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Enantioselective, potentiometric membrane electrodes for enantioanalysis of amino acids of clinical and pharmaceutical importance

by

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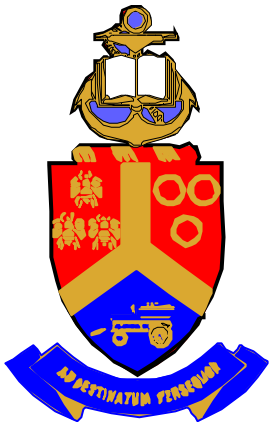

Introduction

It is very important to have means to monitor substances of pharmaceutical and clinical importance to help patience in the intensive care unit and it is better to follow procedures that could be relatively fast and reliable. Chiral recognition has become an area of considerable interest because of its importance in almost all fields of chemical, pharmaceutical, biological and environmental sciences. The presence of chiral compounds in human fluids (spinal, serum, urine) as normal metabolites of human metabolism or drug metabolism has given ways to assess levels of the substances in biofluids. The levels of the clinical molecules are markers of human body abnormalities. Carbohydrates, urinary organic acids and amino acids are excreted in the human body and their change in the body can lead to many kinds of diseases such as cancer, inborn metabolic disorder etc.

It happens that some substances exist in enantiomeric form, however this can be a problem because this can lead into different kinds of diseases because of the existence of each enantiomer, so it will be better to diagnose such illnesses by finding more reliable and effective methods that could discriminate between two enantiomers (L and D or R and S enantiomer) and such analysis should be fast, reliable and highly sensitive.

It is better to focus on molecular recognition that plays the vital role in chiral discrimination around asymmetric center. There is a need to find substances that can be used in the enantioanalysis for the diagnosis, prevention and treatment of human diseases due to the importance of enantiomeric discrimination.

Popular techniques for chiral discrimination are based on chromatography, capillary zone electrophoresis, mass spectrometry and more recently electrochemistry. Amongst all these techniques, electrochemistry feature some advantages, simplicity, and high efficiency, low cost, high reliability that is given by high precision, high reproducibility and rapidity.

The aim of the work is to construct reliable enantioselective electrode (enantioselective, potentiometric membrane electrode) that can easily be applied in diagnosis and pharmaceutical analysis. Carbon paste is proposed as the matrix for the sensors' design. Chiral recognition principle that is based on selective binding is considered for the selection of the best chiral selector. The amino acids of clinical and pharmaceutical importance selected were: clenbuterol, cysteine and histidine.

Potentiometry was used for the direct assay of enantiomers in pharmaceutical tablets and/or in urine and serum samples. The best selection of the type of electrode to be used was done in concordance with the complexity of the structure of the enantiomer to be determined leading to good performance of the electrode in analysis.

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Department of Chemistry

University of Pretoria

Degree: Magister Scientiae

SYNOPSIS

The enantioanalysis of compounds of clinical and pharmaceutical became increasingly important because the enantiomers of the same substance may be markers for different disease or are having a different pathway in the body. The utilization of enantioselective, potentiometric membrane electrodes made the assay of a single enantiomer easier and faster. Also the reliability of the analytical information is higher than that obtained using chromatographic techniques.

The proposed electrodes are made by mixing graphite powder with paraffin oil to give carbon paste, which is modified by the addition of a chiral selector (e.g., cylodextrins, maltodextrins, macrocyclic antibiotics and fullerenes). This design is reliable.

The high sensitivity, selectivity, enantioselectivity, accuracy and precision made them suitable to be used for the enantioanalysis of different compounds of clinical and pharmaceutical importance (e.g., L-histidine, L-cysteine and R-clenbuterol) in pharmaceutical tablets, and/or serum and urine samples.

Acknowledgements

It is my honour and a privilege to acknowledge few people who have made my year to be successful with the great contribution they have given me. I would personally thank my supervisor Prof Raluca-Ioana Stefan-van Staden for the time she had sacrificed to assist me, guiding me in every step of the way with writing papers and everything. I appreciate the support of Prof J.F. van Staden, being encouraging, sharing ideas with me that really meant a lot.

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Chapter 1

Amino Acids

1.1 Introduction

Amino acids are building blocks of proteins and are linked together by peptide (amide) bonds with a positively charged nitrogen-containing amino group at one end and a negatively charged carboxyl group at the other end. A series of different side chains along the chain differs for each of the 20 amino acids. Two amino acids are linked by a dipeptide, three amino acids by a tripeptide. The sequence of the amino acids is of the utmost importance. There are more than a billion possible sequences for a chain of 20 amino acids long and such a sequence is the message.

1.1.1 General formula and 3D structure of amino acids

There are about 20 amino acids found in proteins among all the possible amino acids. Amino group and a carboxylic group gives the general structure of amino acids, both which are bonded to a α-carbon (the next one to the carboxyl group). The α-carbon is bonded to hydrogen and to the side chain group, which is represented by the letter R. The identity of the particular amino acid is given by the R group (Figure 1.1). The most important property of amino acids is their three-3D shape, or stereochemistry.

The fact is that every object has a mirror image (Figure 1.2) many pairs of such objects that are mirror images can be superimposed on each other and a good example is two identical solid-colored coffee mugs. The relation of the right hand to the left

hand is the case whereby two mirror image objects cannot be superimposed on one another and however such nonsuperimposable mirror images are said to be chiral (from Greek cheir, “hand”). Many biomolecules are chiral.

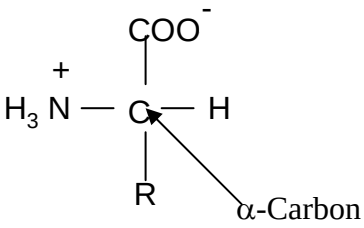

Figure 1.1 General formula of amino acids

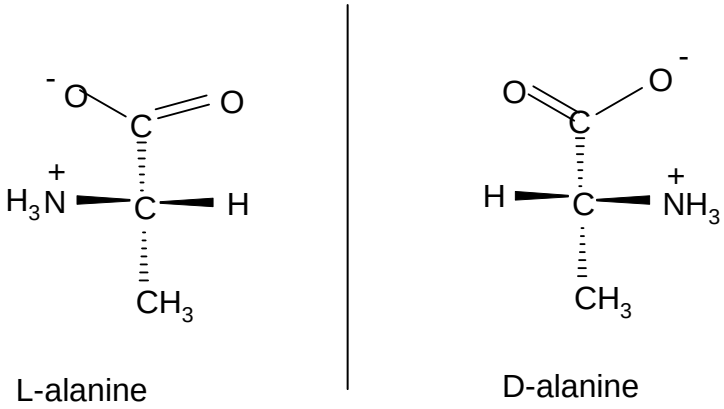

Figure 1.2 Stereochemistry of alanine

A carbon atom is encountered as a chiral center in biomolecules with four different groups attached to it. In all amino acids except glycine such center occurs and this is because glycine has two hydrogen atoms bonded to the α-carbon and in other words, the side chain (R group) of glycine is hydrogen and this makes glycine achiral because of this symmetry. The α-carbon of all other occurring amino acids has four different groups attached to it and giving rise to two nonsuperimposable mirror image forms. The stereoisomers of alanine are in comparison with the corresponding forms

of L- and D-glyceraldehydes, which are two possible stereoisomers of another chiral compound. The basis of classification of amino acids into L and D forms is shown by the two forms of glyceraldehydes. The terminology comes from the Latin laeveus and dexter, meaning “left” and “right”, respectively. If the hydroxyl group is on the left hand side of the molecule it gives the L-form (e.g., L-glyceraldehyde) while for the D-form the hydroxyl group is on the right side of the molecule (Fischer projection). L- and D-designation is determined by the position of the amino group, either on the left or right side of the α-carbon in an amino acid. The form that is common in biological systems or occurs in proteins is the L-form. D-form is not found in proteins, it is found in bacterial cell walls, in some antibiotics, or occurs in nature.

1.2 The structures and properties of the individual amino acids

There are two important ways of classifying the individual amino acid from several criteria. First thing that needs to be considered is their nature of side chain (polar or nonpolar). Secondly, the existence of a group in their side chain either its acidic or basic needs to be considered and also the functional groups that are present in the side chain and their nature is of utmost importance [1-4].

All other amino acids have a larger side chain and are referred to as complex side chains if one is comparing with glycine, which has one of the simplest side chains and is having two hydrogen atoms bonded to the α-carbon and in this case the R-group in glycine is the hydrogen atom. There is a way in which amino acids are designated and letters (Greek alphabet) are used for this kind of classification and first count is from α-carbon and follows the order β-, γ-, and ε-carbons and finally a terminal carbon that is the last carbon in the order is termed ω-carbon since this is the last letter of the

Greek alphabet. A simple way of identifying amino acids is useful and abbreviation of the names of amino acids is common and a three or one letter abbreviation of their names is used. The side chains of individual amino acid verify the nature of those groups and however according to this nature of their side chains they are classified into groups.

1.2.1. Group 1- Amino acids with nonpolar side chains

The first group of amino acid classification is of nonpolar side chains. It consists of alanine, leucine, valine, isoleucine, proline, tryptophan, phenylalanine and methionine. A certain case whereby the amino acid has no polar side chain (e.g., glycine) it is then placed in this group.

Amongst this group, each side chain of the following amino acids valine, alanine, leucine, and isoleucine is an aliphatic hydrocarbon group and by “aliphatic” means the absence of the benzene ring or related structure. An amino acid that has an aliphatic structure is proline and its nitrogen is bonded to two carbon atoms making the amino group of proline a secondary amine compared to the amino groups of all other amino acids that are primary amines. Certain amino acids contain a cyclic group that is similar to a benzene ring and such a hydrocarbon group is aromatic rather than aliphatic and a good example is phenylalanine and also a side chain that contains an indole ring is present in tryptophan that is also aromatic and in addition to this a side chain that contains a sulfur atom in addition to aliphatic hydrocarbon groupings is present in methionine.

1.2.2 Group 2-Amino acids with electrically neutral polar side chains

At neutral pH other group of amino acids has polar side chains that are electrically neutral (uncharged). Tyrosine, cysteine, serine, threonine, glutamine and asparagines are included in this group and in addition to this group. Glycine is also included because it lacks a nonpolar side chain but this amino acid can belong to group 1 as well because of the C-H bond that is nonpolar. The polar groups of some of these amino acids is a hydroxyl group (-OH) that is bonded to an aliphatic hydrocarbon group and this side chain cannot lose a proton (e.g., serine and threonine). A hydroxyl group that is bonded to an aromatic hydrocarbon group is found in tyrosine, which can eventually lose a proton at higher pH, however this hydroxyl group is a phenol that is a stronger acid compared to an aliphatic alcohol.

The polar side chain that consists of an –SH (thiol) group is found in cysteine and a possible self-addition of cysteine (-SH groups) results in the formation of a disulfide (-S-S-) bridges that occurs in oxidation reactions of proteins and the thiol group can also lose a proton. There are certain amino acids that have amide groups on their side chains and amide bonds do not ionise in the range of pH. Such amino acids are derived from carboxyl groups are asparagines and glutamine and they are considered to be derivatives of group-3 amino acids glutamic acid and aspartic acid due to the carboxyl groups in their side chains.

1.2.3.Group 3-Amino acids with carboxyl groups in their side chains

There are some amino acids that are derived from this group (e.g., asparagines and glutamine). Group-3 amino acids have a carboxyl group in their side chain in addition to the one present in all amino acids. A carboxylate anion (glutamate and aspartate)

results when a carboxyl group loses a proton and the presence of the carboxylate side chain at neutral pH become negatively charged. These two amino acids (glutamate and aspartate) differ only by a $–CH_2$ group in their side chain.

1.2.4.Group 4-Amino acids with basic side chains

Histidine, lysine, and arginine are the three amino acids that have basic side chains. At neutral pH (lysine and arginine) are having side chains that are positively charged. A good example is histidine with an imidazole side chain that is highly reactive with a pKa of 6.0 that is not far from the physiological pH.

Many properties of proteins depend on the reactivity imidazole group. A side-chain amino group that is attached to an aliphatic hydrocarbon tail is found in lysine.
A more complex guanidine group in structure than the amino group is the side-chain basic group of arginine also bonded to an aliphatic hydrocarbon tail.

1.3 Titration curves of the amino acids

At neutral pH the free amino acid carboxyl group and amino group are charged giving a negative carboxylate portion and a positive amino group. In neutral solutions, amino acids without charged groups on their side chains exists as zwitterions with no net charge. An ion that has equal number of positive and negative charges and is electrically neutral in solution is called zwitterion. By neutral (or without charges), it does not mean amino acids exist in the form NH_2-CHR-COOH.

The titration curve indicates the reaction of each functional group with hydrogen ion when an amino acid is titrated. In histidine, the imidazole group that is so reactive

contributes a titratable group and a histidine molecule has a net positive charge of 2 at very low pH values because both the imidazole and the amino groups have positive charges.

A carboxyl group loses a proton and become a carboxylate as mentioned earlier when the base is added and the pH increases and histidine now bears a positive charge of 1. A charged imidazole group loses its proton when more base is added and at this point histidine molecule has no net charge. Finally histidine molecule bears a negative charge of 1 when the amino group loses its proton at higher values of pH.

1.4 References

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Chapter 2

Enantioselective, Potentiometric Membrane Electrodes

2.1 Introduction

Enantioselective, potentiometric membrane electrodes (EPMEs) are particularly one of the best choices for enantioanalysis [1-5]. They can be used for real time monitoring of enantiomers concentration. Also the sampling is very simple and the sample needs only to be buffered. The EPMEs produce reliable analytical information, the cost of analysis is low, and the analyses are rapid and simple to perform.

For clinical purposes, EPMEs offer many advantages and it is most preferable compared to other techniques e.g., chromatography, mass spectrometry, capillary zone electrophoresis etc. [6. 7]. The complexity of the structure of the enantiomer to be determined gives information for selection of the type of electrode and chiral selector to be used for analysis. It is better to follow the principle of molecular recognition for EPMEs: the selective binding between a molecule with a special chemical architecture (chiral selector) and the enantiomer. Both enantiomers may interact with the chiral selector:

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

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

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

The equations showing the dependence of the stability constants (K_L and K_D) on the free energy are the following:

$$K_L = e^{-\frac{\Delta G_L}{RT}} \qquad (2.1)$$

$$K_D = e^{-\frac{\Delta G_D}{RT}} \qquad (2.2)$$

where ΔG_L and ΔG_D are the free energies recorded for the L-and D-enantiomer reactions with chiral selector, CS. R is the gas constant = 8.314J/mol K and T is the temperature in Kelvin. The log K_L is directly proportional to ΔG_L and log K_D is also directly proportional to ΔG_D, respectively. Therefore the 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, leading to the stability of the complexes correlating with the response (slope) of the EPMEs [8]. Accordingly, the slope is a measure of enantiorecognition.

2.2. Design of enantioselective, potentiometric membrane electrodes

The design of enantioselective, potentiometric membrane electrodes (EPMEs) play a crucial role in the reliability of analytical information.

The evolution about the design of EPMEs made their utilization an accurate and precise alternative to structural analysis techniques [9]. The reliability of the analytical information and the reliability of the response characteristics obtained using EPMEs are strictly correlated to the reliability of the electrode design [8].

