the top part to be filled with a modified diamond paste.

The diameter of the biosensor was 3 mm. Electrical contact was obtained by inserting a silver wire into the diamond paste. The biosensor tip was gently rubbed on fine abrasive paper to produce a flat surface. The surface of the biosensor was wetted with de-ionized water and then polished with alumina paper (polished strips 30144-011, Orion) before use. The biosensor was stored at 4^{o}C, when not in use. The enzyme solution used for the biosensor design was prepared in 0.1 mol/L phosphate buffer of pH = 8.0.

A modified diamond paste was prepared as follows: 400 mg of natural monocrystalline diamond powder was mixed with 30 µL paraffin oil to form a diamond paste. 50 µL from the enzyme solution (1 mg enzyme/mL phosphate pH = 8.0) was added to the diamond paste.

10.4 Apparatus

A663 VA Stand (Metrohm, Herisau, Switzerland) in combination with a PGSTAT 100 and software Ecochemie (version 4.9) were used for all chronoamperometric

measurements. A Pt electrode and an Ag/AgCl electrode served as the counter and reference electrodes in the cell.

10.5 Recommended Procedures

The chronoamperometric technique was used for the measurement of the intensity of current at 650 mV. The electrode were dipped into a cell containing 10 mL of phosphate buffer, pH = 8.0 and different volumes of L-lysine solution were added. The intensity of current measured was plotted versus the concentration of L-lysine. The unknown concentrations of L-lysine in serum and tablet solutions were determined from the calibration graph.

10.6 Determination of L-lysine in serum samples

Five serum samples were collected from healthy volunteers. The serum samples were buffered with (pH = 8.0) and spiked with different volume of L-lysine. Direct amperometry was involved to determine the concentration of L-lysine in serum samples.

10.7 Results and discussion

10.7.1 Response characteristics of the amperometric biosensors

The response characteristics of the biosensors were measured at 650mV. The reason of the selection of the potential is to obtain the maximum sensitivity and selectivity for the assay of hydrogen peroxide produced by the enzymatic reaction (Figure 10.2) [25]. The biosensor exhibits a linear concentration range from 1 to 100 nmol/L with limit of detection of 4 pmol/L magnitude order. The biosensor response was highly stable and reproducible over two months, when the biosensor was intensively used everyday for measurement and was kept into the fridge at 4 ^{o}C when not in use. The

low limits of detection and high reliability of the biosensor is due to the diamond paste matrix used for its design.

The response of the biosensor to different L-lysine concentrations was linear over wide concentration ranges and can be described by the following calibration equation:

$$I = 0.23\ C + 2.28; \qquad r = 0.9866$$

where I is the intensity of the current in μA, C is the concentration of L-lysine in nmol/L, and (r) is the regression coefficient.

The limits of detection were calculated using the equation proposed by Otto [26]

$$DL = \frac{I_B + 3\sigma_S - a}{S}$$

where I_B is the background current, σ_S is the standard deviation for the measurement of the background current, a is the intercept of the calibration equation and S is the slope of the calibration equation.

10.7.2 Enantioselectivity of the amperometric biosensor

The selectivity of the biosensor was checked using both separate and mixed solution methods over D-lsyine, L-(+)-serin, L-cystine, L-leucine, L-glutamic acid, asparagine, histamine dihydrochloride, glycine, β-alanine, creatine and creatinine. Amperometric selectivity coefficients, K_{sel}^{amp} , were determined following the method proposed by Wang [27] for the same potential (650 mV) that was used for the determination of response characteristics of the proposed amperometric biosensor. The ratio between the concentrations of L-lysine and the interfering species was 1:10. All the values of

the amperometric selectivity coefficients (pK_{sel}^{amp}) obtained by using the mixed solution method, for the biosensor designed for L-lysine are higher than 2 (Table 10.1) demonstrating the enantioselectivity and selectivity of the proposed biosensor.

