Chapter 1

Enantioselective analysis using amperometric biosensors and immunosensors

1.1. Introduction

In the last few years, enantioselective high throughput screening analysis became increasingly important, especially for the pharmaceutical industry [1]. It was found that only one of the enantiomers has the pharmacodynamics and pharmacokinetics required. The other enantiomer may have a different pharmacodynamics or it may considerably reduce the activity of the requested enantiomer. Sometimes, the assay of the one enantiomer is not enough and there is a need for the simultaneous assay of enantiomers in the raw material. For some drugs, a certain ratio between the enantiomers can be tolerated.

The evolution concerning the design of enantioselective electrochemical sensors made their utilization a very accurate and precise alternative for chromatographic techniques in high throughput enantioselective screening analysis [2-5]. The best chromatographic technique for enantiomers separation is capillary zone electrophoresis (CZE) [6], but in most of the cases its sensitivity is lower than for amperometric biosensors [4]. Because the preparation of samples for CZE is very laborious and the separation step is not always

reliable, the accuracy and precision for this technique are lower than for electrochemical sensors [7].

The reliability of the response characteristics as well as the analytical information obtained by using electrochemical sensors is strictly correlated with the design of sensors [8]. Only a reliable construction of sensors will give reliable response characteristics and reliable analytical information. The design proposed for sensors is based on PVC, imprinting polymers, carbon paste and diamond paste matrices. The most reliable designs are that of carbon paste and diamond paste sensors. It is also necessary to look for more reliable chiral selectors and enzymes.

The utilization of electrochemical sensors in molecular recognition of the enantiomers is not laborious if one compares it with structural analysis and with chromatographic techniques [9]. The method is rapid, precise and not expensive. The enantioselective sensors are also used reliably for the determination of the best chiral selector for the assay of a certain enantiomer. By using a multiplexer, a maximum of 256 chiral selectors can be screened in less than two hours. Immunosensors assure the enantiospecificity when used in molecular recognition of enantiomers. The high reliability of analytical information obtained using these sensors made automation of amperometric techniques possible, by integration of enantioselective sensors as detectors in flow injection and sequential injection analysis techniques.

There is, however, no electrochemical sensor that can be reliably used for all types of enantiomers. The type of sensors (in addition to the chiral selector, the enzyme or the antibody) must be selected in concordance with the complexity of the structure of the enantiomer. The best selection of electrochemical sensor will assure the precision of molecular recognition of enantiomers. For electrochemical sensors with good response characteristics, the molecular recognition based on kinetics (integration of the sensors in the flow injection and sequential injection analysis systems) can be applied. This will increase the objectivity as well as the precision of molecular recognition.

1.2. Principles of enantiomer recognition

In molecular recognition of enantiomers, electrochemical sensors are a very good alternative for structural analysis (IR, NIR, Raman, MS, X-Ray Diffraction, Neutron Diffraction). The advantage of using these sensors, over the techniques used in structural analysis, is the high reliability that is given by high precision, high reproducibility, and rapidity [3,4]. Due to the fact that electrochemical sensors can be used directly for measurements of the compounds in solution, without any prior separation of the substance that has to be determined, their precision is higher than that obtained using a chromatographic method [2].

The electrochemical sensors proposed for molecular recognition in chiral discrimination are: potentiometric, enantioselective membrane electrodes, amperometric biosensors and immunosensors. There are two ways to use electrochemical sensors in molecular recognition of chiral substances: selective binding [mainly valuable for potentiometric,

enantioselective membrane electrodes (PEME) and for immunosensors] and catalyst selectivity (mainly valuable for biosensors). A molecule with a special chemical architecture is required for selective binding: a lock for a key. The catalyst selectivity has the same recognition mechanism, but the lock is the enzyme and the key is the chiral center of the molecule.

1.2.1. Molecular recognition of enantiomers using amperometric biosensors

For the design of amperometric biosensors, enzymes are used as they catalyze only the reaction of one of the enantiomers. It was shown that this type of electrode is more precise for the complex molecules than the potentiometric, enantioselective membrane electrode [4]. This enzyme can be combined with different electrochemical transducers: field effect transistor, potentiometric or amperometric.

The high stereo-, regio-, and enantioselectivity enzymes are used for both biotransformation and biosynthesis. However, this potential of biosensors can only be used occasionally, because it is not easy to find appropriate enzyme pairs for analysis that have high specificities and similar optimum pH and temperature ranges but different enantioselectivities. The most common enzyme pairs are [10]:(i) a non- enantioselective enzyme and an enantioselective one for the same analyte; (ii) an enzyme pair with high analyte selectivity, but with different enantiomeric specifity.

Due to the high importance of the assay of the enantiomers of amino acids and amino acids derivatives, numerous papers are dedicated to the assay of the enantiomers of these

substances by using enantioselective amperometric biosensors based on L- amino acid oxidase (L-AAOD) and D- amino acid oxidase (D-AAOD) [4] for the assay of S- and R-enantiomers, respectively. A simultaneous screening of S and R enantiomers can be done by using the multiplexer.

1.2.2. Molecular recognition of enantiomers using immunosensors

The molecular recognition of enantiomers using immunosensors is based on the reaction between antigen and antibody. The antibody can recognize the chirality center of the antigen. This type of reaction is the only one that may be considered to be enantiospecific, because the antibody is not reacting with the other enantiomer. It is one of the advantages that can be used for screening of a particular enantiomer in a racemic mixture. They are also very sensitive, and they can be used for trace analysis of enantiomers.

1.3. Design of amperometric biosensors and immunosensors

1.3.1. Amperometric biosensors

The construction of biosensors is first related to the immobilization techniques for the enzymes, cells, or other biological active substances and second to the transducer that will be used. The evolution of biosensors design is influenced by the evolution of the transducers, in terms of design and sensitivity. When a biosensor is designed, a direct correlation between the type and quantity of the products formed in the enzymatic reaction and the type of transducer must be done. The first reaction that is taking place is the biochemical reaction. Most of the biosensors are based on the enzymatic reaction.

When an enzyme is immobilized, a transformation of the substrate under action of the enzyme (that acts as catalyst) into products is taking place:

$$Substrate \xrightarrow{Enzyme} Product$$

The biochemical reaction is very selective and sensitive. Mainly, the enzyme is selective for a group of substances that are enclosed in the same class of organic compounds. Another one or two enzymes (bienzymatic or trienzymatic biosensors) can be used in biosensor design to improve the selectivity and sensitivity of the initial enzymatic reaction as well as to favor the obtaining of the compounds that can be detected by a certain transducer.

The type of the products resulted in the enzymatic reaction determined the type of the transducer, e.g., when H^+ is forming, a pH electrode can be used as transducer, when H_2O_2 is forming, the amperometric transducer is recommended, when carbon dioxide or ammonia are forming, the corresponding gas sensors are utilized. The sensitivity of the enzymatic reaction must be correlated with the sensitivity of the transducer. It follows that the best electrochemical transducer type that can be utilized in the construction of biosensors is the amperometric one [4]. This type of transducer competes with the chemiluminescences and fluorescence ones from the class of optical transducers.

Due the fact that the equilibria of biotransformation of the substrate are taking place at the membrane-solution interface, it follows that the nature of the matrix will influence the behavior of the enzyme. In this regard, the evolution of the design of biosensors is

interconnected with the evolution of the materials used for matrix (film) construction, as well as with the evolution of the technology involved in the (film) construction.

The best matrices for biological material (e.g., enzymes) immobilization proved to be carbon paste [4] (graphite powder and oil) and diamond paste (diamond powder and oil). Usually, nujol oil and paraffin oil are used for preparation of the paste. Lately, it has been found that the nature and quantity of oil can influence the response of the biosensor.

For the immobilization of the enzyme, the following facts must be taken into account:

1. The type of the enzyme in terms of its stability with the temperature, and its activity;
2. The type of the matrix where the enzyme will be immobilized;
3. The possible types of immobilization.

There are two types of enzyme immobilization:

I. Physical immobilization

II. Chemical immobilization

1.3.1.1. Physical immobilization

The most reliable immobilization of the biological material consists of the physical mixture between the carbon or diamond paste and biological materials [4]. 100 μL of enzyme solution (1 mg enzyme in the optimum buffer of certain pH) are mixed with 100 mg graphite or diamond powder and 40 μL paraffin oil (IR purity). The utilization of

carbon or diamond pastes for physical immobilization of enzyme hasn't only the advantage of a reliable construction of the sensor but also they are ensuring for the enzyme high thermostability. The extreme thermostability in the graphite or diamond paste is attributed to the unusual conformational rigidity in the non-polar binding environment [11].