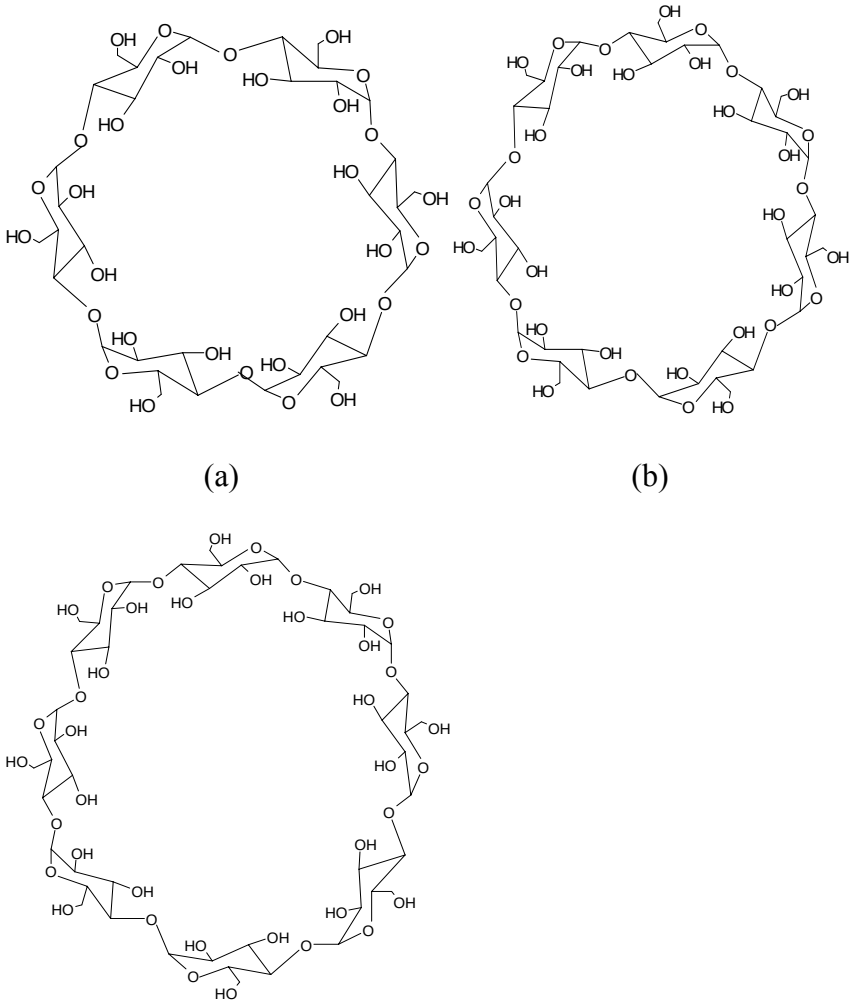
2.2.1. Modified carbon paste electrode design

Graphite powder proved to be a very good material for electrode design. Paraffin/Nujol oil was mixed with graphite powder forming the carbon paste. Stefan et al [10-11] has proposed one of the most reproducible designs for EPMEs based on carbon paste. Graphite powder (100 mg) and paraffin oil (40µL) were mixed in a ratio 1:4 (w/w) followed by the addition of a solution of chiral selector (10^{-3} mol/L) (100µL of a chiral selector solution). A quantity of carbon paste free of the chiral selector was also prepared and placed in a plastic pipette, leaving 3 to 4 mm empty in the top to be filled with the carbon paste containing the chiral selector. The diameter of the potentiometric, enantioselective membrane electrode 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. The surface of the electrode was wetted with deionised water and polished with alumina paper (polishing strips 30144-001, Orion) before use in each experiment.

The carbon paste prevents the leaching of the chiral selector from the membrane into solution. The electrode response is directly proportional to the concentration of the complex formed at the membrane solution interface. Different types of chiral selectors were proposed for the design of EPMEs such as cyclodextrins and its derivative, maltodextrins, macrocyclic antibiotics (vancomycin and teicoplanin) and fullerenes.

2.2.1.1. Cyclodextrins as chiral selector in the EPMEs design

Cyclodextrins (Fig. 2.1) are cyclic, non-reducing oligosaccharides of six, seven and eight α-D-glucose units, which are commonly referred to as α-, β-, and γ-cyclodextrins respectively and obtained from starch by enzymatic degradation by Bacillus Amylobacter [12-17].



(a) (b)

(c)

Figure 2.1 The chemical structures of (a) α-, (b) β-, (c) γ-cyclodextrin

Their cyclic linkage of their glucose units is through C-O-C α-1,4 bonds that gives them a toroidal or truncated molecular shape of relative hydrophobic cavity [12-17]. An important property of cyclodextrins is their ability to form inclusion complexes with a large number of organic and inorganic compounds, an important property that has been extensively exploited in pharmaceutical formulations of certain drugs thereby reducing their side effects and increasing the bioavailability and solubility in water [12-17]. Their cavities are suitable for enantioanalysis of chiral compounds, with the possibility of achieving double selectivity: an internal selectivity (i.e., inclusion type, dependent on the cavity size and guest molecule) and external selectivity (dependent on functional groups) [12-17]. Cyclodextrin derivative are developed to modify their properties such as cavity shape and hydrophilicity.

2.2.1.2. Maltodextrins as chiral selectors in the EPMEs design

Maltodextrins represent a class of very powerful chiral selectors among the chiral selective substances, e.g., cyclodextrins, crown ethers, macrocyclic antibiotics, proteins. Maltodextrins (Fig. 2.2) are complex malto-, oligo-, and polysaccharide mixtures formed by hydrolysis of starch, with DE lower than 20 [18-20]. Possible types of maltodextrins have different DE values [I (4.0-7.0), II (13.0-17.0), and III (16.5-19.5)]. Variations in DE values result in maltodextrins with varying physico-chemical properties: solubility, hydroscopicity, osmolality and their effectiveness to reduce the freezing point increase with increasing DE, while viscosity, cohesiveness and coarse-crystal prevention increase as DE decreases [21,22]. Maltodextrins were intensively investigated as chiral selectors for enantiomeric separations by capillary zone electrophoresis, the maltodextrins with the highest DE values being the best chiral selectors [19,20,23-27], and they were also used in the design of

enantioselective, potentiometric membrane electrode for the enantioanalysis of several drugs.

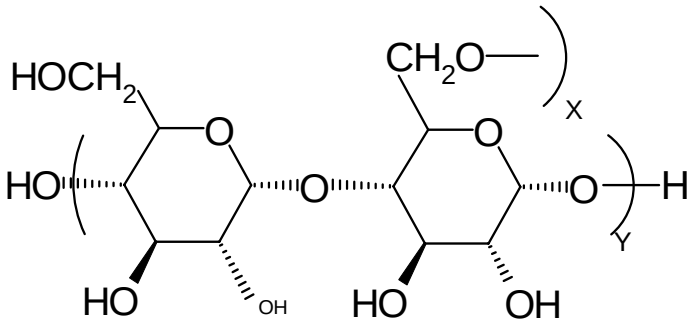
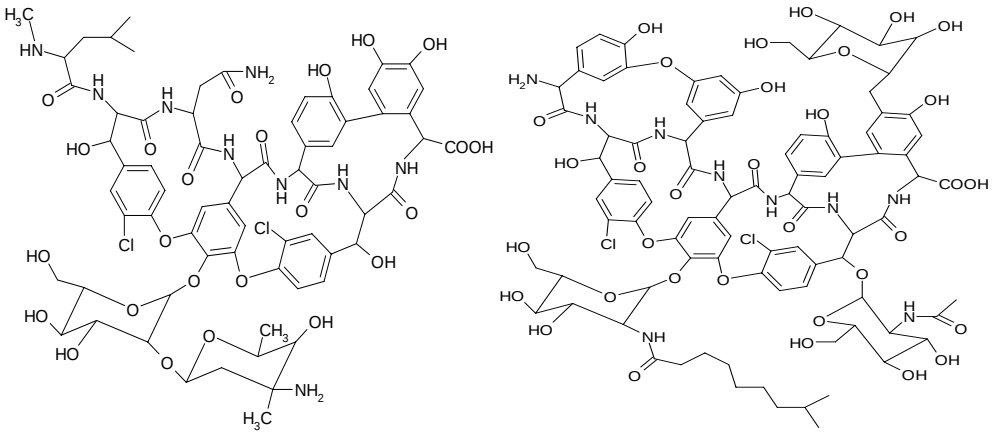


Figure 2.2 Maltodextrin

2.2.1.3. Macrocyclic antibiotics as chiral selectors for EPMEs design

Enantiorecognition of several classes of pharmaceutical drugs and molecules of biological importance has been successfully achieved by using macrocyclic antibiotics as chiral selectors. Macrocyclic antibiotics have several functional groups that are responsible for multiple stereoselective interaction.

Many macrocyclic antibiotics exhibit similar physico-chemical properties, on the hand showing different stereoselective power [28]. The most commonly used macrocyclic antibiotics are vancomycin and teicoplanin [29-31].



(a) (b)

Figure 2.3 Chemical structures of (a) Vancomycin and (b) Teicoplanin

Vancomycin is "basket" shaped (Figure 2.3a) having three fused macrocyclic rings and two side chains, a carbohydrate dimmer and a N-methyl leucine moiety [31]. There are 18 asymmetric centers and several and several functional groups such as carboxylic, hydroxyl, amino, amido, and aromatic rings [28].

It is very soluble in water and can dimerize in aqueous solutions depending on vancomycin concentration [32]. Vancomycin solutions are very stable at low temperatures and in buffered solutions (pH 3.0-6.0) [32-33].

Teicoplanin (Figure 2.3b) is obtained from fermentation by Actinoplanes teichomyceticus. It is structurally related to the antibiotics vancomycin, and ristocetin A, but differs from these antibiotics in several ways [34], it contains the carbohydrates D-glucoseamine and D-mannose, and the amino group of the glucoseamine is substituted with a long fatty acid chain that contains 10 or 11 carbons, and is more hydrophobic than vancomycin and it aggregates in aqueous solution [35].

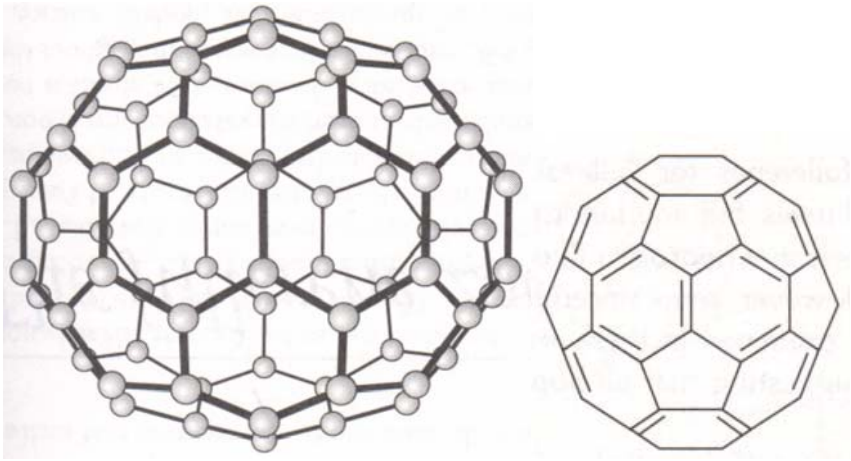
Teicoplanin contains one free amine and one free carboxylic acid group. However it contains 23 stereogenic centers, four phenolic groups and seven aromatic groups and is obtained as a mixture of five analogous compounds containing different fatty acid chains (C_{10}-C_{11}) attached to the amine of 2-amino-2-deoxy-β-D-glucopyranozyl groups. Self-association of teicoplanin with micellization favoured by lower pH [35] is caused by this hydrophobic tail.

The most important functional groups for amino acid recognition are the $–NH_2$ and the –COOH groups ionised over pH 3.5-8.0 range [36]. Teicoplanin is soluble in water, slightly soluble in methanol and ethanol and insoluble in non-polar organic solvents.

2.2.1.4. Fullerenes

A long search for molecular allotropic forms of carbon other than graphite and diamond culminated in 1985 in the discovery of a C_{60} molecule. Fullerenes (Figure 2.4) are “cage” molecules that are named after eccentric architect R. Buckminster Fuller, the inventor of the truncated icosahedron-shaped geodesic dome. The soccer-ball shaped C_{60} molecule and the rugby-ball shaped C_{70} were soon followed by higher-order fullerenes of 76, 84, 90, and 94 carbon “cages” as large as C_{240} and C_{540}.

The fullerenes appear as a whole system able to form a completely new type of chemistry with many surprising arrangements and applications, including analytical ones and it is also said that, when a new atom is putted in a fullerene cage, or when an atom (or group) is attached to the outside, a new molecule is formed [37,38].



(a) (b)

Figure 2.4 Fullerene (a) C-70 and (b) C-60

Since the discovery of the fullerenes (C_{60}, C_{70} and higher fullerenes), electrochemistry has played a significant part in investigations of the properties of these fascinating new types of carbon. Initially, some important physiochemical properties, including the standard redox potentials, and confirming theoretical predictions of these molecules [39,40] were considered. Fullerenes (C_{60} and C_{70}) display anomalous behaviour in solution due to the formation of aggregates [41-49]. Since the specific surface energies of interactions of fullerene molecules are very close in magnitude [41], fullerenes in solution often tend to form aggregates

2.3. Response characteristics of EPME

2.3.1 Electrode function

The electrode function is given by, the functional relation between the potential, E measured at I=0 (chronopotentiometry at zero current), and the activity, a, of the ion (Figure 2.5).

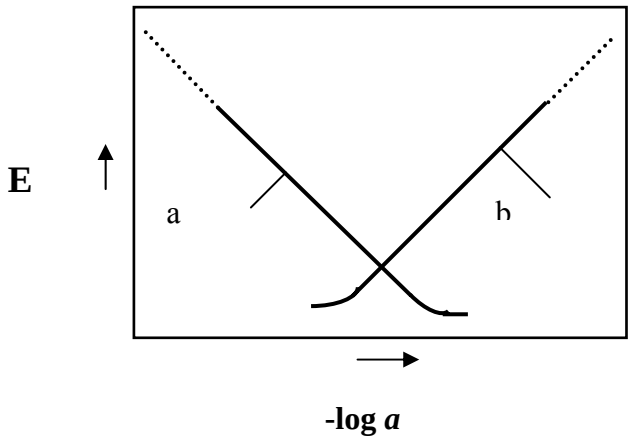


Figure 2.5 Electrode function. (a) cation-selective electrode, (b) anion-selective electrode.

From Figure 2.5, the potential is not depending on the activity, a of the ion but on –loga.

$$E=f(-\log a) \qquad (2.3)$$

The ionic strength can be kept constant by addition of strong electrolytes 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.

By maintaining the ionic strength at a constant value, the activity can be substituted with the concentration, and further more for an ion M^{z+}, $pM= -\log C_M^{z+}$ is used. The electrode function is given by:

$$E=f(pM) \qquad (2.4)$$

2.3.2 Standard electrode potential, E^o

The International Union of Pure and Applied Chemistry (IUPAC) has defined the standard electrode potential as the value of the standard emf of a cell in which the molecular hydrogen is oxidized to solvated protons at the left-hand electrode [50].

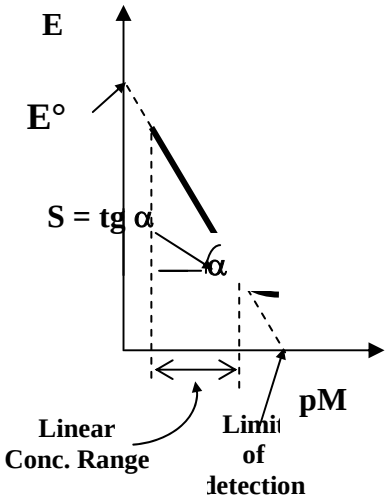


Figure 2.6. Response characteristics of enantioselective, potentiometric membrane electrodes

It does not depend on the concentration of the ions in solution. The calibration graph of the potentiometric electrodes can be of great help to find the standard electrode potential, E^o (Figure 2.6).

The linear regression method is recommended to be used in order to determine the value of the standard electrode potential, using the following equation:

$$E=E^o \pm S \times pM \qquad (2.5)$$

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

2.3.3 Slope (Response) of the electrode

The main characteristic of the potentiometric electrodes is the slope, S (also called the response of the electrode). Nernst gives the ideal value of the slope: 59.16/z mV/decade of concentration, where z is the charge of the ion that has to be determined. This can be deducted from the Nernst equation:

$$E=E^{o}\pm\left(\frac{RT}{zF}\right)\log a \qquad (2.6)$$

where E is the potential of the electrode, E^{o} is the standard electrode potential; R is the gas constant, 8.314 J/Kmol, F=96500 C, T=298K, and a, is the activity of the ion. The slope of the potentiometric electrode can be deducted from this equation.

$$S=\frac{RT}{zF} \qquad (2.7)$$

Nernstian response implies ideal sensitivity but not necessarily ideal selectivity because interfering ions are most likely to interfere with the main ion during analysis and may also give Nernstian response when present as a sole potential determining species. An electrode with the slope of 50/z mV/decade of concentration may be admitted for bioanalysis [51]. The slope is dependent on the stability of the compound formed at the membrane solution interface [52].

Accordingly the slope of the potentiometric electrodes can be improved by choosing the selector that forms the compound with higher stability or by changing the composition of the membrane matrix.

From the experimental data, the slope can be determined as follows:

(i) tangent of the angle made by the calibration curve and pM axis.

(ii) as a parameter of the equation (figure 2.6) of calibration by using the linear regression method and this is known as the most precise method to determine the slope of the electrode.

2.3.4 Limit of detection

This is defined by the 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 [50].

For potentiometric electrodes the limit of detection is dependent on the stability of the compound formed at membrane-solution interface. It depends on the value of the standard electrode potential and slope.

Although, both the slope and the standard electrode potential can be correlated with the stability of the compound formed at membrane-solution interface of the potentiometric electrode [52-58].

The value of the limit of detection is influenced by the internal solution of the potentiometric electrodes. When the analyte is substituted by a solution of a strong electrolyte (e.g., KCl 0.1 mol/L), very low limits of detection are obtained [59].

The limit of detection can be deducted from the calibration graph of the potentiometric electrode in (Figure 2.6).

It may also be taken as the activity (or concentration) of the ion at the point of intersection of the extrapolated linear calibration curve and the activity (or concentration) axis. The most precise value is obtained when a statistical method is applied [60].