Table 10.1 Amperometric selectivity coefficients of the biosensor[a]

Interfering Species (j)	pK_{sel}^{amp}
D-lyine	2.55
L-serine	3.36
L-leucine	2.15
L-aspartic acid	2.01
L-glutamic acid	3.15
Histamine	2.09
Glycine	2.07
β-alanine	3.15
Creatine	2.37
Creatinine	2.91

[a]All measurements were made at room temperature; all values are the average of ten determinations

10.7.3 Analytical applications

The recovery test of L-lysine was conducted in the presence of its antipode, D-lysine. The results obtained (Table 10.2) demonstrated the suitability of the proposed biosensor for the enantioanalysis of L-lysine due to the high recovery values obtained for the assay of L-lysine in the presence of D-lysine. No significant differences in the recovery values were recorded for the ratio between L:D varying from 1:9 to 1:99.9.

Table 10.2 Recovery of L-lysine in the presence of D-lysine[a]

L:D	% L-lysine, Recovery
2:1	99.19±0.07
1:1	99.89±0.06
1:2	99.93±0.06
1:4	99.82±0.07
1:9	99.84±0.06

[a]All measurements were made at room temperature; all values are the average of ten determinations.

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The results obtained for the analysis of L-lysine in serum samples are shown in Table 10.3. Blood samples were collected from healthy volunteers and spiked with different aliquots of L-lysine solutions. The results obtained using the proposed biosensor at 650 mV working potential are in good concordance with the values added to the serum samples.

Table 10.3 Recovery of L-lysine in serum samples

Sample No.	% L-lysine, Recovery
1	99.01±0.05
2	98.99±0.06
3	99.12±0.05
4	99.07±0.05
5	99.18±0.06

[a]All measurements were made at room temperature; all values are the average of ten determinations.

10.8 Conclusion

The proposed design of the amperometric biosensor is simple and reproducible. The analytical information obtained using the proposed biosensor is highly reliable. One of the features of the biosensor is its utilization for *in vivo* determination of L-lysine.

10.9 References

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Chapter 11

Conclusions

The application of electrochemical sensors and biosensors for enantioanalysis of compounds of biological importance (clinical analysis) is increasingly important. Such real-time measurements are highly desirable in intensive-care unit and surgery. The availability of more portable, precise and reliable measurements devices will inevitably be a further stimulus to the demands of the medical research establishment. The researchers involved in the development of biosensors and electrodes are approached for devices of high accuracy and precision for use in clinical and fundamental physiological research. The requirements for an ideal *in vivo* probe are high sensitivity, high selectivity, fast response time, long-term stability, and independence of variations in the biological sample (e.g., pH), biocompatibility, sterilizability and small size.

The aim of the thesis was to design new biosensors and enantioselective, potentiometric membrane electrodes; for the enantioanalysis of metabolites that are markers for different diseases. Three types of electrochemical techniques have been used for enantiomer recognition: amperometry, differential pulse voltammetry and potentiometry. L- and D-enantiomers of pipecolic acid, fucose, glyceric acid, 2-hydroxyglutaric acid, vesamicol and lysine are markers for different diseases. Therefore an enantioanalysis of these substances in serum or/and in urine samples is necessary. Pipecolic acid was analyzed using amperometric and potentiometric methods. Amperometric biosensor based on diamond and carbon pastes impregnated with L- and/or D-AAOD have been designed for the enantioanalysis of pipecolic acid in serum samples. The biosensors based on carbon

paste were utilized as detectors in sequential injection system. The amperometric biosensors described have excellent features in the assay of enantiomers in biological fluids.

The reliability obtained for the electrical properties of single-crystal diamond is encouraging for research in the electrochemical sensors based on the monocrystalline diamond. Monocrystalline diamond (natural diamond, synthetic-1 and synthetic-2) pastes were utilized for the design of electrochemical electrodes for the analysis of L- and D-pipecolic acids and L- and D-fucose using differential pulse voltammetry. An amperometric biosensor based on diamond paste impregnated with L-lysine oxidase has been designed and investigated for the assay of L-lysine in blood samples. The biosensor exhibits high reliability, low limits of detection, good stability and reproducibility due to the use of diamond paste. One of the features of the biosensor is its utilization for *in vivo* assay of L-lysine.