1.3.1.2. Chemical immobilization

Chemical modified carbon paste was prepared as follows: 0.4 g graphite powder were mixed with 400 µL (0.03 mol/L) solution of 1-ethyl-3-3 (3-diamethylaminopropyl) carbodiimid (EDAC) and heated to 700^{0}C for 60 s in a muffle furnace. After cooling down, to ambient temperature, 10 µL polyethylanimine (PEI) and 10 µL of glutaraldehyde were added. 100 µL solution containing (1mg/mL enzymes or mixture of enzymes) were added to the pre-treated graphite powder. The graphite enzyme mixture was allowed to react at 4^{0}C for 2 h and then it was mixed with 40 µL of paraffin oil to produce the modified pastes.

A covalent bond of the enzyme to matrix will decrease its activity. Usually, after a covalent immobilization, it is necessary to check the activity of the enzyme. Also, this type of immobilization will decrease the sensitivity of the electrode as well as its lifetime. Sometimes, a covalent bond of the enzyme to the matrix is done to a temperature higher than the temperature of the body (37^{0}C) that is the optimum working temperature for most of the enzymes. In this case, knowledge concerning the stability of the enzyme with

the temperature is necessary. Although the decrease in the sensitivity of the sensor as well as of the enzyme activity are also due to the temperature applied for immobilization.

1.3.2. Amperometric immunosensors

The main reaction that is taking place at the membrane-solution interface of the immunosensor is:

$$Ag + Ab \Leftrightarrow [Ag - - - Ab]$$

where Ag is the antigen and Ab is the antibody. The Ab is an extraordinarily selective and versatile reagent. It can be synthesized, as part of the immune defense system in animals, by an organism in reasonable quantity within weeks of injecting of a foreign species called an Ag. Therefore, it is necessary to select the type of the transducer in concordance with the sensitivity of the immunoassay that will take place at the electrode surface. It was demonstrated that the stability of shelf life of the sensor elements could be increased for multianalyte immunoassay by using different transducer elements [12,13]. The reproducibility of the construction of the membrane plays an important role in the reliability of response characteristic of the electrode. It was proved for different types of membrane electrodes that the best reproducibility can be obtained using carbon or diamond paste electrodes [8]. Due to the fact that the quality is directly related to the affinity of the Ab for the target analyte, special attention should be given to the selection of the Ab. Multianalyte sensors based on utilization of antibodies with different cross-reactivities produce response patterns which allow structurally similar analytes to be identified and quantified.

In developing an immunosensor, it is necessary to immobilize an Ab onto the surface of a transducer. This can be carried out by physical (e.g., adsorbtion) and chemical (covalent binding) immobilization. Ab immobilization can significantly change its reactivity. Therefore, careful attention was given to achieve oriented coupling of antibodies on solid supports that can increase the Ag binding capacity [14-16]. Covalent binding leads to a decrease in reactivity.

1.3.2.1. Physical immobilization

For the carbon or diamond paste immunosensor, the graphite or diamond powder was heated to 700^{0}C for 15 min in a muffle furnace and cooled to ambient temperature in desiccators [17]. The paraffin oil and graphite or diamond powder were mixed in a ratio of 1:4 (w/w) followed by addition to the diluted Ab to obtain a final composition of 0.9% (w/w) in Ab. The graphite (diamond)–paraffin oil paste was filled with the modified carbon (diamond) paste that contains Ab. Electric contact was made by inserting a silver wire in the paste. The surface can be renewed by simple polishing with an alumina paper.

1.3.2.2. Chemical immobilization

The design where the Ab is chemically immobilized is applied mainly for the ELISA technique. For chemical immobilization, different supports are proposed, e.g., gold, plastic membranes, conducting composite materials, sol-gel derivatives, etc.

The following procedure is adopted for the immobilization of Ab in gold: the freshly prepared electrodes were immersed for 1 h into solutions of 0.01 mol/L 3,3'-

dithiodipropionic acid bis-(N-hydroxysuccinimide) (DSP, Pierce) in dry dimethyl sulfoxide (DMSO), washed with DMSO and dried in a stream of nitrogen. For the immobilization of Ab, the surface must be covered with 15 μL droplets of buffer, containing the Ab, and incubated for 2 h with the Ag. After incubation, the sensor must be washed with buffer solution.

For Ab immobilization on the surface of a plastic membrane, a freshly prepared solution containing the Ab (10 ng/mL) and 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDAC) (5 mg/mL) was deposited and left for 24 h. After this time, the sensor must be washed in a vigorously stirred buffer to remove the excess of unbound Ab.

When the composite materials are used as immobilization matrices for immunosensors design, the Ab is first mixed with the monomer and graphite to prepare the immunocomposite. The ratio between the monomer and graphite must be 1:1 (w/w). The final proportion of Ab is 0.3 % (w/w). The resulting paste is introduced into a 6 mm i.d. PVC tube, until it makes contact with an electrical connector located at the other end of the tube. After this step, the immunocomposite is cured in a nitrogen atmosphere at room temperature for 3 days.

Sol-gel type immunosensors can be constructed using a one-step sensor fabrication technique, based on the coupling of sol-gel and screen-printing technologies. A low temperature cured ink is prepared by dispersion of Ag, graphite powder, and a binder in the sol-gel solution. The enzyme-labeled Ab can readily diffuse towards the encapsulated

Ag, which retains its binding properties, and the association reaction is easily detected at the dispersed graphite surface.

A high reproducible procedure utilized in immunosensors design is the *in situ* Ag immobilization. The procedure was applied for the first time, for the assay of herbicides, and it was based on an enzymatic single-step *in situ* synthesis of hapten-carrier conjugates on electrodes. Catalyzed by transglutaminase from a variant of *Streptoverticillium mobaraense*, the reaction proceeds in aqueous solution with and without addition of organic media in only 1.5 h.

1.4. Response characteristics of the amperometric biosensors and immunosensors

The functional relation between the intensity of the current, I, measured at a certain potential, E, and concentration of the analyte, c, is given in Figure 1.1:

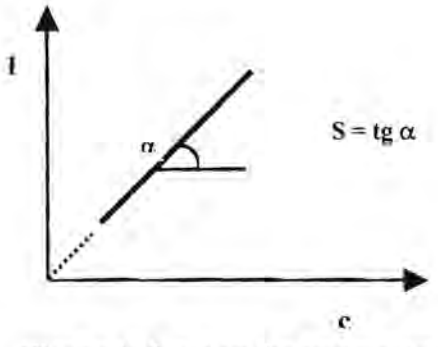

Figure 1.1. Electrode function for amperometric electrodes.

As can be seen from Figure 1.1, it is a linear correlation between the intensity of the current and concentration of the analyte:

$$I=f(c)$$

1.4.1. Slope (Response) of the electrode

The slope is one of the most important response characteristics for amperometric electrodes. A minimum value of 100 nA/decade of concentration is requested for amperometric electrodes to be considered for bioanalysis. The determination of the slope can be done from the graphic method as tangent of the angle formed between the calibration curve and the concentration axis. However the linear regression method is the most recommended for the determination of the slope.

1.4.2. Limit of detection

IUPAC defined the limit of detection as the concentration at which, under specified conditions, the intensity of the current, I, deviates from the average value by a multiple of the standard error of a single measurement of the intensity of the current in this region [18]. For amperometric electrodes an experimental determination of the limit of detection is recommended, rather than a statistical treatment of the experimental data obtained in the calibration of the electrode. In this case, the limit of detection depends on the type of pH of the solution used as blank. The limit of detection can be considered as [19]:

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

(ii) The concentration where the limiting current intensity value is double than the one obtained for the blank solution;

(iii) The concentration below the one where the intensity of the limiting current remains constant.

The amperometric electrodes are very sensitive. Their limits of detection can reach values in the nmol/L-pmol/L magnitude order, or even less. For this reason, they are recommended as transducers in biosensors and immunosensors construction.