The limits of detection for potentiometric electrodes that are using as internal solution a strong electrolyte solution have very low values (up to pmol/L magnitude order).

2.3.5 Linear concentration range

The linear concentration range is the range of concentration of a substance (or ion) over which the sensitivity of the electrode is constant within a specified variation, usually ±5%. It can also be determined from the calibration plot of the cell potential difference versus the logarithm of responsive ionic activity for potentiometric electrodes as it is shown in Figure 2.6.

For potentiometric electrodes, it is important that all solutions that had to be measured must have the activity or the concentration of the substance (or ions) within this range. The reproducibility of the linear concentration range is connected with the working conditions of the electrode, e.g., stirring rate of the solution, composition of the solution in which the quantities are measured, pH of the solution, composition of the solution where the electrode was exposed prior to the measurements, the history and preconditioning of the electrode, and the temperature [53].

2.3.6 pH Range

The pH plays a very important role in the response of the potentiometric electrodes. It can influence the formation of protonated and unprotonated species of the same substance, or the electrode can become pH-selective under certain conditions. It is very important for an electrode to determine the dependence of the potentials on the pH.

For this kind of electrode, a range where the potential does not depend on the pH maybe found [61-63]. Therefore it is better to buffer all the standard and sample solutions at a pH situated within this range.

2.3.7 Ionic strength and activity coefficients

The ionic strength and the activity coefficients also play a very important role in the accuracy of the measurements. It is necessary to work at the same ionic strength to avoid the differences in the potentials, which can cause another source of error due to the variations of the activity coefficients of the ions in solution [64].

A utilization of the strong electrolyte ensures a constant ionic strength in the preparation of standard and sample solutions. The main strong electrolytes used are NaCl, KCl, and some of buffers that can also ensure, through their composition, a constant ionic strength.

2.3.8 Response time

IUPAC defined the response time as the time which elapses between the instant when the electrodes of the potentiometric cells are brought into contact with the 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 [50].

For potentiometric electrodes, the processes that are occurring at the membrane solution interface influence the response time. The response time of such electrodes is represented by the sum between the time necessary for the ion to be extracted in the membrane solution interface and the time necessary to reach the equilibrium (e.g., of complexation, precipitation, or redox) stage. The extraction usually takes longer.

Response time of low concentration levels is high. The response time in the presence of the interferences is modified due to the competitive equilibrium that takes place at the electrode interface.

2.3.9 Influence of the temperature on the response of the electrodes

The temperature has a high effect on the slope of the electrode. The increase of temperature will favour the kinetics as well as the thermodynamics of the processes that take place at the electrode surface. Accordingly the slope will increase. For potentiometric electrodes, the temperature was controlled and maintained constant during the measurement of the standard and sample solutions. A temperature of 298K was recommended for the characterization of the electrode.

2.3.10 Selectivity

Selectivity is one of the basic characteristics of the electrochemical sensors. It depends on the composition of the membrane (active sites as well as matrix), ratio between the activities of the main ion and interfering ion in the solution, complexity of the matrix of the sample that is analysed, current applied, and the pH of the solution. This property of electrochemical sensors restricts their utilization for the assay of an ion from a complex matrix (e.g., environment). The selectivity of EPMEs is high when these are utilized for clinical analysis including pharmaceutical analysis and for the assay of some substances/ions in food.

IUPAC defined the interfering substance as any substance, other than the ion being determined, whose presence in the sample solution affects the measured emf of a cell. There are two classes of interfering substances:

(i) "electrode/electrochemical" (substances whose response is similar to that ion being determined, or electrolytes present at high concentration) interferences;

(ii) "chemical" interferences (substances that interact with the ion being determined, so as to decrease its activity or apparent concentration, e.g., H^+, OH^-, or substances that interact with the membrane surface).

The value of potentiometric ($K_{i,j}^{pot}$) selectivity coefficient gives the degree of selectivity of electrochemical sensors, respectively as follows:

(i) For magnitude order higher than 10^{-3}, the ion tested for interference interfere strongly.

(ii) For a magnitude order of 10^{-3}, the ion tested for interference is not a strong interferent;

(iii) For a magnitude order less than 10^{-3}, the ion does not interfere.

The Nicolsky-Eisenman equation is the main equation that gives the relation between the potentials of the electrode measured in the presence of the interfering ions and the potentiometric selectivity coefficients:

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

where E is the experimentally recorded emf of the cell when the only variables are the activities in the test solution; R is the gas constant that equals to 8.314 J/Kmol; T is the temperature (in degrees Kelvin), F is the Faraday constant which equals to 96500 C/mol; a_i is the activity of the main ion and a_j is the activity of the interfering ion; N is the number of the interfering species in the solution, $K_{i,j}^{pot}$ is the potentiometric selectivity coefficient.

The Nicolsky-Eisenman equation was modified by Buck, by substituting the charge numbers through their absolute values [53]:

$$E = \text{constant} + \left(\frac{2.303RT}{z_j F}\right) \log\left(a_i^{\frac{1}{|z_i|}} + \sum_{j=1}^{N} K_{i,j}^{pot}\, a_j^{\frac{1}{|z_j|}}\right) \qquad (2.9)$$

For cation sensors the sign of the log term is positive and for negative sensors the sign is negative and the standard electrode potential is represented by the constant term.

2.3.10.1 Methods for determining potentiometric selectivity coefficients

Two methods are recommended for determining the potentiometric selectivity coefficients: mixed solution method and separate solution method. The most preferable for the determination of potentiometric selectivity coefficients is the mixed solution method because it shows the behaviour of the electrode in the real sample.

2.3.10.1.1 Mixed solution method

It is recommended that a ratio between the main ion and interfering ions of 1:10 be used for the determination of potentiometric selectivity coefficients. The potential of the solution that contains both the main and interfering ion, is compared with the one recorded for the solution that contains only the main ion provided that the main ion has the same activity in both solutions.

The equation used for the calculation of the potentiometric selectivity coefficient is:

$$K_{i,j}^{pot} = (10^{\frac{\Delta S}{E}} - 1) \times \frac{a_i}{a_j^{\frac{z_i}{z_j}}} \qquad (3.10)$$

where ΔE is the difference between the potentials recorded for mixed solution ($E_{i,j}$) and for the solution that contains only the main ion (E_i), $\Delta E = E_{i,j} - E_i$ (all in mV); S is the slope of the electrode from the calibration graph or from the linear

regression equation (in mV/decade of concentration); a_i and a_j are the activities of both the main ion and the interfering ion, i, and j; z_i and z_j are the charges of both the main and interfering species, i, and j.

2.3.10.1.2 Separate solution method

It is far less desirable than the mixed solution method since it does not represent as well as the actual conditions under which the electrodes are used. The separate solution method determines the potentiometric selectivity coefficient in two ways:

(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 the main ion of the activity, a_i, and the other one containing interfering ion at the same activity, a_j as the main ion from the first solution (a_i=a_j).

The following equation gives the potentiometric selectivity coefficient:

$$\log K_{i,j}^{pot} = \frac{\Delta E}{S} + (1 - \frac{z_i}{z_j}) \log a_i \qquad (2.11)$$

where ΔE is the difference between the potentials recorded for the solution of only the interferent, E_j, and the for the solution that contains the main ion only, E_i and $\Delta E = E_j - E_i$ (all in mV) and all other terms have the same significance definition as in equation (2.10).

(ii) The activities of two different solutions that are introduced separately into the cell comprised of the enantioselective, potentiometric membrane

electrode and a reference electrode are adjusted with each of two different solutions, one containing only the main ion of the activity a_i, and the other containing only the interfering ion, of the activity a_j, with the aim of measuring the same potential. The following equation can be used to calculate the potentiometric selectivity coefficient:

$$K_{i,j}^{pot} = \frac{a_i}{a_j^{\frac{z_i}{z_j}}} \qquad (2.12)$$

where all the terms have the same significance as in equation (2.10)

2.4 Analytical methods that use electrochemical sensors

There are two methods that can be used, a direct method (direct potentiometry) and an indirect method (potentiometric titrations).

2.4.1 Direct potentiometric method

It is compulsory for electrochemical sensors to be calibrated before use. The preparation of solution is very important in terms of concentration, ionic strength, and pH [53,65].

It is better to adjust the ionic strength and the pH of the samples from where the analyte will be determined to the same values as the solutions used for calibration and these solutions must be buffered and also the addition of a strong electrolyte will be keeping the ionic strength constant.

A minimum of five solutions is necessary to obtain good calibration plot. The emf of the cell comprising the working electrode and the reference electrode versus pM gives the plot of calibration [50]. The values of emf are interpolated on the calibration plot from where the concentrations obtained for the sample can be determined.

2.4.2 Standard addition method

A standard addition method is recommended. The direct measurement of concentration situated near the detection limit is far less accurate. Therefore before the measurements of samples, the calibration is required. The standard addition method is used in two ways:

(i) known volumes of the unknown concentration sample are added to a standard solution having a certain volume;

(ii) known volumes of standard solution are added to a certain volume of solution of unknown concentration.

The calibration plot is used for the interpolation of values of measurements, from where the total concentration of the analyte is obtained. The concentration of the sample will be given by the difference between the value of the total concentration that is obtained from the calibration plot and to the one that was added to the sample solution (or the one to which the sample solution was added).

2.4.3 Potentiometric titrations

In titration there is no need for calibration of electrodes before use. The electrochemical cell contains only the sample that has to be titrated.

Step by step addition of the reagent is used and the potential is recorded versus the volume of the reagent added.

For electrochemical titrations, the law of equivalence is applied:

$$C_R V_e = C_A V_A \qquad (2.13)$$

where C_R is the concentration of the reagent used for titration of the analyte, C_A is the concentration of the analyte, V_e is the volume at equivalence and V_A is the volume of the sample.

The rate of change of potentials for potentiometric titrations is slow at the beginning of the titration, increases to a maximum as the equivalence point is reached, and reduces again when the equivalence point is passed. When the measured potential is plotted versus the added volume of reagent, a double curve, like an elongated letter S, is obtained, and the equivalence point is indicated at the steepest point of the curve, where the potential changes most rapidly.

2.4.3.1. Gran plots

The instead of plotting the electrode potential versus the volume of the titrant is assumed by the Gran plots, the concentration of the remaining analyte at each point in the titration is plotted. A straight-line plot in which the concentration would decrease to zero would result. Practically, a logarithmic response to a linear plot is converted by Gran plots.

Gran plot is prepared by the first derivative titration, by plotting the reciprocal of a first derivative curve that is E/V versus V, where E, is the cell potential and V, is the volume of the titrant used. Since in a derivative titration V/E goes to infinity at the equivalence point, the reciprocal will go to zero where the intersection of the two lines occurs, and a V-shape plot will result.

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Chapter 3

Enantioanalysis of R-clenbuterol Using Enantioselective, Potentiometric Membrane Electrodes Based on Cyclodextrin

3.1 Introduction

A β_2-adrenergic drug, clenbuterol (4-amino- (t-butylamino) methyl)-3,5-dichlorobenzyl alcohol hydrochloride) (Figure 3.1) is used as a bronchodilating agent in both human and veterinary medicine. This drug has been utilized illegally, to promote animal growth, as it substantially increases muscular mass and at the same time decreasing fat accumulation [1].

The use of clenbuterol did not become widespread until 1988 (even though, first studies, which confirmed the properties, began appearing in 1984) due to the European directive forbidding the use of hormones in animals [2]. There was no kind of authorization that was given to clenbuterol, or to other β_2-adrenergic agonists for such purposes; contrary clenbuterol and similar products were included in the list of substances formally banned for zootechnical use [3].

Proliferation of the illegal use of clenbuterol to promote animal growth raises new concerns about its safety/toxicity, particularly regarding consumers. Ingestion of liver and meat containing clenbuterol residues has been described for various intoxifications in Spain [4,5], France [6], Italy [5-7] and Portugal [8]. Hypokalemia has already been

described as a relevant element in this kind of intoxification since blood collection is normally undertaken in these emergency clinical situations [4,7,9] and a method is developed to evaluate clenbuterol in blood, in order to allow an equivalent and quick diagnosis of clenbuterol food poisoning.

Apart from its use as a growth promoting in food producing animals, clenbuterol is also abused as a doping agent in human athletes. Athletes promote their performance by using clenbuterol and as a result, its use has been forbidden by the International Olympic Committees. Clenbuterol has been shown to have thermogenic properties by increasing basal metabolic rate.

The mechanism for these changes is incompletely eliminated, but these effects are most likely the result of adrenergic effects on skeletal muscle, although effects on typical adrenoreceptors or nonreceptor-mediated pathways may be involved [10].

Clenbuterol also has been shown to induce a slow-to-fast fiber type transition and increase force generation and twitch concentration speeds in the rat [11]. Literature data indicate that the liver is, among all edible tissues, the most target organ for monitoring the use of beta-agonist [12], because it contains the highest concentrations throughout the withdrawal period [13-16]. A maximum residue limit (MRL) of 0.5 $mg.Kg^{-1}$ for clenbuterol in the liver of cattle and horses is proposed by law [17,18].

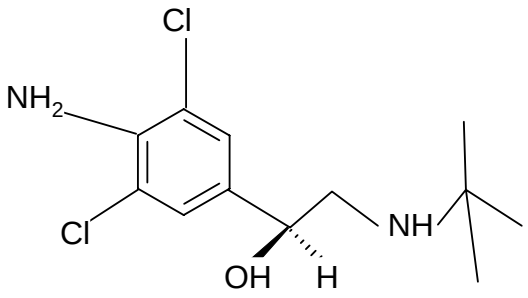

Figure 3.1 R-clenbuterol

3.2 Reagents and materials

Graphite powder (1–2 μm), 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrin (as chlorine salt), creatine, creatinine, S- and R-Clenbuterol were purchased from Sigma Aldrich. Paraffin oil was purchased from Fluka. The phosphate buffer (pH 6.4) was prepared from potassium phosphate and sodium hydrogen phosphate.

The clenbuterol solutions were prepared by serial dilutions from stock S-Clenbuterol and R-Clenbuterol (both 10^{-3} mol/L). All diluted and stock solutions of clenbuterol were buffered at pH 6.4 using phosphate buffer.

Three serum samples were spiked with different amounts of R-clenbuterol. All spiked serum samples were buffered at pH = 6.4 using phosphate buffer.

3.3 Apparatus

A 663 VA Stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 100 computer-controlled potentiostat (Eco Chemie, Utrecht, the Netherlands) and software

version 4.9 were used for all potentiometric measurements. Ag/AgCl served as reference electrode in the cell.

3.4 Electrode design

2-Hydroxy-3-trimethylammoniopropyl-β-cyclodextrin (as chlorine salt) (β-cyclodextrin derivative) was used as chiral selector for the design of the enantioselective, potentiometric membrane electrodes. Paraffin oil, graphite powder and β-CD derivative were mixed in a ratio of 1:4:1 w/w/w. A certain quantity of carbon paste (paraffin oil: graphite powder = 1:4 w/w) free of β–CD derivative was prepared, and it was placed in a plastic pipette peak, leaving the tip of the pipette to be filled with the carbon paste that contained the chiral selector [19]. The diameter of the enantioselective, potentiometric membrane electrode was 3 mm. Electric contact was obtained by inserting the Ag/AgCl wire in the carbon paste. A solution of 0.1 mol/L KCl was used as the internal solution. The surface of the electrode was polished with alumina paper before it was used for each experiment.

3.5 Recommended procedures: direct potentiometry

Direct potentiometry was employed for the measurement of the potential of each standard solution (10^{-10} to 10^{-3} mol/L). The electrodes were placed in stirred standard solutions, and graphs of E(mV) vs. pR-Clen were plotted. The unknown concentrations of R-Clen were determined from the calibration graphs.

3.6 Results and discussion

3.6.1 Response of the electrodes

The response characteristics exhibited by the β–CD derivative based carbon paste electrode are given below. The electrode showed linear and near-Nernstian response for R-Clen with correlation coefficient of 0.9999. The response of the electrode for S-Clen was non-Nernstian. Accordingly, the proposed electrode was not working for the determination of S-Clen.

The equation of calibration is:

$$E = 349.7 - 53.6 \text{ pR-Clen}; r = 0.9999$$

where E is the standard electrode potential and pR-Clen = -log [R-Clen].

The standard electrode potential obtained for the β–CD derivative based electrode was 349.70 mV. The slope obtained for the β–CD derivative based electrode was 53.60mV/pR-Clen. The limit of detection obtained for the electrode was 2.99×10^{-7} mol/L. The linear concentration range for the β–CD derivative based electrode was 10^{-3} to 10^{-6} mol/L.