The results obtained in this work revealed the need to employ diamond paste based electrodes to diagnose the abnormal metabolites in biological fluids. One of the features is utilization of diamond paste electrodes for *in vivo* continuous monitoring of chiral molecules in biological fluids. Amperometric biosensor based on diamond paste impregnated with L-AAOD exhibited the lowest limit of detection for the analysis of L-pipecolic acid; for the assay of D-pipecolic acid, the lowest limit of detection was recorded when the amperometric biosensor based on carbon paste impregnated with D-AAOD and HRP was used. For L-fucose assay the amperometric electrode based on

natural diamond exhibited the lowest limit of detection when NaCl was used as electrolyte, while for the assay of D-fucose, amperometric electrode based on diamond synthetic-2 has the lowest limit of detection when $NaNO_3$ was used as electrolyte.

Enantioselective, potentiometric membrane electrodes based on carbon paste impregnated with maltodextrins (I, II, and II), cyclodextrins (α-CD, γ-CD β-CD, and 2-hydroxy-3-trimethylammoniopropyl-β-CD) and macrocyclic antibiotics (vancomycin, teicoplanin and teicoplanin modified with acetonitrile) have been designed for the analysis of the enantiomers of glyceric acid, 2-hydroxyglutaric acid and L-vesamicol. The proposed enantioselective, potentiometric membranes electrodes have excellent features in the real time enantioselective analyses of glyceric acid in biological fluids, e.g., serum and urine samples. The lowest limit of detection of L-glyceric acid determination was obtained using EPME based on α-cyclodextrin, while EPME based on 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrin exhibited the lowest limit of detection for the assay of D-glyceric acid. EPME based on maltodextrin (I) has the lowest limit of detection for the analysis of L-2-hydroxyglutaric acid and EPME based on teicoplanin modified with acetonitrile exhibited the lowest limit of detection for the assay of D-2-hydroxyglutaric acid. EPMEs based on maltodextrins have only worked for the assay of L-2-HGA, while EPMEs based on antibiotics have worked only for the assay of D-2-HGA. In the determination of L-vesamicol, EPME based on β-CD exhibited the lowest limit of detection.

The construction of the electrodes is simple, fast and reproducible. One of the main advantages of the proposed methods is that the serum and urine samples did only need to

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be buffered before the assay of any of the enantiomers and that makes the method simple, fast and highly reliable.

One can conclude, that the proposed amperometric (bio)sensors and enantioselective, potentiometric membrane electrodes can be directly used in clinical analysis. Application of these biosensors and electrodes for *in vivo* enantioanalysis is very important for fast and continuous assay of abnormal metabolites during surgery or in the intensive care units.

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APPENDIX

APPENDIX A

PUBLICATIONS

1. *Biosensors for the enantioselective analysis of pipecolic acid*

   R.I. Stefan, R.M. Nejem, J.F. van Staden and H.Y. Aboul-Enein

   Sens.Actuators B, 94(3), 271-275, 2003.

2. *Determination of L- and D-pipecolic acid using diamond paste based amperometric biosensors*

   R.I. Stefan and R.M. Nejem

   Anal.Lett., 36(2), 2635-2644, 2003.

3. *Determination of L- and D-pipecolic acids using a diamond paste based electrode*

   R.I. Stefan and R.M. Nejem

   Instrum. Sci. & Technol., 32(3), 309-318, 2004.

4. *New amperometric biosensors based on diamond paste for the assay of L- and D-pipecolic acids in serum samples*

   R.I. Stefan, R.M. Nejem, J.F. van Staden and H.Y. Aboul-Enein

   Prep.Biochem.& Biotechnol., 34(2), 135-143, 2004.

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5. *Enantioselective, potentiometric membrane electrodes for the determination of L-pipecolic acid in serum*

R.I. Stefan, R.M. Nejem, J.F. van Staden and H.Y. Aboul-Enein

Electroanalysis, In Press.