1.4.3. Linear concentration range

The linear concentration range represents the range of concentration of a substrate (or ion) over which the sensitivity of the electrode is constant within a specific variation, usually ± 5 %. The linear concentration range can be determined from the plot of current versus the concentration. This response characteristic is very important because all the solutions that had to be measured must have the activity or the concentration of the substances (or ions) within this range. The reproducibility of the linear range is connected with the working conditions of the electrode, like 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 [19].

1.4.4. pH range

The pH plays a very important role in the response of amperometric electrodes. For biosensors and immunosensors, the pH plays the main role in the biochemical reaction. In these cases it is very important to work at a certain pH value and to use a certain composition for the buffer. It is very important for all kinds of electrodes to determine the dependence between intensity of the limited current and the pH. For some of these electrodes a range where the intensity of the limited current does not depend on the pH

was found [20,21,2]. In these situations it is necessary to buffer all the standard and sample solutions at a pH situated within this range. There are electrodes that did not have any range where the intensity of the current is not dependent on the pH. In this case, special care must be accorded to the buffering of each solution, because a small difference on pH will cause a change in the intensity of the current and that will be an error in the measurement.

1.4.5. Ionic strength and activity coefficients

The ionic strength and the activity coefficients are also playing a very important role in the accuracy of the measurements. To avoid the differences in the intensity of the limiting current readings, which can cause another source of error due to the variations of the activity coefficients of the ions in the solution, it is necessary to work at the same ionic strength [22].

1.4.6. Response time

IUPAC defined the response time as the time which elapses between the instant when the electrodes of the amperometric cells are brought into contact with a sample solution and the first instant at which the slope of the working electrodes becomes equal to a limiting value selected on the basis of the experimental conditions and /or requirements concerning the accuracy [18]. For amperometric electrodes, the response time is a function of the kinetics of the processes that takes place at the surface of the electrode. For all types of electrodes the response time increases with the decreasing of the concentration of the ion that has to be determined. In the presence of the interferences,

the response time is modified due to the competitive equilibrium that takes place at the electrode interface. The electrodes with short response time are preferred to be used in bioanalysis, especially if they are used as detectors in flow systems.

1.4.7. Influence of the temperature on the response of the electrodes

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

1.4.8. Lifetime (t_L)

This parameter is very important for biosensors and immunosensors. The mode of assessment of lifetime should be specified, i.e. by reference to initial sensitivity, upper limit of the linear concentration range for the calibration curve, accuracy, or reproducibility. Lifetime can be defined as the storage or operation time necessary for the sensitivity, with the linear concentration range, to decrease by a factor of 10% (t_{L10}) or 50% (t_{L50}) [23]. For the storage lifetime, it is necessary to compare the sensitivities of different biosensors, derived from the same production batch, after different storage times under identical conditions.

1.4.9. Michaelis-Menten constant (KM)

The Michaelis-Menten constant represents the analyte concentration yielding a response equal to half of its maximum value for the current measured for infinite analyte concentration [23]. When the apparent KM is much larger than its value for soluble enzyme, it means either that a significant substrate diffusion barrier is present between the sample and reaction layer, or that the rate of reaction to the co-substrate with the enzyme is increased. As for enzyme solution kinetics the apparent KM is usually determined using line Weaver-Burk reciprocal plots, i.e. $1/\Delta I$ versus $1/c$, where $\Delta I = I - I_b, I$ is the value of the current recorded for the analyte and I_b is the current recorded for the blank solution, and c is the concentration of the analyte.

1.5. Enantioselectivity of the amperometric biosensors and immunosensors

Selectivity is one of the basic characteristics of 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 analyzed, current applied, and pH of the solution. The selectivity can be improved by modifying these parameters or by using a biochemical reaction before the electrochemical detection (e.g., biosensors, immunosensors). The most utilized biochemical reactions are based on enzymes or antigen-antibody (Ag-Ab) pairs. This property of the electrochemical sensors restricts their utilization for the assay of an ion from a complex matrix (e.g., environment). Usually, these electrodes have group selectivity. Their selectivity is high when they 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 current [18]. There are two classes of interfering substances:

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

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

The degree of selectivity of the electrochemical sensors is given by the value of amperometric ($K_{i,j}^{amp}$) selectivity coefficients as follows:

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

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

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

These coefficients defined the ability of the electrodes to distinguish a particular ion from other (interfering ions) [18].

Wang [24] proposed, for the amperometric electrodes, an equation for the correlation of the total current response and amperometric selectivity coefficient:

$$I_t = b\left[c_i + \sum_{j=1}^{N} K_{i,j}^{amp} c_j\right]$$

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

$$b = \frac{n_i A F D_i}{\delta_i}$$

where n_i is the number of electrons transferred per mol of analyte, A is the surface of the electrode (cm^2), F is Faraday number (96500 C), D_i is the diffusion coefficient of the analyte (cm^2 /s), and δ_i is the thickness of diffusion layer (cm). For a biosensors and immunosensors, $K_{i,j}^{amp}$ is a complex function responsible for the recognition of the substrate as well as of the selectivity of transducer over the species formed in the biochemical reaction and the species belonging to the matrix of the sample.

1.5.1. Methods for determining amperometric selectivity coefficients

In the case of amperometric electrodes there are two methods for determination of the amperometric selectivity coefficient: mixed and separate solutions methods. The mixed solution method shows the actual conditions under which the electrode is used and therefore recommended for the determination of the amperometric selectivity coefficients rather the separate solution method.

1.5.1.1. Mixed solution method

The equation for the determination of the selectivity coefficient using this method can be deduced from the equation proposed by Wang [25]. Practically the current recorded from a solution that contains only the main species that has to be analyzed is compared with the total current given by the equation proposed by Wang [25] for a solution containing both the main and the interfering species. The amperometric selectivity coefficient may be calculated from the equation:

$$K_{i,j}^{amp} = \left[\frac{\Delta I_t}{\Delta I_i} - 1\right] \times \frac{c_i}{c_j}$$

where $\Delta I_t = I_t - I_b$ and $\Delta I_i = I_i - I_b$ are the values of the current recorded for the mixed solution and for the solution containing only the main species, and c_i and c_j are the concentrations of the main and interfering species, respectively. I_b is the current recorded for the blank solution. I_t is the current recorded for the mixed solution, and I_i is the current recorded for the solution containing only the main species.

1.5.1.2. Separate solution method

The intensity of the current is recorded for the solution that contains only the main species and separately for the solution containing only the interfering species, and the values obtained are compared with each other. The following equation is proposed to be used for the determination of the amperometric selectivity coefficient [26]:

$$K_{i,j}^{amp} = \frac{\Delta I_j c_i}{\Delta I_i c_j}$$

where $\Delta I_j = I_j - I_b$ and $\Delta I_i = I_i - I_b$, I_j and I_i are values of the current recorded for the interfering and main ion/substance, respectively, and I_b is the current recorded for the blank solution.

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Chapter 2

Sequential injection analysis

2.1. Introduction

Sequential injection analysis (SIA) is an analytical technique that was conceived from flow injection analysis [1,2]. This was due to the demand for mechanically simple and robust systems for process analyses that were able to process a large number of samples in a certain period of time. In contrast to FIA, SIA is based on discontinuous flow and consumes reagents only when the sample is being treated by exploiting a combination of stopped flow, reversed as well as forward flow in the microlitre scale [3].

The sequential injection system is assembled from the following: a pump, a multi position selection valve, a holding coil and appropriate detector (Figure 2.1). The versatility of the sequential injection technique is centered around the selection valve where each port of the valve allows a different operation to be performed. Each port of the valve is dedicated to a specific purpose and the combinations of standards, reagents and detectors around the valve are easily modified to suit particular analysis [4].

The principle of SIA is based on sequential aspiration of a sample zone and reagent zone into a holding coil through a selection valve. The aspiration of zones is achieved by means of a pump, which is capable of controlled stop-go-forward-reverse movement [5].