3.6.2 Effect of pH on the response of the electrode

The effect of pH on the response of the potential readings was checked by recording the electromotive force (emf) of the cell, using the direct potentiometric method. This was done using solutions containing 10^{-5} mol/L R-Clenbuterol at various pH values (1–12). These solutions were obtained by the addition of small amounts of HCl and/or NaOH

solution (0.1 mol/L or 1 mol/L of each) to the R-Clen stock solution. The E (mV) vs. pH graph (Figure 3.2) shows that the emf is not dependent on the pH in the pH range 5-9.

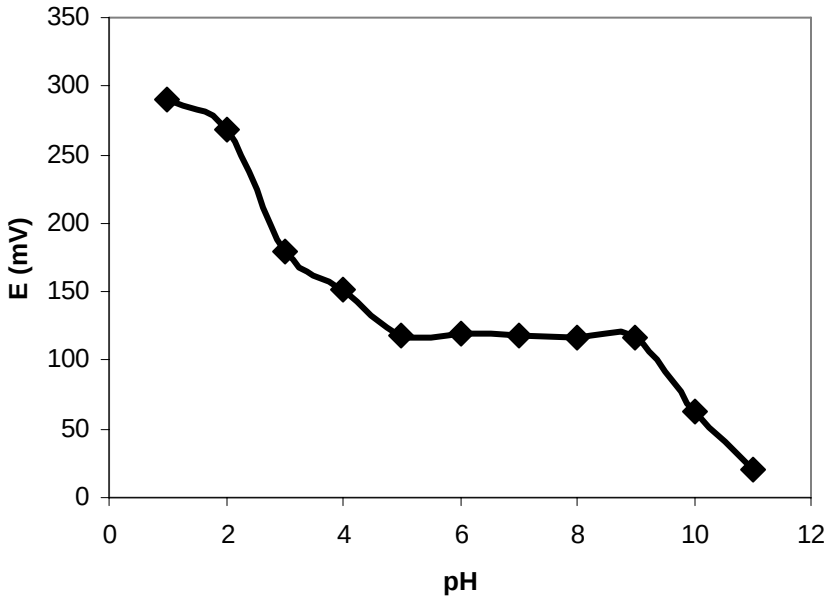

Figure 3.2 Effect of pH on the response of the enantioselective, potentiometric membrane electrode ($C_{R\text{-}clen} = 10^{-5}$ mol/L)

3.6.3 Selectivity of the electrode

The selectivity of the electrodes was investigated over S-Clen, PVP, creatine, creatinine using the mixed solutions method. The ratio between the concentration of R-Clen and interfering species was 1:10. The values of the potentiometric selectivity coefficient are given in Table 3.1.

Table 3.1. Potentiometric selectivity coefficients for the enantioselective, potentiometric membrane electrode for R-Clenbuterol.

Interferent	K_{sel}^{pot}
S-Clen	$4.20x10^{-3}$
PVP	$8.20x10^{-3}$
Creatine	$8.00x10^{-3}$
Creatinine	$4.00x10^{-3}$

All measurements were done at 25 ˚C; values are the average of 10 determinations.

The values of the potentiometric selectivity coefficients show that the proposed electrode is enantioselective and selective over PVP, creatine, and creatinine. Inorganic ions such as Na^+, K^+, Ca^{2+} and Mg^{2+} do not interfere.

3.6.4 Analytical aplications

The results obtained proved to be useful for the determination of the enantiopurity of R-Clen as well as for the determination of the level of R-Clen in the serum samples. The assay of R-Clen was performed in the presence of S-Clen using different ratios between R and S-Clen.

Table 3.2. Determination of R-Clenbuterol in the presence of S-Clenbuterol.

R:S mol:mol	R-Clenbuterol, Recovery, (%)
2:1	99.28±0.10
1:1	99.11±0.20
1:2	99.17±0.10
1:4	99.56±0.10
1:9	99.48±0.10

All measurements were made at 25 °C; all values are the average of 10 determinations

The results presented in Table 3.2 show that R-Clen can be determined in the presence of S-Clen. No significant differences were recorded between R:S ratios of 1:9 and 1:99.9. Accordingly, the electrode can be used successfully for the enantiopurity test of clenbuterol.

The results obtained for the recovery of R-Clen in serum samples showed the suitability of this method for such assay (Table 3.3). The method can be used successfully in clinical analysis for the assay of R-Clen in serum samples.

Table 3.3 Recovery of R-Clenbuterol in serum samples.

Sample no.	R-Clen added	S-Clenbuterol, Average Recovery (%)
1	40	99.93±0.12
2	100	99.92±0.12
3	120	99.94±0.16

All measurements were made at 25 °C; all values are the average of 10 determinations.

3.7 Conclusion

The β–CD derivative proved to be a powerful chiral selector for the construction of enantioselective, potentiometric membrane electrodes. The enantioselective, potentiometric membrane electrode provides excellent features for enantioselective *in vivo* assay of R-Clenbuterol. The construction of the electrode is simple, fast and reproducible. The enantioselectivity of the electrode made it suitable for the enantiopurity test of the clenbuterol used in pharmaceutical formulations.

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Chapter 4

Enantioanalysis of L-cysteine Using Enantioselective, Potentiometric Membrane Electrodes

4.1 Introduction

The thiol group of cysteine (Cys) residues (Figure 4.1) is the most reactive of any amino acid side chain [1]. The Cys thiol usually ionises at slightly alkaline pH values with an intrinsic pK_a in the region of 9.0 to 9.5. The thiolate anion formed is the reactive species in most instances. It reacts rapidly with alkyl halides, such as iodoacetate, iodoacetamide, and methyl iodide, to give the corresponding stable alkyl derivatives.

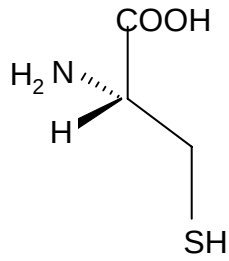

Figure 4.1 L-Cysteine

The thiols of Cys residues form complexes of varying stability with a variety of metal ions. The most stable are those with divalent mercury, Hg^{2+}, but complexes with a variety of stochiometries are formed. Consequently, univalent organic mercurials of the type $R\text{-}Hg^{+}$ tend to be used instead because they form more reproducible 1:1 complexes with thiols.

Thiol complexes with silver are less stable than those with mercury, but univalent Ag^{+} reacts stochiometrically and can be used to titrate thiols. Thiols are readily oxidized by oxygen, especially in the presence of trace amounts of metal ions such as Cu^{2+}, Fe^{2+}, Co^{2+}, and Mn^{2+}. It is likely that the metal complexes are the actual reactants with oxygen.

The sulfur atom of Cys residues can exists in a variety of oxidation states, but some of them are unstable. Besides the thiol form, only two oxidation states are generally encountered, disulfide and the sulfonic acid. Disulfide is usually the end product of air oxidation.

Disulfide bonds between Cys residues occur in some proteins [2]; two such residues linked by a disulfide bond are often designated as a cystine residue, after the amino acid cystine and individual Cys residues are often designated as $\frac{1}{2}$-cystines. It is now clear that cystine is not incorporated into proteins as such. Instead the thiol form, cystine, is used in protein biosynthesis, and the disulfide bonds between Cys residues can be added later.

4.1.1 Biosynthesis of cysteine

In bacteria and plants, cysteine is synthesized by a two-step reaction. Serine is 0-acetylated by a reaction with acetyl CoA that is catalysed by the pyridoxal phosphate dependent serine acetyltransferase. The synthesis of this enzyme is repressed when bacteria grow on cysteine-rich media, although the activity of the performed enzyme is unaffected by cysteine.

The acetyl group is then displaced by the reaction with inorganic sulfide, either free in solution or bound to a protein such as thioredoxin. In Salmonella typhimurium and some other microorganism, these two enzymes occur together as a multi-enzyme complex.

In some species, including the chick embryo (but not the adult chicken), there is an alternative pathway for cysteine synthesis: direct displacement of the hydroxyl group of serine by inorganic sulphide, without prior acetylation. The same enzyme catalyses and exchange between inorganic sulphite and the sulphydryl group of cysteine, forming cysteic acid and liberating H_2S.

The formation of cysteine and its onward metabolism would provide a pathway for methionine catabolism. This trans-sulphuration pathway has the characteristics of a biosynthesis rather than a catabolic pathway. It has a relatively high requirement for metabolic energy, and the initial, rate-limiting step, cystathione β-synthetase, has a low activity, and is induced by feeding a high protein diet. The Mitchell's Menten constant (K_m) of the enzyme is low compared with normal intracellular concentrations of methionine or homocysteine [3,4], so that it functions at a more or less constant rate under normal physiological conditions.

Impaired activity of cystathione synthetase in man results in homocystinuria, an inborn error of metabolism in which large amounts of homocysteine, its disulphide homocystine and the mixed cysteine-homocysteine disulphide are excreted in the urine. Since the synthesis of cysteine from methionine is impaired, cysteine becomes a dietary essential amino acid for these patients.

Different methods of analysis were proposed for the analysis of cysteine in biological samples: chromatographic methods e.g., capillary electrophoresis [5], liquid chromatography [6-8]; chemiluminescence [9] and an electrode based on oxovanafium(IV) complex of Salen [10]. These methods of analysis can only determine the full amount of L- and D-cysteine and are not able to discriminate between the two enantiomers.

In this chapter, nine enantioselective, potentiometric membrane electrodes (EPMEs) based on cyclodextrins, maltodextrins, and antibiotics (vancomycin and teicoplanin) have been designed for the enantioanalysis of L-cysteine. The main advantage of the proposed electrodes versus existing methods is their possibility for the enantioanalysis of L-cysteine in urine samples.

4.2 Reagents and materials

L- and D-Cysteine, vancomycin and teicoplanin were supplied by Sigma-Aldrich (St. Louis, MO, USA). α-, β-, 2-hydroxyl-3-trimethylammoniopropyl -β- (as chloride salt) and γ-cyclodextrins were supplied by Walker-Chemie GmbH (München, Germany). Maltodextrins (DE 4.0-7.0, 13.0-17.0, 16.5-19.5) were purchased from Aldrich (Milwaukee, WI, USA). Graphite powder (1-2 μm, synthetic) was supplied by Aldrich.

Deionised water from a Modulab system (Continental Water Systems, San Antonio, TX, USA) was used for the preparation of all solutions. The L- and D-cysteine solutions necessarily in the characterization of the enantioselective, potentiometric membrane electrodes were prepared from standard L- and D-cysteine solutions (10^{-2}

mol/L), respectively, by serial dilutions. All standard and diluted solutions were buffered with phosphate buffer (pH 2.40, 0.1mol/L) from Merck (Darmstadt, Germany) (1:1, v/v, buffer:deionised water).

4.3 Enantioselective, potentiometric membrane electrodes based on cyclodextrins for the enantioanalysis of L-cysteine

4.3.1 Apparatus

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

4.3.2 Electrodes 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 cyclodextrin (CD) (α-, β-, 2-hydroxyl-3-trimethylammoniopropyl -β-(as chloride salt) (β-CD-derivative) or γ) (10^{-3} mol/L) (100 μL chiral selector solution to 100 mg carbon paste) [11]. A certain quantity of carbon paste free of cyclodextrin was prepared and it was placed into a plastic pipette peak leaving 3-4 mm empty in the top to be filled with the carbon paste that contains the chiral selector. The diameter of the EPMEs was 3mm. Electric contact was obtained by inserting Ag/AgCl wire in the carbon paste. As internal solution it was utilized a solution of 0.1 mol/L KCl.

The surface of the electrode was wetted with deionised water and polished with alumina paper (polishing strips 30144-001, Orion) before using them for each experiment. The oil from the carbon paste prevents the leach of the cyclodextrin from the membrane into solution.

4.3.3 Recommended procedures

The direct potentiometry was used for measurements of the potentials of each standard solution (10^{-10}-10^{-2} mol/L). The electrodes were placed in stirred standard solutions and graphs of E(mv) versus pL-Cys were plotted. The unknown concentrations were determined from the calibration graphs.

4.3.3.1 Determination of L-cysteine in urine samples

Urine samples were collected from different patients and buffered with phosphate buffer (pH 2.40, 0.1mol/L) (1:1, v/v, buffer:urine sample). Direct potentiometry was applied to determine L-cysteine in urine samples.

4.3.4 Results and discussion

4.3.4.1 Electrodes response

The response characteristics exhibited by the proposed electrodes towards the detection of L-cysteine are summarized in Table 1. For all the calibration plots, the membrane electrodes showed linear and near-Nernstian responses for L-cysteine, with correlation coefficients of 0.9999.

The proposed EPMEs showed non-Nernstian responses for D-cysteine. Amongst all the electrodes, EPME based on γ-CD showed the best response 59.0 mV/decade of concentration.

Table 4.1 Response characteristics of enantioselective, potentiometric membrane electrodes designed for the assay of L-cysteine.

EPME based on	Slope (mV/pL-cys)	Intercept, E^0 (mV)	Linear conc. range (mol/L)	Detection limit (mol/L)
α-CD	58.9	637.8	10^{-5}–10^{-10}	$1.5x10^{-11}$
β-CD	58.4	627.8	10^{-3}–10^{-10}	$1.8x10^{-11}$
β-CD-deriv.	58.3	602.8	10^{-3}–10^{-11}	$4.6x10^{-11}$
γ-CD	59.0	615.0	10^{-5}–10^{-11}	$3.8x10^{-11}$

All measurements were made at 25^0C. All values are average of 10 determinations.

The proposed electrodes were highly stable and reproducible over a month test period.

4.3.4.2 The effect of pH on the response of the electrodes

The influence of pH on the response of the proposed electrodes was investigated by recording the emf of the cell for the solutions containing 10^{-5} mol/L L-cysteine at different pH values (pH 1-12). These solutions were prepared by adding small volumes of HCl and /or NaOH solutions (0.1-1 mol/L of each) to a L-cysteine solution. The plots of E (mV) versus pH (Fig. 4.2) showed that the response of the electrodes is not depending on the pH, in the ranges 2.0-6.0 (I), 2.0-7.0 (II), 2.0-5.0 (III) and 2.0-5.0 (IV).

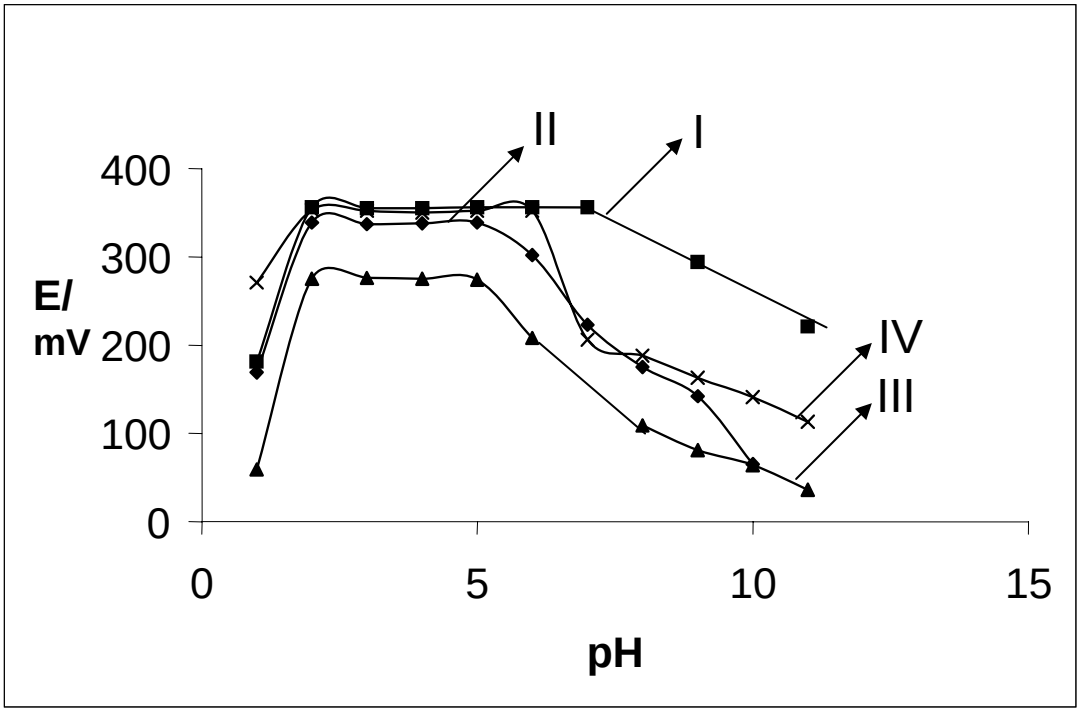

Figure 4.2. The influence of pH on the response of the enantioselective potentiometric membrane electrodes ($C_{L\text{-}cys}=10^{-5}$); (EPME based on α-(I), β-(II), γ-(IV), and β-(III) derivative-cyclodextrins).