6. *Sequential injection analysis utilizing amperometric biosensors as detectors for simultaneous determination of L- and D-pipecolic acid*

R.I. Stefan, R.M. Nejem, J.F. van Staden and H.Y. Aboul-Enein

Sensors, In Press.

7. *Enantioselective, potentiometric membrane electrodes based on antibiotics for the determination of L- and D-glyceric acids.*

R.M. Nejem, R.I. Stefan, J.F. van Staden and H.Y. Aboul-Enein

Talanta, Submitted.

8. *Diamond paste-based electrodes for the determination of L- fucose*

R.I. Stefan, R.M. Nejem, J.F. van Staden and H.Y. Aboul-Enein

Anal.Chem., Submitted.

9. *Enantioselective, potentiometric membrane electrodes based on cyclodextrines for the assay of L- and D-hydroxyglutaric acids*

R.M. Nejem, R.I. Stefan, J.F. van Staden and H.Y. Aboul-Enein

Anal.Lett., Submitted.

10. *Enantioanalysis of L-hydroxyglutaric acid in urine samples using enantioselective, potentiometric membrane electrodes based on maltodextrins*

R.M. Nejem, R.I. Stefan, J.F. van Staden and H.Y. Aboul-Enein

Talanta, In Press.

11. *Determination of D-hydroxyglutaric acid in urine samples using enantioselective, potentiometric membrane electrodes based on antibiotics*

R.M. Nejem, R.I. Stefan, J.F. van Staden and H.Y. Aboul-Enein

Sensors and Actuators B, Submitted.

12. *Enantioanalysis of glyceric acid in urine samples using enantioselective, potentiometric membrane electrodes based on maltodextrins*

R.M. Nejem and R.I. Stefan

J.Pharm.Biomed.Anal., Submitted.

13. E*nantioselective, potentiometric membrane electrodes based on cyclodextrines for the assay of glyceric acid in urine samples*

R.M. Nejem and R.I. Stefan

Sensors, Submitted.

14. Cyclodextrins based e*nantioselective, potentiometric membrane electrodes for L-vesamicol assay in serum samples*

R.M. Nejem and R.I. Stefan

Sensors and Actuators B, Submitted.

15. E*nantioselective, potentiometric membrane electrodes based on maltodextrins and their applications for the determination of L-vesamicol in serum samples*

R.M. Nejem and R.I. Stefan

The Analyst, Submitted.

16. *Determination of L-vesamicol in serum samples using enantioselective, potentiometric membrane electrodes based on antibiotics*

R.M. Nejem and R.I. Stefan

Anal.Lett., Submitted.

17. *Amperometric biosensor based on diamond paste for the enantioanalysis of L-lysine*

R.M. Nejem, R.I. Stefan, J.F. van Staden and H.Y. Aboul-Enein

Anal.Chem., Submitted.

18. *Determination of D-fucose using amperometric electrodes based on diamond paste*

R.I. Stefan and R.M. Nejem

Sensors, In Press.

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APPENDIX B

PRESENTATIONS

Oral presentations

1. *Enantioselective, potentiometric membrane electrodes for the enantioanalysis of L- and D-2-hydroxyglutaric acids in urine samples*
   R.M. Nejem, R.I. Stefan, J.F. van Staden and H.Y. Aboul-Enein
   37th Convention SA Chemical Institute. Pretoria. 4-9 July 2004

Poster presentations

1. *Biosensors for the enantioselective analysis of pipecolic acid*
   R.I. Stefan, R.M. Nejem, J.F. van Staden, H.Y. Aboul-Enein
   IMA'2003. The 3rd International Conference of Instrumental Methods of Analysis. (Modern trends and Applications). Thessaloniki. Greece. 23-27 September 2003.

2. *Biosensors for the enantioselective analysis of pipecolic acid*
   R.I. Stefan, R.M. Nejem, J.F. van Staden, H.Y. Aboul-Enein
   ICFIA'2003. 12th International Conference on Flow Injection Analysis, including related techniques. Merida. Venezuela. 7 - 13 December 2003.