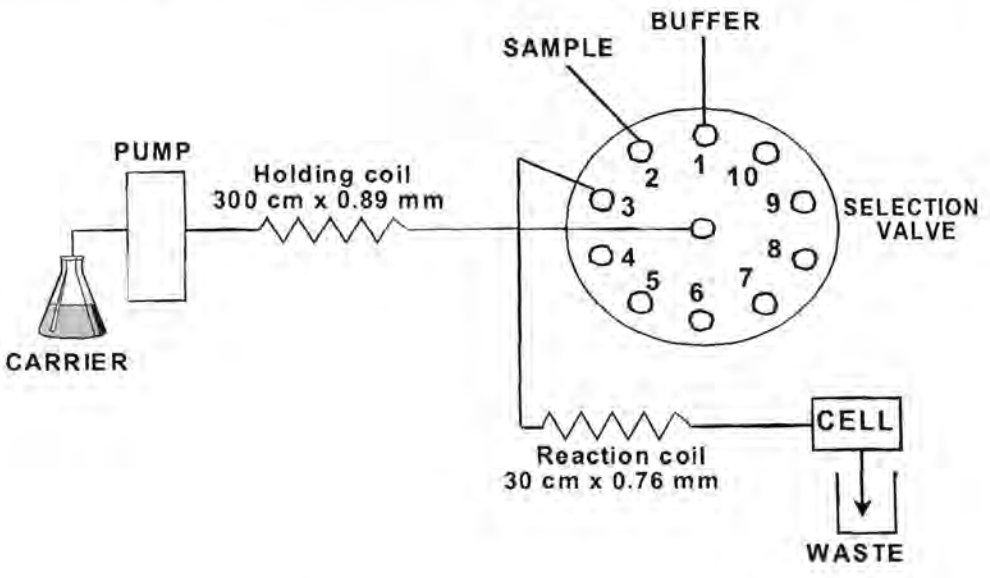


Figure 2.1. Schematic flow diagram of SIA system

A stack of well-defined zones adjacent to each other are formed in the holding coil (Figure 2.2). The selection valve is then switched to the detector position and flow reversal creates a composite zone in which the sample and reagent zone mutually disperse and penetrate each other due to combined axial and radial dispersion.

This creates the formation of a product zone, which is monitored by the detector in the form of a peak whose height or width is related to the concentration of species determined. The reagent is usually in excess to ensure effective mixing [6]. By varying the different operational parameters in a SIA flow conduit, it is possible to control the

amount of dispersion within the system [7]. The order in which sample and reagent zones are stacked is in accordance with the reactions sought. The degree of mixing and length of the reaction time as well as the sample and reagent volumes may be changed without physical re-configuration of flow channel by means of programming the piston and valve movements [8,9]. Zone penetration was found to be a key parameter in a SIA system [7]. Its importance is ascribed to the fact that this influence has a dramatic impact on the surface area over which a concentration gradient exists and therefore over which axial mixing takes place [10,11].

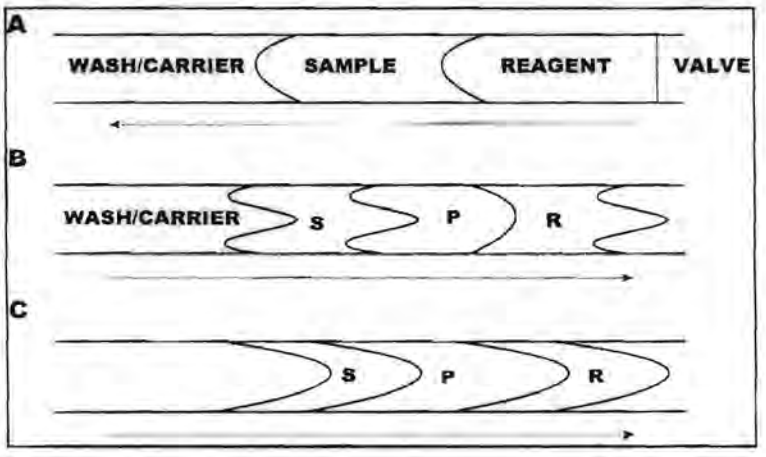


Figure 2.2. Principles of sequential injection analysis. Profile zones after injection (A), immediately after flow reversal (B) and in reaction coil (C) S-sample R-reagent and P-formed product.

The holding coil is considered the heart of the system as it substitute the mixing chamber and its volume could be adjusted by changing the length and diameter as required. A high reproducible flow pattern is obtained by computer control of the SIA system because an

analytical procedure often requires a complex and high reproducible pattern. This technique can employ any detector capable to operate successfully in a flowing stream. SIA uses a single channel pump to move the liquid in reverse and forward steps [12], as compared to a multichannel pump in FIA. Peristaltic pumps are preferred over syringe and sinusoidal flow types. The advantages of peristaltic pumps are: (i) that the configuration is easier and simpler to design, initiate and operate; (ii) they are widely available and easy to handle and (iii) the sampling cycle is considered shorter as there is no need for the aspiration of a wash solution. Syringe pumps on the other hand offer a pulse-less and highly precise flow but they require priming before use, and have a limited reservoir volume and are relatively expensive. The technique of SIA has the advantages of reduced reagent consumption and easy and fast change of solutions.

2.2. The role of amperometric biosensors and immunosensors as detectors in flow systems

Amperometric biosensors and immunosensors can be easily incorporated in the conduits of a flow system (FIA, SIA) to form a simple, low-cost analyzer [13-15]. The hydrodynamic conditions can be controlled in such a way that a very reliable electrochemical sensor/flow system is obtained. The use of amperometric biosensors and immunosensors in flow systems has advantages also for amperometric detection itself [16], because:

(i) As the sample being in contact with the electrode surface moving continuously, the electrode cannot influence the sample concentration and

furthermore, the electrode surface is renewed by "on-line polishing" with the carrier;

(ii) As the diffusion layer of small thickness is established at the electrode surface, the linear concentration range, the sensitivity, the limit of detection, and the response time of the indicator are improved;

(iii) The reference electrode usually is placed down-stream in respect to the indicator one, so the ionic species flowing out of the reference electrode cannot influence the response of the indicator electrode;

(iv) Reference electrodes with flowing inner solution can easily be employed to overcome problems arising from the alteration of liquid junction potential;

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

Among amperometric biosensors and immunosensors parameters, their dynamic response characteristics are very important for flow systems. Therefore, efforts have been done devoted in developing appropriate measuring techniques in order to study their dynamic response properties [17]. Amperometric biosensors and immunosensors were used as detectors in FIA and SIA. The advantages of using SIA in the place of FIA are to decrease the consumption of samples and buffers, a cheap electrolyte (e.g., NaCl 0.1-1 mol/L solution) can be used as carrier, lower cost, and increasing the precision, accuracy, and reliability of the analysis.

2.3. Designs of SIA/electrodes systems for the assay of enantiomers

The sequential injection system (SIA) used for the assay of enantiomers, is illustrated in Figure 2.1. The system is constructed from:

1. A Gilson Minpuls peristaltic pump;
2. A ten-port electrically actuated selection valve (Model ECSD10P, Valco Instruments, Houston Texas).
3. Flow detection cell.

Data acquisition and device control are achieved using a PC 30-B interface board (Eagle Electric Cape Town, South Africa). The FlowTek software package (obtained from Mintek) was used throughout for device control and data acquisition [18]. Tygon tubing were used for the holding coils (HC) and reaction coils (RC).

2.3.1. Optimization of flow system

It is necessary to optimize a number of physical and chemical parameters to obtain the highest sensitivity and precision. Accordingly, van Staden [19] evaluated different SIA sample-buffer configurations (sample-buffer, buffer-sample, sample-buffer-sample, and buffer-sample-buffer, the last two being in the sandwich mode) that can be used with electrochemical sensor as detection system. The peak profiles of these SIA configurations were evaluated using a conventional fluoride selective membrane electrode. The best response characteristics and peak shapes as well as recovery and precision values were obtained for the buffer sample configuration. For very low concentration levels, sandwich SIA configurations are more suitable, when optimum buffer and pH are used. The

original optimization was done using the configuration where the aspiration order into a SIA holding coil was buffer-sample. The optimum conditions where applied to all the other configuration [19].

2.3.1.1. Flow rate

The influence of the flow rate on sensitivity and precision was evaluated. A total sample of 270 µL was used during these evaluations. The volume was adjusted to remain constant for every flow rate studied. This resulted in longer times for lower flow rates and shorter times for higher flow rate. The optimum flow rate was chosen to be 3.61 mL/min [19].

2.3.1.2. Sample volume

It is important to optimize this parameter to ensure that effective mixing with the buffer solutions was obtained. Different sample volumes were investigated and a sample volume of 270 µL was selected for optimum working conditions [19].

2.3.1.3. Buffer volume

Different volumes of buffer solution were aspirated into the SIA system to evaluate the volume of the buffer solution. The optimum volume is 300 µL [19].