4.3.4.3 The selectivity of the electrodes

The selectivity of the electrodes was investigated over D-cysteine, PVP, creatine and creatinine using mixed solutions method. The concentration of the interfering ions and L-cysteine were 10^{-4} and 10^{-5} mol/L, respectively. The values of the potentiometric selectivity coefficients proved that the electrodes are selective and enantioselective. The best results were obtained by using the β-CD-derivative based EPME. Inorganic ions such as Na^{+}, K^{+}, Ca^{2+} did not interfere with the analysis of L-cysteine.

Table 4.2 Potentiometric selectivity coefficients K_{sel}^{pot}, of the enantioselective, potentiometric membrane electrodes.

EPME based on	K_{sel}^{pot}			
	Interfering species (j)			
	D-Cys	PVP	Creatine	Creatinine
α-cyclodextrin	$8.1x10^{-4}$	$1.2x10^{-3}$	$8.0x10^{-4}$	$1.3x10^{-3}$
β-cyclodextrin	$1.0x10^{-4}$	$3.9x10^{-3}$	$4.0x10^{-3}$	$4.0x10^{-3}$
β- derivative-cyclodextrins	$1.0x10^{-4}$	$4.0x10^{-4}$	$4.1x10^{-4}$	$3.9x10^{-4}$
γ cyclodextrin	$8.1x10^{-4}$	$4.0x10^{-4}$	$8.0x10^{-4}$	$1.0x10^{-4}$

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

4.3.4.4 Analytical applications

To assess the feasibility of the proposed direct potentiometric procedure, recovery test were performed for the assay of L-cysteine in the presence of D-cysteine by useing of different ratios between L-cysteine and D-cysteine. The results obtained (Table 4.3) demonstrated the suitability for the proposed enantioselective, potentiometric membrane electrodes for enantioanalysis of L-cysteine in urine samples. No significant differences in the recovery values were recorded for the ratios between L:D enantiomers varying from 1:9 to 1:99.9.

The average recoveries of L-cysteine in urine samples were: 99.55±0.02, 99.51±0.02, 99.47±0.02 and 99.76±0.02, when the electrodes based on α-, β-, 2-hydroxyl-3-trimethylammoniopropyl -β-(as chloride salt) (β-CD-derivative) and γ-CD were used.

Table 4.3 Determination of L-cysteine in the presence of D-cysteine.

L: D (mol/mol)	L-Cys, Recovery (%)			
	EPME based on			
	α-CD	β-CD	β-CD-der	γ-CD
2:1	99.49±0.02	99.92±0.02	99.99±0.01	99.14±0.02
1:1	99.50±0.01	99.94±0.02	99.91±0.02	99.84±0.01
1:2	99.35±0.01	99.90±0.01	99.24±0.02	99.94±0.02
1:4	99.47±0.01	99.85±0.01	99.99±0.01	99.89±0.02
1:9	99.43±0.02	99.51±0.01	99.98±0.02	99.96±0.01

All measurements were made at 25^0C. All values are average of ten determinations.

4.4 Enantioselective, potentiometric membrane electrodes based on maltodextrins for the enantioanalysis of L-cysteine

4.4.1 Apparatus

A 663 VA stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 20 (Eco Chemie, Utretch, Netherlands) and a software version 4.9 was used for all the potentiometric measurements. Ag/AgCl (0.1 mol/L KCl) served as reference electrode in the cell.

4.4.2 Electrodes 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; 100μL chiral selector solution to 100mg carbon paste) [12]. A quantity of carbon paste free from maltodextrin was also prepared and placed

in a plastic pipette, leaving 3 to 4 mm empty in the top to be filled with the carbon paste containing the chiral selector. The diameter of the potentiometric, enantioselective membrane electrode was 3 mm. Electric contact was obtained by inserting a Ag/AgCl wire into the carbon paste. The internal solution was 0.1 mol/L KCl.

The surface of the electrode was wetted with deionised water and polished with alumina paper (polishing strips 30144-001, Orion) before use in each experiment.

4.4.3 Recommended procedures

The procedures are the same as described in 4.3.4. Determination of L-cysteine in urine samples was done as described in 4.3.4.1.

4.4.4 Results and discussion

4.4.4.1 Electrodes response

The response characteristics of the electrodes are shown in table 1. For all the calibration equations, the correlation coefficient was 0.9999. The limits of detection are very low and all the electrodes have a near-Nernstian response for L-cysteine. The electrodes did not exhibit a near-Nernstian response for D-cysteine.

The proposed electrodes were highly stable and reproducible over a month test period. The response time was 20s for concentration range 10^{-5} – 10^{-3} mol/L and 1min for concentrations lower than 10^{-5} mol/L.

Table 4.4 Response characteristics of enantioselctive, potentiometric membrane electrodes for the assay of L-Cysteine.

Maltodextrin	Slope (mV/decade of conc.)	Intercept, E^o (mV)	Linear conc. range (mol/L)	Detection limit (mol/L)
I	-58.5	587.5	10^{-10}-10^{-3}	$9.0x10^{-12}$
II	-59.0	659.8	10^{-10}-10^{-3}	$5.2x10^{-12}$
III	-59.2	600.7	10^{-10}-10^{-3}	$7.1x10^{-11}$

All measurements were made at 25°C. All values are averages of ten determinations.

4.4.4.2 The effect of pH on the response of the electrodes

The influence of pH on the response of the proposed electrodes was investigated by recording the emf of the cell for solutions containing 10^{-5} mol/L L-Cys at different pH values (pH 1-12). These solutions were prepared by adding small volumes of HCl and/or NaOH solution (0.1-1 mol/L of each) to a L-Cys solution. The plots of E (mV) versus pH (Fig. 2) show that the response of the electrodes is not depending on pH, in the following ranges 2.0-5.0, 2.0-7.0, and 2.0-6.0 for the EPMEs based on maltodextrins I, II, and III, respectively.

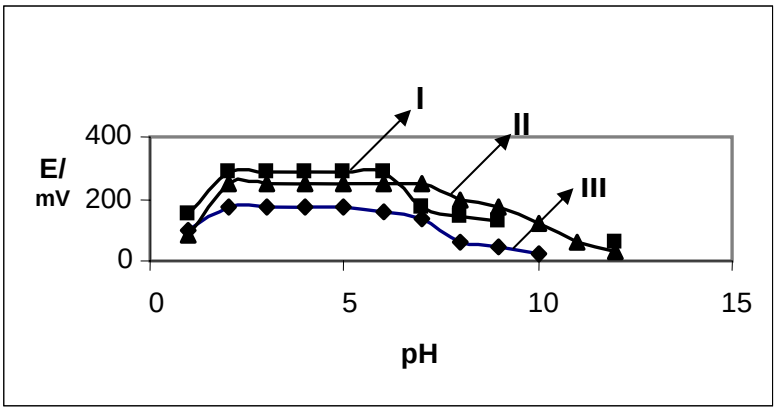

Figure 4.3 The influence of pH on the response of the enantioselective, potentiometric membrane electrodes ($C_{L\text{-}Cys}$=10^{-5}mol/L); I - MD-I, II - MD-III and III - MD-II based EPMEs.

4.4.4.3 The selectivity of the electrodes

The selectivity of the proposed electrodes was investigated over D-Cys, PVP, creatine and creatinine, using mixed solution method. The concentration of the interfering ions and L-Cys were 10^{-4} and 10^{-5} mol/L, respectively. The EPMEs based on maltodextrins are selective over PVP, creatine and creatinine and enantioselective, as shown by the values of the potentiometric selectivity coefficients in Table 4.5. Inorganic ions such as Na^{+}, K^{+}, and Ca^{2+} did not interfere with the analysis of L-Cys.

Table 4.5 Potentiometric, selectivity coefficients of the electrodes used for the enantioanalysis of L-cysteine.

Interfering species (J)	$K_{i,j}^{pot}$		
	EPME based on		
	MD-I	MD-II	MD-III
D-Cys	6.0×10^{-3}	8.1×10^{-4}	4.0×10^{-4}
PVP	3.2×10^{-3}	3.2×10^{-3}	8.1×10^{-4}
Creatine	4.0×10^{-4}	4.0×10^{-3}	4.0×10^{-4}
Creatinine	1.0×10^{-4}	4.0×10^{-4}	8.1×10^{-4}

All measurements were made at 25°C. All values are averages of ten determinations.

4.4.4.4 Analytical applications

In order to establish if the proposed electrodes can be used for the enantioanalysis of L-cys, recovery tests of L-cys in the presence of D-cys were performed. The assay of L-Cys in the presence of D-Cys was conducted by use of different ratios between L- and D-Cys. The results obtained (Table 4.6) demonstrated the suitability for the

proposed enantioselective, potentiometric membrane electrodes for the enantioanalysis of L-cys in biological fluids and/or raw material. No significant differences in the recovery values were recorded for the ratios between L: D enantiomers varying from 1:9 to 1:99.9.

Table 4.6 Determination of L-cysteine in the presence of D-cysteine.

L:D mol/mol	L-cys, Recovery, %		
	EPME based on MD		
	I	II	III
2:1	99.63±0.01	99.72±0.02	99.98±0.02
1:1	99.95±0.02	99.99±0.01	99.60±0.02
1:2	99.81±0.02	100.00±0.01	99.24±0.01
1:4	99.90±0.01	99.97±0.02	100.00±0.01
1:9	99.54±0.02	99.99±0.01	99.99±0.02

All measurements were made at 25^{o}C. All values are averages of ten determinations.

L-cysteine was determined in urine samples with average recoveries of: 99.55±0.01, 99.79±0.01, 99.47±0.02 and 99.64±0.02, when the electrodes based on maltodextrins I, II and III were used.

4.5 Enantioselective, potentiometric membrane electrodes based on antibiotics for the enantioanalysis of L-cysteine

4.5.1 Apparatus

A 663 VA stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 20 (Eco Chemie, Utretch, Netherlands) and a software version 4.9 was used for all the potentiometric measurements. Ag/AgCl (0.1 mol/L KCl) served as reference electrode in the cell.

4.5.2 Electrodes 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 vancomycin or teicoplanin (10^{-3} mol/L) (100 µL chiral selector solution to 100 mg carbon paste) [13]. A certain quantity of carbon paste free from chiral selector was prepared and it was placed into a plastic pipette peak leaving 3-4 mm empty in the top to be filled with the carbon paste that contains the chiral selector. The diameter of the potentiometric, enantioselective membrane electrode was 3mm. Electric contact was obtained by inserting Ag/AgCl wire in the carbon paste. As internal solution it was utilized a solution of 0.1 mol/L KCl.

The surface of the electrode was wetted with deionised water and polished with alumina paper (polishing strips 30144-001, Orion) before using them for each experiment.

4.5.3 Recommended procedures

The procedures are the same as described in 4.3.4. Determination of L-cysteine in urine samples was done as described in 4.3.4.1.

4.5.4 Results and discussion

4.5.4.1 Electrodes response

The response characteristics exhibited by the two carbon paste modified with vancomycin and teicoplanin electrodes towards the detection of L-cysteine are summarized in Table 4.7. For all the calibration plots, the membrane electrodes showed linear and near-Nernstian responses for L-cysteine, with correlation coefficient of 0.9999. D-cysteine on the other hand, showed non-Nernstian responses.

Amongst the two electrodes, teicoplanin showed to be the best electrode with a slope of 59.0 mV/decade of concentration.

Table 4.7 Response characteristics of enantioselective, potentiometric membrane electrodes designed for the assay of L-cysteine.

EPME based on	Slope (mV/pL-cys)	Intercept E^0 (mV)	Linear concentration range (mol/L)	Detection limit (mol/L)	Correlation coefficient (r)
Vancomycin	58.0	601.5	10^{-3}–10^{-10}	$4.3x10^{-11}$	0.9999
Teicoplanin	59.0	480.0	10^{-5}–10^{-8}	$7.3x10^{-9}$	0.9999

All measurements were made at 25^0C. All values are average of 10 determinations.

The proposed electrodes were highly stable and reproducible over a month test period. The response time was 30s for concentration range 10^{-5} – 10^{-7} mol/L and 1min for concentrations lower than 10^{-7} mol/L.

4.5.4.2 Effect of pH on the response of the electrodes

The influence of pH on the response of the proposed electrodes was investigated by recording the emf of the cell for the solutions containing 10^{-5} mol/LL-cysteine at different pH values (pH 1-12).

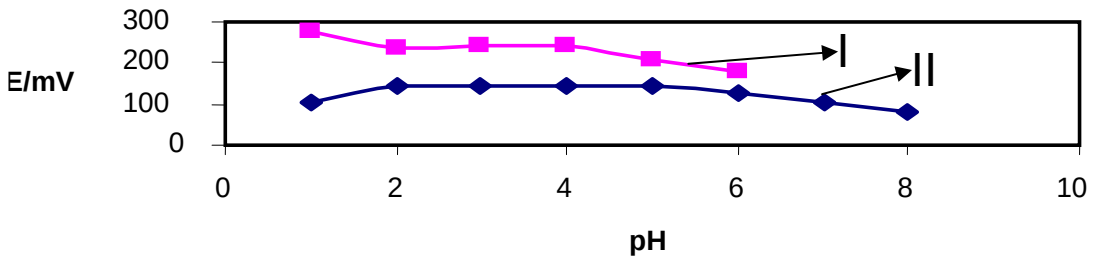

Figure 4.4 The influence of pH on the response of the enantioselective potentiometric membrane electrodes ($C_{L\text{-}cys}=10^{-5}$) based on vancomycin (I) and teicoplanin (II).

These solutions were prepared by adding small volumes of HCl and/or NaOH solutions (0.1-1 mol/L of each) to the L-cysteine solution. The plots of E (mV) versus pH (Fig. 4.4) showed that the response of the electrode is not depending on the pH, in the ranges 2.0-4.0 (I), 2.0-6.0 (II).

4.5.4.3 The selectivity of the electrodes

The selectivity of the proposed electrodes was investigated over D-Cys, PVP, creatine and creatinine, using mixed solution method. The concentration of the interfering ions and L-Cys were 10^{-4} and 10^{-5} mol/L, respectively. The potentiometric, selectivity coefficients shown that the electrodes are selective and enantioselective (Table 4.8). Inorganic ions such as Na^{+}, K^{+}, Ca^{2+} did not interfere with the analysis of L-cysteine.

Table 4.8 Potentiometric selectivity coefficients of the proposed enantioselective, potentiometric membrane electrodes for the assay of L-cysteine.

Interference species (J)	K_{sel}^{pot}	
	Vancomycin	Teicoplanin
D-Cys	$2.2\text{x}10^{-3}$	$1.2\text{x}10^{-3}$
PVP	$1.7\text{x}10^{-3}$	$3.8\text{x}10^{-4}$
Creatine	$4.0\text{x}10^{-4}$	$2.2\text{x}10^{-3}$
Creatinine	$1.0\text{x}10^{-4}$	$4.0\text{x}10^{-4}$

All measurements were made at 25^{0}C. All values are average of 10 determinations.

4.5.4.4 Analytical applications

Determination of L-cysteine in the presence of D-cysteine was performed in order to prove if the electrodes can be used for the enantioanalysis of L-cysteine. The assay of L-cysteine in the presence of D-cysteine was conducted by use of different ratios between L- and D-cysteine. The results obtained (Table 4.9) demonstrated the suitability for the proposed enantioselective, potentiometric membrane electrodes for

testing the enantiopurity of cysteine in urine samples 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 enantiomers varying from 1:9 to 1:99.9.

Table 4.9 Determination of L-cysteine in the presence of D-cysteine.

L: D (mol/mol)	L-Cysteine, Recovery (%) EPME based on	
	Vancomycin	Teicoplanin
2:1	100.00±0.01	99.27±0.01
1:1	99.65±0.02	99.09±0.02
1:2	99.65±0.01	99.55±0.02
1:4	99.82±0.02	99.53±0.02
1:9	99.87±0.02	99.55±0.02

All measurements were made at 25^0C. All values are average of 10 determinations.

L-cysteine was determined in urine samples. The average recoveries were 99.77±0.02 and 99.90±0.02 for vancomycin and teicoplanin based EPMEs.

4.6 Conclusion

Nine enantioselective, potentiometric membrane electrodes were proposed for the enantioanalysis of L-cysteine. The proposed enantioselective, potentiometric membrane electrodes proved to be suitable for the enantioanalysis of L-cysteine in urine samples.

Good selectivities were recorded over polyvinylpyrolidone (PVP), creatine and creatinine. PVP is a component present in the formulations of pharmaceutical products. PVP was checked as possible interferent due to the feature of the proposed electrodes to be used for enantioanalysis of L-cysteine in pharmaceutical products. It was essential to know if creatine and creatinine will interfere in the determination of L-cysteine, because they are part of urine samples. None of these substances interfered in the assay of L-cysteine. Furthermore, some of the potentiometric selectivity coefficients calculated were of 10^{-4}mol/L magnitude order.

Enantioselectivity is a very important characteristic of the proposed electrodes. The values recorded for the potentiometric selectivity coefficients proved that all the proposed electrodes are enantioselective. To prove that the electrodes can be used for the enantioanalysis of L-cysteine, recovery tests were performed for the assay of L-cysteine in the presence of D-cysteine. The values of % recovery of L-cysteine in the presence of D-cysteine were higher than 99.00% when the rations between L- and D-enantiomers were 2:1 to 1:99.9.

The design of the electrodes is reliable, and that also make the response characteristics of the proposed electrodes reliable. The responses recorded for the electrodes are near-Nernstian. The linear concentration ranges are wide. Limits of detection of very low magnitude orders were recorded.

The response characteristics as well as the selectivity and recovery tests proved that the proposed electrodes can be successfully used for the enantioanalysis of L-cysteine in urine samples with recoveries higher than 99.00%.

4.7 References

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Chapter 5

Enantioanalysis of L-histidine Using Enantioselective, Potentiometric Membrane Electrodes

5.1 Introduction

Histidine (His) (Figure 5.1) is synthesized by mammals from common metabolic intermediates and is not referred to as an essential amino acid. It must be supplied constantly because just like arginine, the rate of histidine synthesis in young animals and human infants is inadequate to meet the requirement for growth and human infants is inadequate to meet the requirement for growth.

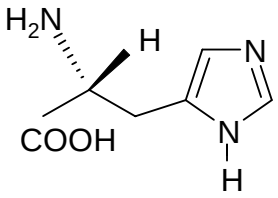

Figure 5.1 L-Histidine

If none is provided in the diet of adult human beings it is also inadequate to synthesize histidine and this was discovered by Kopple and Swendseid (1975) [1].

A fine scaly erythematous dermatitis is developed for patients on long-term total parental nutrition if no histidine is included in the intravenous nutrient solution [2]. It

is necessary for histidine to be considered to be at least partially a dietary essential even though histidine is not nutritionally important.

Histidine also plays a very crucial role as the precursor of the peripherally pharmacologically active and central neurotransmitter amine histamine, and the dipeptides carnosine (β-alanylhistidine) and homocarnosine (γ-aminobutyryl-histidine). An intermediate of the oxidative catabolism of histidine, urocanic acid, is one of the major ultraviolet-absorbing compounds in the skin, and changes in histidine metabolism may be important in the aetiology of the characteristic dermatitis of pellagra and zinc deficiency [3].

The imidazole group of histidine plays an important role in the buffering capacity of tissues and plasma proteins and is reactive at the catalytic site of many enzymes and this makes histidine playing a very important role in many proteins [4]. Histidine is much more basic than hydroxide ion in terms of its basicity. It is a tertiary amine that is intrinsically more nucleophilic than primary or secondary amines.

The enhanced reactivity of tertiary amines is usually cancelled by their greater steric hindrance, but in imidazole the atoms bonded to the nitrogen's are held back in a five-membered ring and cause relatively little steric hindrance. It is one of the strongest bases that can exist at neutral pH with a pK_a value near 7. A weaker base would have a lower nucleophilic reactivity, whereas a stronger base would be protonated to a greater extent at neutral pH and resulting in less reactivity.

The nitrogen with the hydrogen atom of the nonionized form of the imidazole ring is an eletrophile and donor for hydrogen bonding, and the other nitrogen atom is a nucleophile and the acceptor for hydrogen bonding and this side chain is extremely versatile and almost the chemical equivalent of being ambidextrous.

The imidazole group is in principle capable of undergoing numerous reactions, and most of these reactions occur more readily with amino and thiol groups and very few are suitable for modifying His residues specifically. The two nitrogen atoms of His side chain are designated as δ1 and ∈2, but they are also known as π and τ or as N-1 and N-3. Biochemists usually assign the number 1 to the nitrogen atom adjacent to the side chain whereas organic chemists tend to designate this atom as 3.

The nonionized imidazole ring can exist as two tautomers, with the hydrogen atom on either the δ1 or the ∈2 nitrogen atom [5-7]. ^{13}Carbon-NMR studies have shown that in model peptides the hydrogen atom is usually predominantly on the ∈2-nitrogen atom, which has a pK_a value of about 0.6 pH units higher than that of the δ1 atom. The position of the hydrogen atom depends on the relative affinities of the two nitrogen atoms for protons, and can vary with conditions in the local environment. However both forms are found in proteins.

The side chain of His is readily protonated (pK_a value is nearly 7 at the second N-atom) and this destroys the nucleophilicity of the molecule. The two N atoms share the positive charge by resonance. Both of the imidazole nitrogen atoms can also be deprotonated simultaneously, with an apparent pK_a value of about 14.4, giving the

aromatic anion and this anion would be expected to be a potent nucleophile but is rarely present in substantial quantities.

The hydrogen atom of $C^{\epsilon 1}$ atom (often designated as C-2) is most likely to be slowly exchanged with the solvent, indicating that it has a very small probability of being deprotonated. This exchange reaction provides a useful probe of the environment of His residues in proteins.

His residues are especially useful in ^{1}H-NMR studies of proteins because the hydrogen atom on the $C^{\epsilon 1}$ atom is usually well resolved from the multitude of resonance's of the other hydrogen atoms in proteins in one-dimensional spectra [8]. Its resonance is also usually shifted by about 1ppm to lower field strength upon protonation of the side chain, often making it relatively easy to determine the pK_a values of individual His residues even in large proteins.

5.1.1. Biosynthesis of histidine

Histidine is synthesized by a simple pathway, which does not involve any branch points, and does not lead to the formation of any other metabolically important products. The same pathway is involved in all organisms that have been investigated.

The first step in histidine synthesis is the condensation of phosphoribosyl pyrophosphate with ATP to form phosphoribosyl-ATP. This reaction is catalysed by ATP phosphoribosyltransferase, which is freely reversible *in vitro*. *In vivo* the equilibrium is forced in the direction of synthesis of phosphoribosyl-ATP by the action of pyrophosphatase, which hydrolyses the pyrophosphate released into two

molecules of phosphate, removing one of the products of the reaction, and effectively preventing reversal.

ATP phosphoribosyltransferase is strongly inhibited by histidine. The inhibition is non-competitive with respect to both substrates, and involves a conformational change in the hexameric enzyme. On the addition of histidine, it can be shown that there is no conformational change in bacterial mutants in which the enzyme is insensitive to feedback inhibition.

Hydrolysis of phosphoribosyl-ATP to phosphoribosyl-AMP is catalysed by pyrophosphohydrolase. The resultant pyrophosphate is cleaved by pyrophosphatase. The adenine ring of phosphoribosyl-AMP is then cleaved hydrolytically between N-1 and C-6, to yield phosphoribosylformiminoaminoimidazolecarboxamide ribotide (PR-formimino-AIC-RP), by a cyclohydrolase. PR-formimino-AIC-RP undergoes isomerization to the ribulosyl derivative, which is aminated by transfer of the amide group of glutamine. Cyclization of the intermediate takes place and followed by cleavage to yield aminoimidazolecarboxamide ribotide from the ATP moiety, and the imidazole glycerol phosphate from the ribusyl moiety and C-2 and N-1 of the adenine ring. Although C-2 and N-1 of the imidazole ring of adenine are incorporated into histidine, the complete imidazole ring of the purine is not utilized.

Two separate enzymes, an amidotransferase and a cyclase, catalyse this reaction, but no intermediates has been identified, so the two enzymes must act in close concert, and the intermediate must be transferred from one enzyme to the other without appearing in free solution.

Dehydration of the imidazole glycerol phosphate to the 2-oxo derivative, imidazole acetol phosphate takes place, which is a substrate for amino transfer from glutamate to form histidinol phosophate. This is the hydrolysed to release the free amino alcohol, histidinol. Although in Neurospora there is a specific histidinol phosphate phosphatase, in Salmonella it appears that the reaction is catalysed by the same enzyme as catalyses the dehydration of the imidazole glycerol phosphate. Such catalysis of two non-adjacent steps in a pathway by one enzyme is very unusual.

Histidinol is oxidized to histidine in a two-step reaction. Single enzyme catalyses both steps, although the intermediate, histidinal, is a substrate *in vitro*, it is not normally detected *in vivo*, so it must remain firmly enzyme bound, and be oxidized rapidly.

Electrochemical methods for the determination of trace amount of histidine [9-11] were proposed, but none of them described the enantioanalysis of histidine. Several other methods based on capillary electrophoresis [12-17], coulometry [18], fluorimetry [19], chromatography, e.g., HPLC [20-22], GC-MS [23] and fluorescence [24] were used for the analysis and enantioanalysis of histidine, but they are not as simple and accurate as electroanalysis.

In this chapter, eleven enantioselective, potentiometric membrane electrodes (EPMEs) have been designed for the enantioanalysis of L-histidine and two enantioselective, potentiometric membrane electrodes have been designed for the enantioanalysis of D-histidine.

EPMEs based on cyclodextrins, maltodextrins, and C_{60} were used for the analysis of L-histidine while [5-6] fullerene-C_{70} and diethyl (1,2-methanofullerene C-$_{70}$)-70-70-dicarboxylic acid based EPMEs were used for the enantioanalysis of D-histidine.

5.2 Reagents and materials

L- and D-Histidine were supplied by Sigma-Aldrich (St. Louis, MO, USA). α-, β-, 2-hydroxyl-3-trimethylammoniopropyl -β- (as chloride salt) and γ-cyclodextrins were supplied by Walker-Chemie GmbH (München, Germany). Maltodextrins (DE 4.0-7.0, 13.0-17.0, 16.5-19.5) were purchased from Aldrich (Milwaukee, WI, USA). C_{60} fullerene, (1,2-methanofullerene C60)- 61- carboxylic acid, diethyl (1,2-methano fullerene C60)-61-61-dicarboxylate, tert-butyl (1,2-methanofullerene C60)-61-carboxylic acid, [5-6] fullerene-C_{70}, diethyl (1,2 methanofullerene C_{70})-70-70-dicarboxylic acid and paraffin oil were supplied by Fluka (Buchs, Switzerland). Graphite powder (1-2 μm, synthetic) was supplied by Aldrich. Food supplement, energy booster, immunity modulator, capsules (containing 0.72g L-histidine/capsule) were supplied by Hypo-plus Naturals, South Africa.

Deionised water from a Modulab system (Continental Water Systems, San Antonio, TX, USA) was used for the preparation of all solutions. The L- and D-histidine solutions necessarily in the characterization of the enantioselective, potentiometric membrane electrodes were prepared from standard L-histidine and D-histidine solutions (10^{-2} mol/L), respectively, by serial dilutions. All standard and diluted solutions were buffered with phosphate buffer (pH 5.40, 0.1mol/L) from Merck (Darmstadt, Germany) (1:1, v/v, buffer:deionised water).

5.3 Enantioselective, potentiometric membrane electrodes based on cyclodextrins for the enantioanalysis of L-histidine

5.3.1 Apparatus

A 663 VA stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 20 (Eco Chemie, Utrech, The Netherlands) and a software version 4.8 was used for all potentiometric measurements. Ag/AgCl (0.1 mol/L KCl) served as a reference electrode in the cell.

5.3.2 Electrodes 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 cyclodextrin (CD) (α-, β-, 2-hydroxyl-3-trimethylammoniopropyl -β-(as chloride salt) (β-CD-derivative) or γ) (10^{-3} mol/L) (100 μL chiral selector solution to 100 mg carbon paste) [25]. A certain quantity of carbon paste free of cyclodextrin was prepared and it was placed into a plastic pipette peak leaving 3-4 mm empty in the top to be filled with the carbon paste that contains the chiral selector. The diameter of the EPMEs was 3mm. Electric contact was obtained by inserting Ag/AgCl wire in the carbon paste. As internal solution it was utilized a solution of 0.1 mol/L KCl.

The surface of the electrode was wetted with deionised water and polished with alumina paper (polishing strips 30144-001, Orion) before using them for each experiment. The oil from the carbon paste prevents the leach of the cyclodextrin from the membrane into solution.

5.3.3 Recommended procedures

The direct potentiometry was used for measurements of the potentials of each standard solution (10^{-10}-10^{-2} mol/L). The electrodes were placed in stirred standard solutions and graphs of E(mv) versus pL-his were plotted. The unknown concentrations were determined from the calibration graphs.

5.3.3.1 Uniformity content test

Each of ten capsules (4mg perindopril/tablet) was placed in a 250 mL volumetric flask and dissolve in a 1:1 distilled water:buffer (pH = 5.4) solution. The unknown concentration was determined using the direct potentiometric method.

5.3.4 Results and discussion

5.3.4.1 EPMEs response characteristics

The response characteristics exhibited by the proposed EPMEs towards the detection of L-histidine are shown in Table 1. The response of all the electrodes was near-Nernstian for L-histidine and non-Nernstian for D-histidine. The working concentration ranges are large, and the correlation coefficients for the calibration plots are 0.9999. The response time of the proposed electrodes was 30s for concentration between 10^{-5} and 10^{-3} mol/L and 1 min for concentrations lower than 10^{-5} mol/L.

The proposed electrodes were highly stable and reproducible over a month test period. α- and β-cyclodextrin based enantioselective, potentiometric membrane electrodes showed better time stability, their standard potentials varying by ±0.10 mV, compared to that of γ-cyclodextrin based enantioselective, potentiometric membrane electrode, varying by ±4.0 mV during the one month test period.

Table 5.1 Response characteristics of enantioselective, potentiometric membrane electrodes designed for the assay of L-histidine.

EPME based on	Slope (mV/decade of conc.)	Intercept E^0 (mV)	Linear conc. range (mol/L)	Detection limit (mol/L)
α-CD	57.30	413.10	$10^{-3} – 10^{-7}$	$6.17x10^{-8}$
β-CD	57.76	587.95	$10^{-3} – 10^{-10}$	$6.62x10^{-11}$
β-CD-deriv.	53.89	556.25	$10^{-4} – 10^{-10}$	$4.77x10^{-11}$
γ-CD	56.36	576.21	$10^{-3} – 10^{-10}$	$5.97x10^{-11}$

All measurements were made at 25^0C. All values are average of 10 determinations.

5.3.4.2 Effect of pH on the response of the electrodes

The influence of pH on the response of the proposed electrodes was investigated by recording the emf of the cell for the solutions containing 10^{-5} mol/L L-histidine at different pH values (pH 1-12). These solutions were prepared by adding small volumes of HCl and/or NaOH solutions (0.1 or 1 mol/L of each) to an L-histidine solution. The plots of E(mV) versus pH (Fig. 5.2) showed that the response of the electrodes is not depending on pH, in the ranges 4.0-9.0 (β-CD and β-CD-derivative based EPMEs), 4.0-7.0 (γ-CD based EPME), and 3.0-8.0 (α-CD based EPME). This proves the basic behaviour of L-his at pH<4 and its acidic behaviour at pH>8.

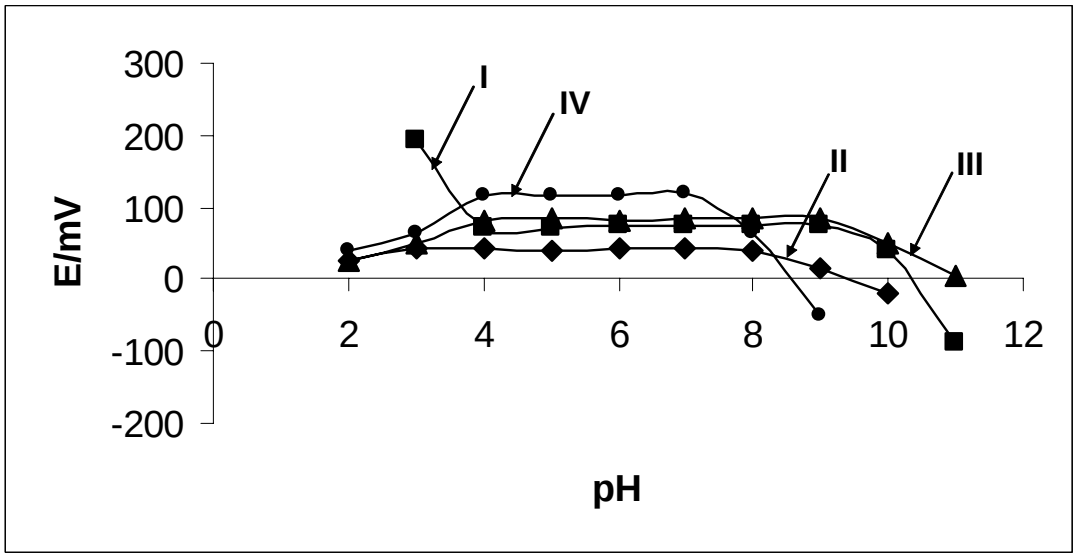

Figure 5.2 The influence of pH on the response of the enantioselective potentiometric membrane electrodes ($C_{L\text{-}his}=10^{-5}$); I - β-CD based EPME, II - γ -CD based EPME, III - β-CD-derivative based EPME, and IV - α-CD based EPME.