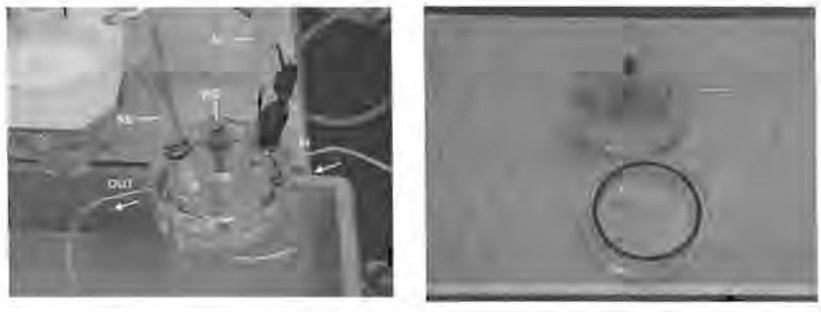
2.3.1.4. Diameter and length of tubing

i. **Holding coil.** The holding coil serves as a holding reservoir that prevents the stack of zones from entering the conduit of the pumping tube in the peristaltic

pump where deformation could take place. The length and diameter of the holding coil did not have a large influence and 3.5 m (1.6 mm i.d.) Tygon tubing was used [19].

ii. **Reaction coil.** The length and diameter of reaction coil where optimized using different lengths and diameters of Tygon tubing. The optimum length and diameter for the reaction coil were found to be 0.3 m and 0.89 mm i.d., respectively [19].

Improving of the quality of the analyses when a certain electrochemical sensor has to be used in flow system can be done not only through the optimization of the parameters of the flow system, but also through optimization of the size and geometry of the flow cell. In this regard, Stefan [20] proposed a new cell to be used in flow systems (Figure 2.3).



(A) (B)

Figure 2.3. The electrochemical cell for FIA and SIA measurements. (A) Complete cell during working runs. WE, working electrode; RE, reference electrode; AE, auxiliary electrode. (B) View of top and bottom parts of the cell [20].

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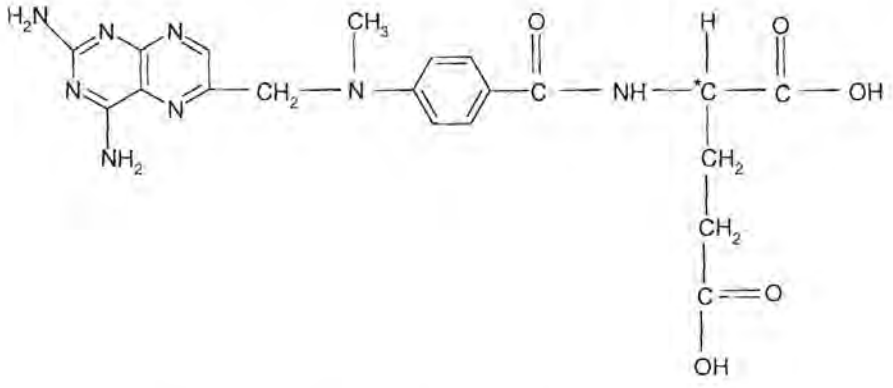
Chapter 3

On-line enantioselective analysis of methotrexate

3.1. Introduction

Methotrexate (Mtx) is a compound with a glutamate moiety. L-Mtx, N- [4-[[2,4-Diamino-6-pteridinyl) methyl] methylamino]-benzoyl] L-glutamic acid (Scheme 1) is an antifolate drug that shows significant antitumor activity in acute leukemia and other neoplastic diseases. It functions by interfering with the growth of cancer cells.



Scheme 1. The structure of methotrexate

These cells are then eventually destroyed. L-Mtx inhibits the enzyme dihydrofolate reductase causing a lack of reduced folates, which are donors of one-carbon units in the biosynthesis of nucleic acids and some amino acids. Like the folates, Mtx is converted intracellularly to polyglutamates, which inhibits further enzymes of the folate metabolism and thus takes part in the cytostatic action of the drug [1,2]. D-Mtx

is the antipode, and its pharmacokinetic behavior is significantly different from that of L-Mtx [2,3]. Although D-Mtx exhibits similar inhibitory effect to dehydrofolate as L-Mtx, vet D-Mtx is much less toxic (40-fold lower LD_{50}) and its antitumor effect is proportionately reduced [2].

Many analytical methods have been reported to analyse Mtx in its pharmaceutical formulations and in biological fluids by using chromatographic techniques, e.g., HPLC [4-10] and capillary zone electrophoresis [11], and spectrometric techniques, e.g., chemiluminscence [12] and fluorescence [13], yet only few methods had been reported discriminating between L- and D-enantiomers of Mtx [11].

Among the electrochemical biosensors, amperometric biosensors represent the best combination of sensitivity and selectivity. Amperometric biosensors are capable of direct sensing without additional reagents. Thus the sensors could be used when a fast analysis or a continuous *in situ* monitoring of analyte concentrations are required [14].

Ten amperometric biosensors based on different enzymes are proposed for the assay of L-Mtx and D-Mtx. Physical and chemical immobilization techniques were used for the sensors design: glutamate oxidase (Glox) based biosensor was used to determine L-Mtx by direct electrochemical reduction/oxidation of the co-substrate (O_2/or formed H_2O_2). The detection principle of the biosensor modified with Glox is usually based on the amperometric detection of enzymatically produced H_2O_2 [15]. L-amino acid oxidase (L-AAOD) and D-amino acid oxidase (D-AAOD) is catalyzing only the conversion of the L- or D-enantiomer of the amino acid into their keto- acids and the hydrogen peroxide formed is measured by the amperometric transducer [16].

Horseradish peroxidase (HRP) is usually accompanied by Glox, L-amino acid or D-AAOD in the design of bi- or trienzyme sensors, and it is used in order to improve the reduction of H_2O_2 formed from the enzymatic reaction [17]. The reliability of biosensor construction is influencing the reliability of the analytical information and it will also contribute to the validation of the biosensor for pharmaceutical analysis.

3.2. Experimental section

3.2.1. Reagents and materials

Graphite powder, 1-2 μ, synthetic was supplied by Aldrich (Milwaukee, WI, USA). Paraffin oil was supplied by Fluka (Buchs, Switzerland); phosphate buffer (pH =7.00) was supplied by Merck (Darmstadt, Germany). De-ionized water from a Modulab system (Continental water systems, San Antonio, TX, USA) was used for all solution preparations: L-amino acid oxidase (L-AAOD) (E.C.1.4.3.2.Type I crude Dried Venom from Crotalus adamanteus, Sigma, St. Louis, MO) solution, L-Glox (L-Glutamate: oxygen oxido reductase (deaminating) (E.C.1.4.3.11 from steptomyces sp. Sigma) solution, D-amino acid oxidase (D-AAOD) (E.C.1.4.3.3.Type I: from porcine kidney Sigma) solution, horseradish peroxidase (HRP) (EC 1.11.1.7 Type I from Horseradish) solution, polyethylenimine (PEI) [(9002-98-6) (50%w/v) aqueous solution, (Sigma)], glutaraldehyde [EC 203-856-5(111-30-8) grade I 8% aqueous solution, (Sigma), 1-ethyl-3-3(3-dimethylaminopropyl)carbodiimid (EDAC) (EC 247-361-2). The L- and D-methotrexate (L (D) amethopterin hydrated, 98%) were supplied by Aldrich (Milwaukee, WI, USA). Solutions of L- and D-Mtx (10^{-4} mol/L), respectively, were also prepared in de-ionized water.

Methotrexate tablets (2.5 mg Mtx/tablet) were supplied by David Bull Laboratories, Warwick, UK. Methotrexate LPF® Sodium injections were supplied by Immunex Corporation, Seattle, WA, USA.

3.2.2. Amperometric biosensors design

Ten plastic tips were filled with plane carbon paste leaving an empty space of 3-4 mm in the top part to be filled with carbon paste containing the different enzyme mixtures as shown below. The diameters of all biosensors were 3 mm. Electric contacts were obtained by inserting silver wires into the carbon paste. The biosensors tips were gently rubbed on fine abrasive paper to produce a flat surface. The surface of the biosensors were wetted with de-ionized water and then polished with an alumina paper (polished strips 30144-001, Orion) before use. The biosensors were stored dry at 4^0C, when not in use.