5.3.4.3 The selectivity of the electrodes

The enantioselectivity of the electrodes was investigated over D-histidine, using mixed solution method. The concentration of the interfering ion and L-histidine were 10^{-4} and 10^{-5} mol/L, respectively. The values of pK^{pot} ($pK^{pot} = -\log K^{pot}$, where K^{pot} is the potentiometric selectivity coefficient) were 3.0, 2.2, 2.4, and 2.7 for EPMEs based on β-CD, γ-CD, β-CD-derivative and α-CD. Inorganic ions such as Na^+, K^+, Ca^{2+} as well as polyvinylpyrolidone did not interfere with the analysis of L-histidine (pK^{pot} >>4).

5.3.4.4 Analytical application

To assess the feasibility of the proposed direct potentiometric procedure, recovery test were performed for histidine-raw material. The assay of L-histidine in the presence of D-histidine was conducted using different ratios between L- and D-histidine. The

results obtained (Table 5.2) demonstrated the suitability for the proposed enantioselective, potentiometric membrane electrodes for testing the enantiopurity of histidine-raw material 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 enantiomers varying from 1:9 to 1:99.9.

Table 5.2 Determination of L-histidine in the presence of D-histidine.

L:D (mol/mol)	L-His, Recovery (%)			
	EPME based on			
	α-CD	β-CD	γ-CD	β-CD-derivative
2:1	99.14±0.03	99.98±0.02	99.95±0.03	99.59±0.02
1:1	99.12±0.02	99.94±0.02	99.97±0.03	99.47±0.02
1:2	99.12±0.02	99.96±0.02	99.98±0.02	99.52±0.03
1:4	99.16±0.03	99.95±0.03	99.96±0.02	99.47±0.03
1:9	99.14±0.02	99.96±0.03	99.97±0.02	99.57±0.02

All measurements were made at 25^0C. All values are average of 10 determinations.

Uniformity content tests were performed for the capsules containing histidine. The results shown that the capsules contain: 96.78 ± 0.21, 96.07 ± 0.14, 96.95 ± 0.19, and 96.93 ± 0.20% L-histidine when. α-, β-, 2-hydroxyl-3-trimethylammoniopropyl -β- (as chloride salt) and γ-cyclodextrins based electrodes were used for this tests.

5.4 Enantioselective, potentiometric membrane electrodes based on maltodextrins for the enantioanalysis of L-histidine

5.4.1 Apparatus

A 663 VA stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 20 (Eco Chemie, Utretch, Netherlands) and a software version 4.8 was used for all the potentiometric measurements. Ag/AgCl (0.1 mol/L KCl) served as reference electrode in the cell.

5.4.2 Electrodes 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; 100µL chiral selector solution to 100mg carbon paste) [26]. A quantity of carbon paste free from maltodextrin was also prepared and placed in a plastic pipette, leaving 3 to 4 mm empty in the top to be filled with the carbon paste containing the chiral selector. The diameter of the potentiometric, enantioselective membrane electrode was 3 mm. Electric contact was obtained by inserting a Ag/AgCl wire into the carbon paste. The internal solution was 0.1 mol/L KCl. The surface of the electrode was wetted with deionised water and polished with alumina paper (polishing strips 30144-001, Orion) before use in each experiment.

5.4.3 Recommended procedure: direct potentiometry

The direct potentiometry was used for the measurement of the potential of each standard solution (10^{-10}-10^{-3}mol/L). The electrodes were placed in stirred standard solutions and graphs of E (mV) versus pL-His were plotted. The unknown concentrations were determined from the calibration graphs.

5.4.3.1 Uniformity content test

This test was performed accordingly with the same procedure as described in 5.3.3.1.

5.4.4 Results and discussion

5.4.4.1 Electrodes response

The response characteristics of the electrodes are shown in table 5.3. The proposed electrodes exhibited near-Nernstian response over L-histidine. For all the calibration equations, the correlation coefficient was 0.9999. The limits of detection are low. The electrodes showed non-Nernstian responses for D-histidine.

The response time was 30s for concentration in the 10^{-3} and 10^{-5} mol/L range, and 1min for concentrations lower than 10^{-5}mol/L. The EPMEs responses exhibited a good stability and reproducibility for the tests performed for 6 months, when daily used for measurements (RSD<1.0%).

Table 5.3 Response characteristics of enantioselctive, potentiometric membrane electrodes for the assay of L-Histidine.

Maltodextrin	Slope (mV/pL-His)	Intercept E^o (mV)	Linear conc. range (mol/L)	Detection limit (mol/L)
I	-58.30	681.90	10^{-11}-10^{-3}	$1.97x10^{-12}$
II	-58.90	692.30	10^{-11}-10^{-3}	$1.72x10^{-12}$
III	-58.50	634.40	10^{-10}-10^{-3}	$1.40x10^{-11}$

All measurements were made at 25°C. All the values are averages of ten determinations.

5.4.4.2 The effect of pH on the response of the electrodes

The influence of pH on the response of the proposed electrodes was investigated by recording the emf of the cell for solutions containing 10^{-5} mol/L L-His at different pH values (pH 1-12). These solutions were prepared by adding small volumes of HCl and/or NaOH solution (0.1 mol/L of each) to a L-His solution. The plots of E (mV) versus pH (Fig. 5.3) show that the response of the electrodes is not depending on pH, in the following ranges 4.0-8.0, 4.0-10.0, and 4.0-9.0, for MDI, II, and III based EPME, respectively.

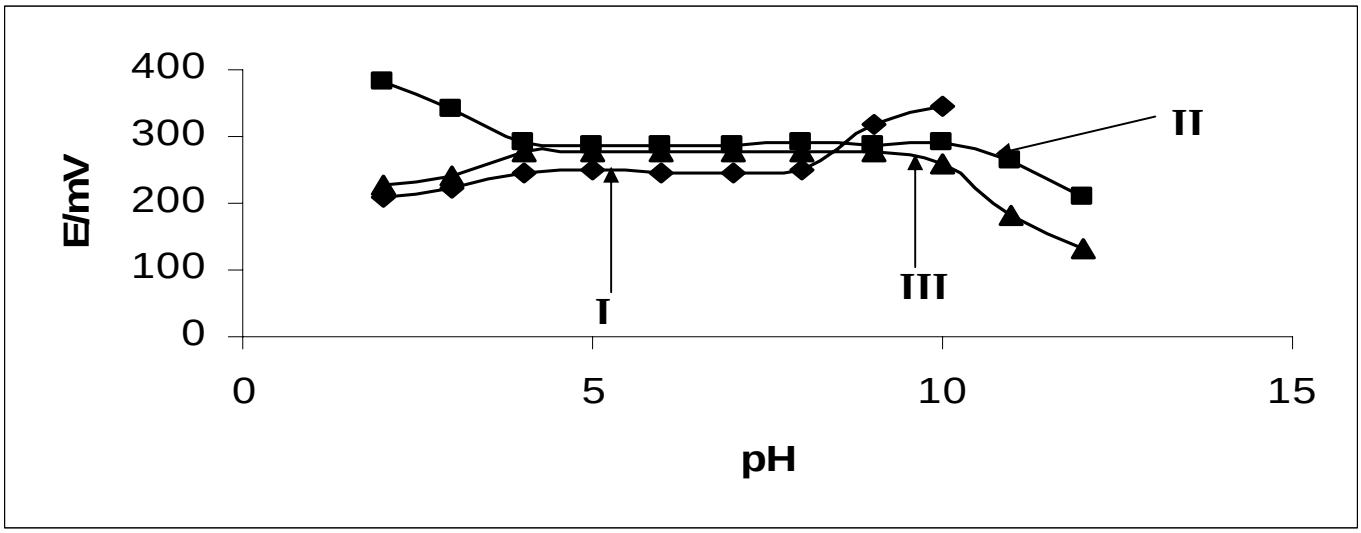

Figure 5.3 The influence of pH on the response of the enantioselective, potentiometric membrane electrodes ($C_{L\text{-}His}=10^{-5}$mol/L); I – MDI based EPME, II – MDII based EPME and III - MDIII based EPME.

5.4.4.3 The selectivity of the electrodes

The enantioselectivity of the electrodes was investigated over D-His, using mixed solution method. The concentration of the interfering ions and L-His were 10^{-4} and 10^{-5} mol/L, respectively. The values of pKpot (pKpot = -logKpot) (where Kpot is the potentiometric selectivity coefficient) were 2.40, 3.09 and 2.40 for MD I, II and III based EPMEs, respectively. The best enantioselectivity being shown by MD-II based electrode. PVP, creatine and creatinine did not interfere in the enantioanalysis of L-his (Table 5.4). Inorganic ions such as Na^+, K^+, and Ca^{2+} did not interfere with the analysis of L-His.

Table 5.4 Potentiometric, selectivity coefficients of the electrodes used for the enantioanalysis of L-histidine.

Interfering species (J)	$K_{i,j}^{pot}$		
	EPME based on		
	MD-I	MD-II	MD-III
D-His	$4x10^{-3}$	$8.1x10^{-4}$	$4x10^{-3}$
PVP	$3.8x10^{-3}$	1.2x10-3	$1x10^{-4}$
Creatine	$1x10^{-4}$	$1x10^{-3}$	$3.8x10^{-3}$
Creatinine	$8.2x10^{-3}$	$7.9x10^{-4}$	$8.1x10^{-3}$

5.4.4.4 Analytical applications

To assess the feasibility of the proposed direct potentiometry procedure, recovery tests were performed for histidine-raw material. The assay of L-His in the presence of D-His was conducted by use of different ratios between L-His and D-His. The results obtained (Table 5.5) demonstrated the suitability of the proposed enantioselective, potentiometric membrane electrodes for testing the enantiopurity of histidine-raw material 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 enantiomers varying from 1:9 to 1:99.9.

Table 5.5 Determination of L-His in the presence of D-His.

L:D (mol/mol)	L-His, Recovery (%)		
	EPME based on		
	MD I	MD II	MD III
2:1	99.19±0.02	99.98±0.02	99.21±0.02
1:1	99.46±0.02	99.97±0.01	100.00±0.01
1:2	100.00±0.01	99.42±0.02	100.00±0.02
1:4	99.12±0.01	99.76±0.02	99.89±0.02
1:9	99.18±0.02	99.77±0.02	99.25±0.02

All measurements were made at 25^{o}C. All values are averages of ten measurements.

Uniformity content tests were performed for the capsules containing histidine. The results shown that the capsules contain: 97.68±0.18, 97.81±0.11, and 97.39±0.20 L-histidine when. MD I, II and III based electrodes were used for this tests.

5.5 Enantioselective, potentiometric membrane electrodes based on C_{60} for the enantioanalysis of L-histidine

5.5.1 Apparatus

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

5.5.2 Electrodes design

Paraffin oil and graphite powder were mixed in a ratio of 1:4 (w/w) followed by the addition of chiral selector [5-6] fullerene- C_{60} (I), (1,2-methanofullerene C_{60})- 61-carboxylic acid (II), diethyl (1,2-methanofullerene C_{60})-61-61-dicarboxylate (III) or tert-butyl (1,2-methanofullerene C_{60})-61-carboxylic acid (IV) to carbon paste [27]. A certain quantity of carbon paste free of chiral selector was prepared and it was placed into a plastic pipette peak leaving 3–4mm empty in the top to be filled with the carbon paste that contains the chiral selector. The diameter of the potentiometric, enantioselective membrane electrode was 3 mm. Electric contact was obtained by inserting a Ag/AgCl wire in the carbon paste. 0.1mol/L of KCl was used 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 experiment.

The carbon paste prevents the leach of the chiral selector from the membrane into solution. When it was not in use, the electrode was immersed in a 10^{-3} mol/L L-his solution.

5.5.3 Recommended procedure: direct potentiometry

The potentiometric method was used for the potential determination of each standard solution (10^{-10}- 10^{-3} mol/L, pH 5.40). The electrodes were placed in the stirred standard solutions and graphs of E (mV) versus pL-His were plotted. The unknown concentrations of L-His were determined from the calibration plots.

5.5.3.1 Uniformity content test

This test was performed accordingly with the same procedure as described in 5.3.3.1.

5.5.4 Results and discussion

5.5.4.1 Electrodes response

The response characteristics exhibited by the proposed electrodes towards the L-his are summarized in Table 5.6. For all the calibration plots, the potentiometric membrane electrodes showed linear and near-Nernstian responses for L-his, with correlation coefficients for the equations of calibration of 0.9997 (I), 0.9999 (II, IV), and 0.9998 (III), respectively. The lowest limit of detection as well as the wider linear concentration range were recorded for the electrode based on fullerene (III) while the best response was recorded for the electrode based on fullerene (II). D-his on the other hand, showed non-Nernstian response. All electrodes displayed good stability and reproducibility over the test period (RSD < 0.1%).

Table 5.6 Response characteristics of the enantioselective, potentiometric, membrane electrodes based on C_{60} fullerene.

EPME based on	Slope (mV/decade of conc.)	Intercept, E^o (mV)	Linear conc. range (mol/L)	Detection limit (mol/L)
I	56.8	465.8	10^{-8}-10^{-4}	6.3×10^{-9}
II	57.7	601.9	10^{-10}-10^{-5}	3.7×10^{-11}
III	56.0	652.4	10^{-10}-10^{-3}	2.2×10^{-12}
IV	57.5	602.4	10^{-10}-10^{-5}	3.3×10^{-11}

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

5.5.4.2 Effect of pH on the response of the electrodes

The influence of pH on the response of the proposed electrodes was checked by recording the emf of the cell for solutions containing 10^{-5} mol/L L-his at different pH values. These solutions were prepared by adding very small volumes of HCl/NaOH solution (0.1 mol/L or 1 mol/L of each) to a L-his solution. The plots of E (mV) versus pH (Figure 5.4) indicate that the response of the electrodes does not depend upon the pH in the following ranges: 4.0-6.0 [I] 3.0-7.0 [II], 3.0-8.0 [III], and 5.0-8.0 [IV], respectively.

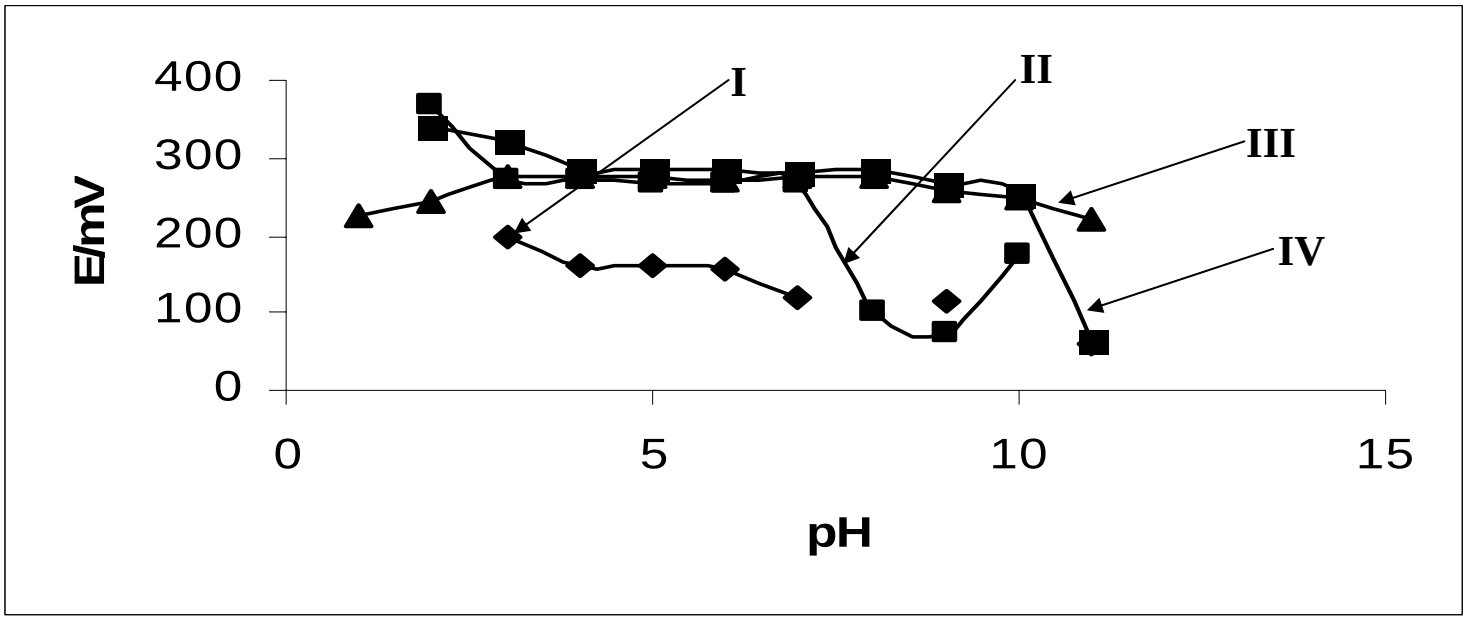

Figure 5.4 The influence of pH on the response of the enantioselective, potentiometric membrane electrodes (L-his =10^{-5} mol/L); for enantioselective, potentiometric electrodes based on (I), (II), (III) and (IV), respectively.