3.2.2.1. Physical immobilization

Mono-, bi- and trienzyme electrodes were constructed. All the enzyme solutions used for the biosensors design were prepared in 0.1 mol/L phosphate buffer of pH=7.00.

3.2.2.1.1. Monoenzyme amperometric biosensors

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

3.2.2.1.2. Bienzyme amperometric biosensors

Four different mixtures of enzymes were used for the design of the amperometric biosensors: (1) 1 mg of HRP was dissolved in 50 µL of Glox (0.25 mg/mL); (2) 25 µL of Glox solution (0.125 mg/mL) was mixed with 25 µL of L-AAOD solution (0.125 mg/mL); (3) 1 mg of HRP was dissolved in 50 µL of L-AAOD solution (0.25 mg/mL); (4) 1mg of HRP was dissolved in 50 µL of D-AAOD solution (0.25 mg/mL). Each mixture was incorporated in the carbon paste (100 mg graphite powder and 40 µL paraffin oil), to obtain four bienzyme electrodes.

3.2.2.1.3. Trienzyme amperometric biosensors

Paraffin oil and graphite powder was mixed in the ratio 1:4 (w/w) to form a carbon paste. 25 µL of Glox solution (0.125 mg/mL) was first mixed with 25 µL of L-AAOD (0.125 mg/mL) solution and then with 50 µL of HRP (0.5 mg/mL) solution. The resulting solution was added to the carbon paste.

3.2.2.2. Chemical immobilization

Two electrodes based on chemical modified carbon paste were prepared as follows: 0.4 g graphite powder were mixed with 400 µL (0.03 mol/L) solution of EDAC and heated to 700^{0}C for 60 s in a muffle furnace. After cooling down, to ambient temperature, 10 µL PEI and 10 µL of glutaraldehyde were added. 100 µL solution containing 1 mg/mL of HRP and 0.25 mg/mL of Glox were added to the pre-treated graphite powder for the determination of L-Mtx, and 100 µL solution containing 4 mg/mL of HRP and 1 mg/mL of D-AAOD was added to the respective pre-treated graphite powder for the determination of D-Mtx. Each graphite enzyme mixture was

allowed to react at 4^0C for 2 h and then it was mixed with 40 µL of paraffin oil to produce the modified pastes.

3.2.3. Apparatus

A663VA Stand (Metrohm, Herisau, Switzerland) in combination with a µAutolab and software (Ecochemie version 4.8) were used for all chronoamperometric measurements. A Pt electrode and an Ag/AgCl electrode served as the counter and reference electrodes in the cell.

3.2.4. Recommended procedures

3.2.4.1. Direct amperometry

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

3.2.4.2. Uniformity content test for Methotrexate tablets and Methotrexate injections

Ten methotrexate tablets (2.5 mg Mtx/tablet) were individually placed in ten 100 mL volumetric flasks, and dissolved in deionized water. 20 µL solution of a Methotrexate injection (25 mg Mtx/mL injection) was poured into a 100 mL calibrated flask and then diluted to the mark with de-ionized water. Different aliquots from the solution prepared from tablets and injection were added to phosphate buffer (pH = 7.00) in the

electrochemical cell. Direct amperometry was used to determine the unknown concentration of L- and D-Mtx in the pharmaceutical formulations.

3.3. Results and discussion

3.3.1. Response characteristics of the amperometric biosensors

The response characteristics of the electrodes were measured at different potentials in order to determine the best working potential (higher sensitivity, lower limit of detection, shorter response time, etc.) for the determination of L- and D-Mtx (Tables 3.1 and 3.2). The electrode response was highly stable and reproducible over one week. The best response characteristics (larger concentration range, lower limit of detection and highest sensitivity) for the assay of L-Mtx and D-Mtx were obtained at 650 mV using the Glox (monoenzyme) based biosensor and physical immobilization of D-AAOD and HRP based biosensor, respectively. The faster response time was recorded for the biosensors based on L-AAOD and D-AAOD and HRP, for the assay of L- and D-Mtx, when the measurement of the intensity of the current was done at 650 mV respectively.

Table 3.1. Response characteristics for the amperometric biosensors designed for L-Mtx

Enzyme(s) used for the design of the biosensor	E (mV)	Linear conc. range	Detection limit	Response time (s)
Glox	150	40-100 pmol/L	2 pmol/L	180
	650	80-1000 fmol/L	4 fmol/L	60
Glox +HRP	250	1-4 nmol/L	100 pmol/L	120
	650	40-400 pmol/L	8 pmol/L	120
	740	6-10 nmol/L	4 nmol/L	300
L-AAOD	650	100-600 nmol/L	80 nmol/l	30
L-AAOD + HRP	650	400-4000 pmol/L	200 pmol/l	120
L-AAOD +Glox	360	200-600 pmol/L	80 pmol/L	240
	650	400-2000 fmol/L	200 fmol/L	120
L-AAOD + Glox + HRP	240	800-2000 fmol/L	200 fmol/L	300
	650	60-100 pmol/L	40 pmol/L	120
Glox + HRP *	650	40-100 pmol/L	20 pmol/L	180

*Chemical immobilization.

Table 3.2. Response characteristics for the amperometric biosensors designed for D-Mtx

Enzyme(s) used for the design of the biosensor	E (mV)	Linear conc. range	Detection limit	Response time (s)
D-AAOD	12	40-80 fmol/L	10 fmol/L	180
	650	4-40 nmol/L	100 pmol/L	60
D-AAOD +HRP	530	1-10 nmol/L	800 pmol/L	60
	650	600-4000 pmol/L	2 pmol/L	30
D-AAOD + HRP*	650	80-600 nmol/L	60 nmol/L	180

*Chemical immobilization.

3.3.2. Enantioselectivity of the amperometric biosensors

The selectivity of all biosensors was checked by both separate and mixed solution method with respect to L- and D- Mtx. Amperometric selectivity coefficients were determined following the method proposed by Wang [18], for the same potentials used for the determination of the response characteristics of the proposed amperometric biosensors. The ratio between the concentration of the main enantiomer and the other enantiomer was 1:10. The values of the amperometric selectivity coefficients (obtained using the mixed solution method) for the biosensors designed for L- and D-Mtx, respectively, are shown in Tables 3.3 and 3.4.

The $pK_{i,j}^{amp}$ values show that the biosensor based on L-AAOD, Glox and HRP enzymes has got the best enantioselectivity for L-Mtx assay, when the measurements are performed at 240mV, and that the biosensor based on physical immobilization of D-AAOD and HRP has got the best enantioselectivity for D-Mtx assay, when the measurements are performed at 650 mV.

Table 3.3. Enantioselectivity of the amperometric biosensors designed for the assay of L-Mtx

Enzyme(s) used for the design of the biosensor	**E (mV)**	**$pK_{i,j}^{amp}$**
Glox	150	2.14
	650	2.29
Glox +HRP	250	2.24
	650	2.10
	740	2.12
L-AAOD	650	2.12
L-AAOD +HRP	650	2.86
L-AAOD +Glox	360	2.18
	650	2.21
L-AAOD +HRP +Glox	240	3.09
	650	2.03
Glox +HRP*	650	2.07

*Chemical immobilization.

Table 3.4. Enantioselectivity of the amperometric biosensors designed for the assay of D-Mtx

Enzyme(s) used for the design of the biosensor	**E (mV)**	**$pK_{i,j}^{amp}$**
D-AAOD	12	2.61
	650	2.35
D-AAOD +HRP	530	2.04
	650	2.91
D-AAOD +HRP *	650	2.26

*Chemical immobilization.

3.3.3. Analytical applications

The amperometric biosensors proved useful for determination of the enantiopurity of L-Mtx raw materials and for testing the content of uniformity of Methotrexate tablets and Methotrexate injections due to the results obtained for the recovery tests of the pure L- and D-enantiomer of Mtx: higher than 99.90% (RSD < 0.20%, n=10) and higher than 99.78% (RSD < 0.30%, n=10), respectively for all biosensors proposed in this chapter. The determinations of L-Mtx and D-Mtx were conducted by use of different ratio of L- to D-Mtx. The results obtained (Tables 3.5 and 3.6) demonstrated the suitability of the proposed amperometric biosensors electrodes for testing the

enantiopurity of Mtx - raw material due to the good recovery values obtained for the assay of L-enantiomer in the presence of D-enantiomer and of D-enantiomer in the presence of L-enantiomer. No differences in the recovery tests were recorded for L: D or D: L ratios between 1:9 and 1:99.9.

Table 3.5. Determination of L-Mtx in the presence of D-Mtx

Enzyme(s) used for the design of the biosensor	**E (mV)**	**Average recovery, (%)**				
		L: D				
		2:1	1:1	1:2	1:4	1:9
Glox	150	99.99	99.98	99.95	99.97	99.98
	650	99.49	99.47	99.82	99.82	99.81
Glox +HRP	250	95.91	96.04	95.82	95.91	95.90
	650	99.35	99.35	99.35	99.68	99.70
	740	94.13	97.16	98.09	96.25	96.47
L-AAOD	650	99.25	99.12	99.32	99.28	99.35
L-AAOD +HRP	650	99.60	99.40	99.42	99.46	99.45
L-AAOD+ Glox	360	95.18	95.31	96.84	98.10	98.08
	650	100.00	100.00	99.98	99.98	99.98
L-AAOD +HRP +Glox	240	99.92	99.98	99.96	99.95	99.96
	650	99.90	99.92	99.91	99.89	99.93
Glox +HRP *	650	93.15	94.50	94.50	93.20	96.27

*Chemical immobilization.
All values are the average of ten determinations and the RSD values were less than 0.25%.

Table 3.6. Determination of D-Mtx in the presence of L-Mtx

Enzyme(s) used for the design of the biosensor	**E (mV)**	**Average recovery, (%)**				
		D: L				
		2:1	1:1	1:2	1:4	1:9
D-AAOD	12	90.68	90.70	90.68	91.00	90.73
	650	98.85	99.00	98.93	98.80	98.81
D-AAOD +HRP	530	99.82	99.82	99.82	99.80	99.84
	650	99.98	99.96	100.00	99.97	99.97
D-AAOD + HRP *	650	92.08	91.27	91.66	92.00	91.98

*Chemical immobilization.
All values are the average of ten determinations and the RSD values were less than 0.30%.

The results obtained for the uniformity content test are presented in Tables 3.7, 3.8, 3.9 and 3.10 for Methotrexate tablets and Methotrexate injections, respectively. The uniformity content tests show that the tested pharmaceutical formulations contain as

main component the L-enantiomer of Mtx and only small amounts of the D-enantiomer of Mtx.

Table 3.7. Determination of L-Mtx in methotrexate tablets (2.5 mg Mtx/tablets)

Enzyme(s) used for the design of the biosensor	**E (mV)**	**Average recovery of L-Mtx, (%)**
Glox	150	95.14 ± 1.61
	650	95.31 ± 1.71
Glox +HRP	250	95.05 ± 1.59
	650	95.14 ± 1.64
	740	95.07 ± 1.58
L-AAOD	650	95.10 ± 1.63
L-AAOD +HRP	650	95.10 ± 1.64
L-AAOD +Glox	360	95.11 ± 1.62
	650	94.99 ± 1.66
L-AAOD +Glox +HRP	240	95.18 ± 1.60
	650	95.13 ± 1.58
Glox +HRP *	650	95.06 ± 1.72

*Chemical immobilization.
All values are the average of ten determinations.

Table 3.8. Determination of D-Mtx in Methotrexate tablets (2.5 mg Mtx/tablets)

Enzyme(s) used for the design of the biosensor	**E (mV)**	**Recovery of L-Mtx, (%)**
D-AAOD	12	3.71 ± 1.75
	650	3.95 ± 1.71
D-AAOD +HRP	530	3.82 ± 1.74
	650	3.81 ± 1.77
D-AAOD +HRP *	650	4.01 ± 1.79

* Chemical immobilization.
All values are the average of ten determinations.

The recovery values for the L-Mtx – which is the active component of the Methotrexate tablets and injections, are within the labeled amount of 90-110%, with RSD values less than 6.00% required by the USP XXV [19].

Table 3.9. Determination of L-Mtx in the methotrexate injection (25 mg Mtx/mL injection).

Enzyme(s) used for the design of the biosensor	E (mV)	Recovery of L-Mtx,(%)
Glox	150	99.04 ± 0.20
	650	98.02 ± 0.18
Glox +HRP	250	99.09 ± 0.17
	650	97.92 ± 0.17
	740	98.40 ± 0.19
L-AAOD	650	98.10 ± 0.19
L-AAOD +HRP	650	98.00 ± 0.18
L-AAOD +Glox	360	98.97 ± 0.15
	650	98.14 ± 0.14
L-AAOD +Glox +HRP	240	98.67 ± 0.17
	650	98.00 ± 0.18
Glox +HRP *	650	98.03 ± 0.19

* Chemical immobilization.
All values are the average of ten determinations.

Table 3.10. Determination of D-Mtx in Methotrexate injection (25 mg Mtx/mL injection).

Enzyme(s) used for the design of the biosensor	E (mV)	Recovery of D-Mtx, (%)
D-AAOD	12	0.92 ± 0.23
	650	0.99 ± 0.20
D-AAOD +HRP	530	0.89 ± 0.21
	650	1.00 ± 0.14
D-AAOD +HRP *	650	0.87 ± 0.18

* Chemical immobilization.
All values are the average of ten determinations.

The amperometric biosensors electrodes described have excellent features in enantioselective analysis. The construction of the electrodes is simple, fast, and reproducible, and it is also assuring reliable response characteristics for the proposed amperometric biosensors. The enantioselectivity of the described biosensor made possible the performance of the enantiopurity assay of L-Mtx as raw material and from its pharmaceutical formulations. The selection of the working potential in the assay of the enantiomers of L- and D-Mtx proved to have a high effect on the

performances of the amperometric biosensors, in terms of sensitivity, limit of detection, linear concentration range, response time and enantioselectivity.

3.4. Sequential injection analysis system for on-line simultaneous assay of enantiomers of methotrexate

3.4.1. Sequential injection system

The biosensors were incorporated into the conduits of a SIA system (Figure 3.1A) constructed from a Gilson Minipuls peristaltic pump and a 10-port electrically actuated selection valve (Model ECSD10P, Valco Instruments, Houston, TX).

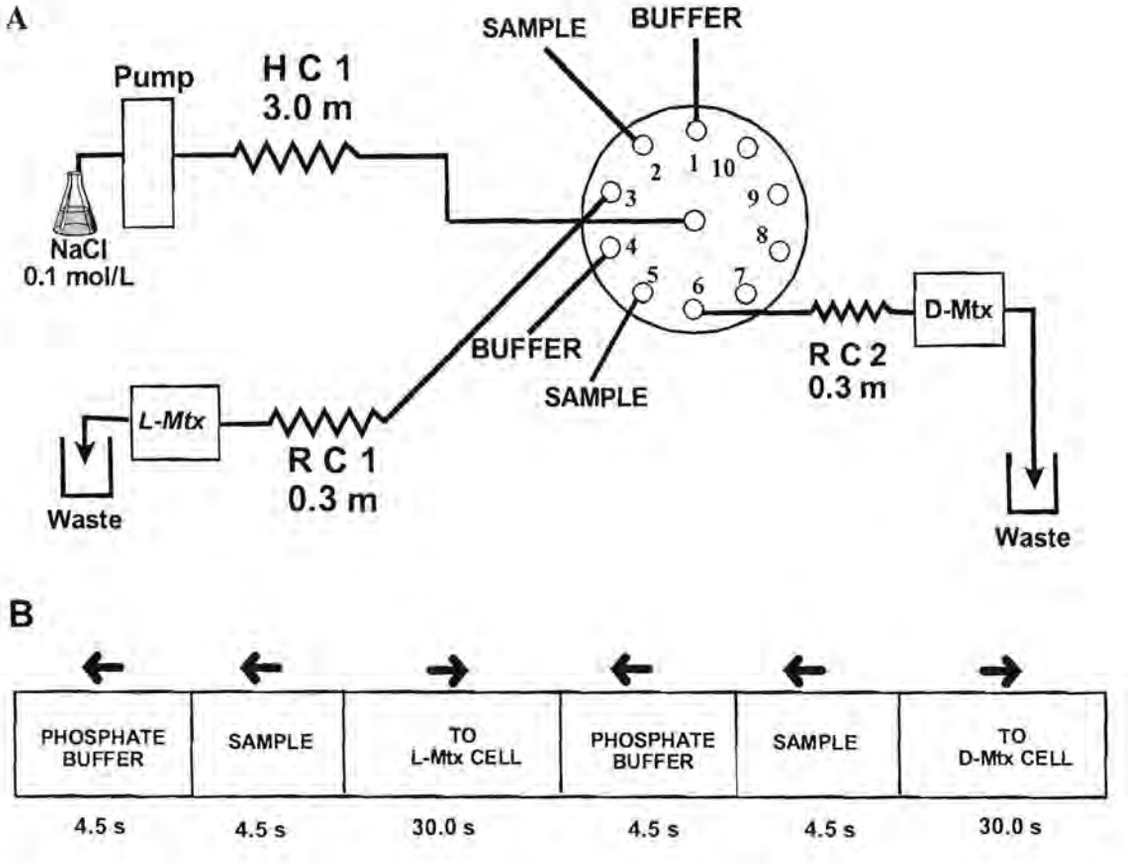


Figure 3.1. SIA system used for the simultaneous determination of L- and D-methotrexate (A) Schematic flow diagram (B) sequence of sample, phosphate buffer, and electrochemical cells for L-Mtx and D-Mtx.