5.5.4.3 Selectivity of the electrodes

The selectivity of the potentiometric membrane electrode was checked using mixed solutions method. The concentrations of interfering ions and L-His were 10^{-4} and 10^{-5} mol/L, respectively. The values of potentiometric selectivity coefficients, K_{sel}^{pot}, shown in Table 5.7 demonstrated the enantioselectivity and selectivity properties of the proposed electrodes for the assay of L-his. Inorganic cations such a Na^{+}, K^{+}, and Ca^{2+} do not interfere in the analysis of L-his ($K_{sel}^{pot} << 10^{-4}$).

Table 5.7 Potentiometric selectivity coefficients for the proposed electrodes.

Interfering species	K_{sel}^{pot}			
	EPME based on			
	I	II	III	IV
D-His	4.0×10^{-3}	4.3×10^{-3}	2.2×10^{-3}	2.4×10^{-3}
PVP	3.7×10^{-3}	5.7×10^{-3}	3.4×10^{-3}	5.7×10^{-3}
Creatine	3.2×10^{-3}	$<< 10^{-4}$	2.9×10^{-3}	1.8×10^{-3}
Creatinine	4.6×10^{-3}	6.2×10^{-3}	2.7×10^{-3}	5.3×10^{-3}

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

5.5.4.4 Analytical applications

The response characteristics as well as the selectivity ad enantioselectoivity of the proposed electrodes made them useful for the determination of enantiopurity of histidine raw material and for performing the content uniformity test of L-his capsules (Food supplement, energy booster, immunity modulator, capsules).

Recovery tests of L-his in the presence of D-his were performed. The results proved that the electrodes are suitable for enantioanalysis of L-histidine (Table 5.8), when the ratio between L- and D-his has got any value. No differences between the recovery values when the ratios L:D were 1:9 and 1:99.9 were recorded.

Table 5.8 Determination of L-His in the presence of D-His.

L:D (mol/mol)	L-Histidine, Recovery (%)			
	EPME based on			
	I	II	III	IV
2: 1	99.50 ± 0.02	99.92 ± 0.02	99.60 ± 0.02	99.99 ± 0.01
1: 1	99.50 ± 0.02	99.90 ± 0.01	99.99 ± 0.01	99.40 ± 0.02
1: 2	99.99 ± 0.03	99.98 ± 0.02	99.89 ± 0.03	99.80 ± 0.02
1: 4	99.50 ± 0.02	99.95 ± 0.02	99.98 ± 0.02	99.98 ± 0.01
1: 9	99.52 ± 0.02	99.94 ± 0.01	99.98 ± 0.02	99.98 ± 0.02

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

Uniformity content test was performed for Food supplement, energy booster, immunity modulator, and capsules. The results obtained with the proposed electrodes: 96.41 ± 0.12% (I), 96.99 ± 0.14% (II), 96.53 ± 0.02% (III), 96.70 ± 0.28% (IV), respectively.

5.6 Enantioselective, potentiometric membrane electrodes based on C_{70} for the enantioanalysis of D-histidine

5.6.1 Apparatus

A 663 VA Stand (Metrohm, Herisau, Switzerland) combined with a PGSTAT 100 and a software (Eco Chemie version 4.4) were used for all potentiometric measurements.

A glassy carbon electrode and a Ag/AgCl (0.1 mol/L KCl) served as the counter and reference electrodes in the cell.

5.6.2 *Electrode design.*

Paraffin oil and graphite powder were mixed in a ratio of 1:4 (w/w) followed by the addition of chiral selector, [5-6] fullerene– C_{70} and diethyl methanofullerene C_{70})-70-70-dicarboxylic acid [28]. A certain quantity of carbon paste free of chiral selector was prepared and it was placed into a plastic pipette peak leaving 3–4mm empty in the top to be filled with the carbon paste that contains the chiral selector. The diameter of the potentiometric, enantioselective membrane electrode was 3 mm. Electric contact was obtained by inserting a Ag/AgCl wire in the carbon paste. 0.1mol/L of KCl was used 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 experiment.

The carbon paste prevents the leach of the chiral selector from the membrane into solution. When it was not in use, the electrode was immersed in a 10^{-3} mol/L L-his solution.

5.6.3 Recommended procedure

5.6.3.1.Direct potentiometry

The potentiometric method was used for the potential determination of each standard solution (10^{-10}- 10^{-3} mol/L, pH 5.30). The electrodes were placed in the stirred standard solutions and graphs of E (mV) versus pD-His were plotted. The unknown concentrations of D- His were determined from the calibration plots.

5.6.4 Results and discussion

5.6.4.1 Electrode response

The response characteristics exhibited by the proposed electrodes towards the detection of D-his are summarized in Table 5.9. For all the calibration plots, the potentiometric membrane electrodes showed linear near-Nernstian responses for D-his, with correlation coefficients for the equations of calibration of 0.9997 (I), 0.9998 (II) respectively. On the other hand L-histidine, showed non-Nernstian response.

The response times for diethyl (1,2 methanofullerene C_{70})-70-70-dicarboxylic acid electrode is 30s. The response time of [5-6] fullerene-C_{70} of concentration is 25s between 10^{-9}-10^{-8} and 10s between 10^{-6}-10^{-5}. All electrodes displayed good stability and reproducibility over the test, as shown by the relative standard deviation values.

Table 5.9. Response characteristics of the potentiometric, enantioselective membrane electrode based on C_{70} fullerene and its derivative.

EPME based on fullerene	Slope (mV/D-his)	Intercept, E^o (mV)	Linear conc. range (mol/L)	Detection limit (mol/L)
I	55.34	713.57	10^{-11}-10^{-5}	$1.27x10^{-13}$
II	52.51	642.19	10^{-11}-10^{-5}	$5.90x10^{-13}$

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

5.6.4.2 Effect of pH on the response of the electrodes

The influence of pH on the response of the EPMEs was checked by recording the emf of the cell for solutions containing 10^{-5} mol/L D-his at different pH values (pH, 1-12). These solutions were prepared by adding very small volumes of HCl/NaOH solution (0.1 mol/L or 1 mol/L of each) to a D-his solution.

The plots of E (mV) versus pH (Figure 5.5) indicate that the response of the electrodes does not depend upon the pH in the following range: 4.0-8.0 (I) 2.0-7.0 (II).

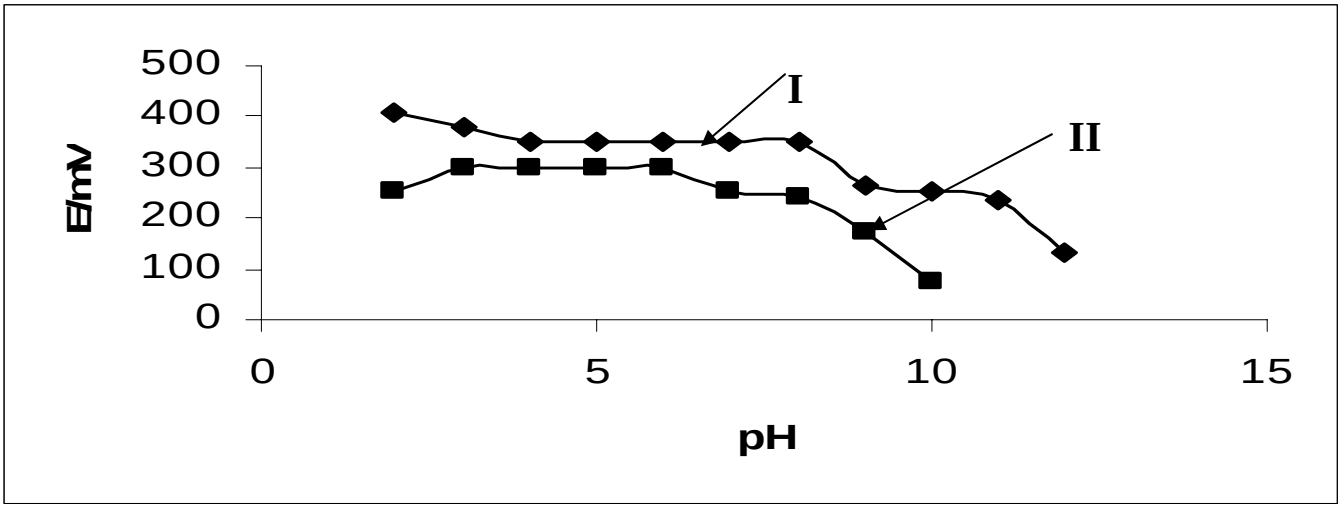

Figure 5.5 The influence of pH on the response of the enantioselective potentiometric membrane electrodes (D-his =10^{-5} mol/L); for [5-6] fullerene-C_{70} based electrode and diethyl (1,2 methanofullerene C_{70})-70-dicarboxylic acid based electrode,

5.6.4.3 Selectivity of the electrodes

The selectivity of the potentiometric membrane electrode was checked by the mixed solutions method. The concentrations of interfering ions and D-His were 10^{-4} and 10^{-5} mol/L, respectively. The values obtained for the L-his, PVP, Creatine and Creatinine demonstrated the enantioselectivity and selectivity properties of the proposed EPMEs for the assay of D-his.

Table 5.10 Potentiometric selectivity coefficients for the enantioselective, potentiometric membrane electrode for D-histidine.

Interfering species	K_{sel}^{pot}	
	EPME based on	
	I	II
L-cys	$1.0x10^{-4}$	$2.3x10^{-3}$
PVP	$4.5x10^{-3}$	$2.0x10^{-3}$
Creatine	$1.3x10^{-3}$	$3.2x10^{-4}$
Creatinine	$9.4x10^{-4}$	$3.0x10^{-3}$

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

5.6.4.4 Analytical applications

The proposed enantioselective, potentiometric membrane electrodes proved to be useful for the determination of enantiopurity of histidine raw material and for performing the content uniformity test of pharmaceutical compounds containing histidine. The assays of D- and L-his were conducted using different ratios between D- and L-his and L-and D-his, respectively.

The good recovery values obtained for the determination of D-enantiomer in the presence of L-enantiomer and for L-enantiomer in the presence of D-enantiomer (Table 5.11) demonstrated the suitability of the proposed enantioselective, potentiometric membrane electrodes for the testing the enantiopurity of Histidine raw material.

Table 5.11 Determination of D-His in the presence of L-His.

L:D mol/mol	D-histidine, Recovery (%)	
	EPMEs based on	
	I	II
2:1	99.93±0.02	99.95±0.02
1:1	99.96±0.02	99.93±0.02
1:2	99.95±0.01	99.97±0.01
1:4	99.97±0.02	99.96±0.02
1:9	99.96±0.02	99.96±0.02

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

The average recoveries of D-his in the capsules were 2.58 ± 0.82% (I) and 2.67 ± 0.14% (II) respectively for the two electrodes.

5.7 Conclusions

Enantioselective, potentiometric membrane electrodes based on carbon paste impregnated with cyclodextrin (α-, β-, and γ-CD) and its derivative 2-hydroxy-3-trimethylammoniopropyl-β-CD (β-CD-derivative), maltodextrins, and fullerenes (C_{60} and C_{70}) have been designed for the enantioanalysis of L- and D-enantiomers of histidine. The proposed enantioselective, potentiometric membrane electrodes have excellent features in real-time enantioselective analysis of histidine in biological

fluids. The construction of the electrodes is simple, fast and highly reproducible and the method used is highly sensitive with good accuracy.

Enantioselective, potentiometric membrane electrodes (EPMEs) based on cyclodextrins has the lowest limit of detection when β-CD-derivative was used as chiral selector. EPMEs based on maltodextrins have the lowest limit of detection when MD II was used for the design of the electrode. EPMEs based on C_{60}-fullerenes have low limits of detection for fullerene-III based electrode. EPMEs based on C_{70}-fullerenes showed low limits of detection for the analysis of D-histidine.

Both selectivity and enantioselectivity of the proposed electrodes were good. The recovery tests of one enantiomers in the presence of its antipode proved that the enantioselective, potentiometric membrane electrodes are suitable for the analysis and enantioanalysis of L- and D-histidine in pharmaceutical formulations.

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Chapter 6

Conclusions

Pharmaceutical and clinical enantioanalysis require high reliable analytical techniques. The utilization of enantioselective, potentiometric membrane electrodes proved to be a good alternative of chromatographic techniques in terms of accuracy, precision, reliability of the analytical information, simplicity, cost and time consuming.

The principle of enantioanalysis using enantioselective, potentiometric membrane electrodes is to find the best chiral selector for the enantiomers to be determined. Accordingly, for the amino acids of clinical and pharmaceutical importance studied (clenbuterol, cysteine and histidine) different chiral selectors were used for the design of the electrochemical sensors. If cyclodextrins and maltodextrins were previously used in the design of the potentiometric electrodes as well as stationary phases for liquid chromatography, fullerenes C_{60} and C_{70} are new chiral selectors that we considered to be used in enantioanalysis.

An enantioselective, potentiometric membrane electrode based on 2-hydroxy-3-trimethylammoniopropyl-β-CD was designed and proposed for the enantioanalysis of R-clenbuterol. The electrode had good response characteristics, and it could have been used for the enantioanalysis of R-clenbuterol in serum samples.

Nine enantioselective, potentiometric membrane electrodes (EPMEs) based on cyclodextrins (α-CD, γ-CD β-CD, and 2-hydroxy-3-trimethylammoniopropyl-β-CD), maltodextrins (of different dextrose equivalence), and antibiotics (vancomycin and teicoplanin) have been designed for the enantioanalysis of L-cysteine. The main advantage of the proposed electrodes versus existing methods is their possibility for the enantioanalysis of L-cysteine in urine samples.

Eleven enantioselective, potentiometric membrane electrodes (EPMEs) have been designed for the enantioanalysis of L-histidine and two enantioselective, potentiometric membrane electrodes have been designed for the enantioanalysis of D-histidine. Enantioselective, potentiometric membrane electrodes based on cyclodextrins, maltodextrins, and C_{60} were used for the analysis of L-histidine while [5-6] fullerene-C_{70} and diethyl (1,2-methanofullerene C-$_{70}$)-70-70-dicarboxylic acid based electrodes were used for the enantioanalysis of D-histidine. The utilization of fullerenes in the electrodes design improved the response characteristics, reliability and accuracy of the analytical information obtained using the proposed electrodes.

The construction of the electrodes is simple, fast and reproducible. One of the main advantages of the proposed method is that the sample (pharmaceutical product, serum and urine) did only need to 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 enantioselective, potentiometric membrane electrodes can be used for the enantioanalysis of enantiomers as raw materials and in their pharmaceutical formulations and the enantiomers can be determine in real time from biological fluids such as serum and/or urine. One of the features of these electrodes is their application for *in vivo* enantioanalysis of the amino acids.

APPENDIX

PUBLICATIONS

1. *Enantioselective, potentiometric membrane electrodes based on cyclodextrins for the determination of L-histidine*

   R.I. Stefan-van Staden, L. Holo

   Sens.Actuators B, Submitted.

2. *Enantioanalysis of L-histidine using enantioselective, potentiometric membrane electrodes based on maltodextrins*

   R.I. Stefan-van Staden, L. Holo

   Anal.Lett., Submitted.

3. *Enantioanalysis of S-clenbuterol*

   R.I. Stefan-van Staden, B Moeketsi, L.Holo, K.Sharma, H.Y. Aboul-Enein

   Anal.Chem., Submitted.

4. *Enantioanalysis of L-cysteine using enantioselective, potentiometric membrane electrodes*

   RI Stefan-van Staden, L. Holo

   J.Pharm. Biomed.Anal., Submitted.

5. *Vancomycin and teicoplanine based enantioselective, potentiometric membrane electrodes for the assay of L-cysteine*

R.I. Stefan-van Staden, L. Holo

Sens.Actuators B, Submitted.

6. *Cyclodextrins based enantioselective, potentiometric membrane electrodes and their applications for enantioanalysis of L-cysteine in urine*

R.I. Stefan-van Staden, L. Holo

Anal.Lett., Submitted.

7. *Enantioselective, potentiometric membrane electrodes based on C_{60} fullerene and its derivatives for the assay of L-histidine*

R.I. Stefan-van Staden, B. Lal, L. Holo

Talanta, Submitted.

8. *C_{70} fullerenes based enantioselective, potentiometric membrane electrodes for the assay of D-histidine*

K.Sharma, R.I. Stefan-van Staden, L. Holo, B. Lal

JACS, Submitted.