Tygon tubing (0.76 mm i.d. for both holding coils and 0.89 mm i.d for both mixing coils) was used to construct the manifold; coils were wound round suitable lengths of glass tubing (15 mm o.d.); 0.1 mol/L NaCl was used as carrier. The capacity of the system is about 34 samples per hour. The device operating sequence is shown in Table 3.11. The device control was achieved using a PC30-B interface board (Eagle Electric, Cape Town, South Africa). The FlowTEK [20] software package (obtained from MINTEK) for computer-aided flow analysis was used though out for device control.

Table 3.11. Device sequence for one cycle of the SIA system

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

An optimum flow rate of 3.61 mL/min was used to propel the solutions. The timing and flow direction is shown in Figure 3.1B. The sample and buffer consumption is

only 270 µL each per measurement of L- and D-enantiomer, which is very economical.

3.4.2. Response characteristics of the amperometric biosensors as detectors in SIA system

The working concentration ranges as well as the limits of detection demonstrated the suitability of the proposed amperometric biosensors for the on-line monitoring of both enantiomers (Table 3.12). The response obtained for all biosensors revealed good stability and reproducibility for tests performed over one week.

Table 3.12. Response characteristics for the amperometric biosensors designed for L- or D-Mtx when the biosensors are used as detectors in SIA system

Enzyme(s) used for the design of the biosensor	E(mV)	Linear conc. Range	Detection limit	Equation of calibration**	Correlation coefficient (r)
Glox	150	2-400 pmol/L	1 pmol/L	^{1,a} H=38.77 +2.66C	0.9999
	650	10-2000 fmol/L	0.6 fmol/L	^{1,b} H=4.41+118.84C	0.9994
L-AAOD	650	10-600 nmol/L	4 nmol/L	^{1,c} H=13.14 +0.91C	0.9921
L-AAOD + HRP	650	100-2000 pmol/L	6 pmol/L	^{2,a} H=0.10 +1.06C	0.9999
L-AAOD +Glox	360	100-8000 pmol/L	40 pmol/L	^{1,a} H=3.12+34.37C	0.9999
	650	40-1000 fmol/L	8 fmol/L	^{1,b} H=0.89 + 90.51C	0.9999
L-AAOD + Glox + HRP	240	60-1000 fmol/L	8 fmol/L	^{1,b} H=7.11 +702.22C	0.9999
	650	0.4-100 pmol/L	200 fmol/L	^{1,a} H=5.59 +25.23C	0.9999
Glox + HRP*	650	6-800 pmol/L	1 pmol/L	^{2,a} H=0.02 +0.03C	0.9999
D-AAOD	650	0.08-80 nmol/L	40 pmol/L	^{1,c} H=3.25 +6.19C	0.9997
D-AAOD+HRP*	650	10-1000 nmol/L	200 pmol/L	^{2,c} H=0.06 +0.02C	0.9999

*Chemical immobilization

**H is the peak height in ^{1}nA and ^{2}µA, and C is the concentration of L- and D-Mtx, respectively, in ^{a}pmol/L, ^{b}fmol/L and ^{c}nmol/L.

3.4.3. Selectivity of the biosensors as detectors in SIA system

The selectivity of all biosensors were checked using both the mixed and separate solutions methods with respect to L- and D-Mtx. Amperometric selectivity coefficient were determined following the method proposed by Wang [18] for the same potential used for the determination of the response characteristics of the proposed

amperometric biosensors. In the evaluation, the concentration of the interferent was selected to be ten times higher than that for the enantiomer of interest. The values for $pK_{i,j}^{amp}$ obtained using mixed solutions method are shown in Table 3.13. Accordingly, the proposed biosensors are enantioselective when used as detectors in a SIA system. Furthermore, at 650 mV, the biosensor based on L-AAOD, Glox and HRP enzymes has got the best enantioselectivity for L-Mtx assay, and that the biosensor based on D-AAOD has got the best enantioselectivity for D-Mtx assay.

Table 3.13. Selectivity coefficients, pK_{amp} for amperometric biosensors as detectors in SIA systems

Enzyme(s) used for the design of the biosensor	**Interfering species (J)**	**E (mV)**	**$pK_{i,j}^{amp}$ ****
Glox	D-Mtx	150	2.20
	D-Mtx	650	2.51
L-AAOD	D-Mtx	650	2.10
L-AAOD +HRP	D-Mtx	650	2.06
L-AAOD +Glox	D-Mtx	360	2.19
	D-Mtx	650	2.01
L-AAOD +HRP +Glox	D-Mtx	240	2.06
	D-Mtx	650	3.05
Glox +HRP*	D-Mtx	650	2.03
D-AAOD	L-Mtx	650	3.09
D-AAOD +HRP*	L-Mtx	650	2.55

*Chemical immobilization
**n=10

3.4.4. Analytical applications of SIA/amperometric biosensors system

The flow systems obtained by incorporation of the amperometric biosensors in the SIA conduits, proved to be useful for on-line simultaneous assay of L-and D-Mtx. The results obtained for the recovery of one of the enantiomers in the presence of its antipode (Tables 3.14 and 3.15) demonstrated the suitability of the proposed flow system for on-line purity tests of Mtx-raw material. No differences were recorded in

the recovery (%) of the enantiomers between 1:9 and 1:99 in a favor of each enantiomer.

Table 3.14. Recovery of L-Mtx in the presence of D-Mtx using SIA/amperometric biosensors system

Enzyme(s) used for the design of the biosensor	E (mV)	Recovery L-Mtx, (%)** L: D				
		2:1	1:1	1:2	1:4	1:9
Glox	150	99.85	99.87	99.90	99.89	99.90
	650	99.85	99.99	99.90	99.45	99.46
L-AAOD	650	99.58	99.60	99.51	99.58	99.28
L-AAOD +HRP	650	99.95	99.98	99.97	99.98	99.98
L-AAOD+ Glox	360	99.20	100.02	99.25	99.30	99.30
	650	100.00	99.95	99.97	99.96	99.97
L-AAOD +HRP +Glox	240	99.88	99.85	99.90	99.87	99.88
	650	99.09	99.26	99.51	99.64	99.65
Glox +HRP*	650	99.98	99.92	99.98	99.95	99.95

*Chemical immobilization; **n=10

Table 3.15. Recovery of D-Mtx in the presence of L-Mtx using SIA/amperometric biosensors system

Enzyme(s) used for the design of the biosensor	E (mV)	Recovery D-Mtx, (%)** D: L				
		2:1	1:1	1:2	1:4	1:9
D-AAOD	650	99.85	99.90	99.92	99.93	99.92
D-AAOD +HRP *	650	99.75	99.80	99.82	99.87	99.88

*Chemical immobilization
**n=10

Uniformity content tests were performed for Methotrexate tablets and Methotrexate injections. Ten Methotrexate tablets (2.5 mg Mtx/tablet) were individually placed in ten 100 mL volumetric flasks, and dissolved in de-ionized water. 20 µL solution of a Methotrexate injection (25 mg Mtx/mL injection) was poured into a 100 mL calibrated flask and then diluted to the mark with de-ionized water. The solution prepared from tablets and injection were diluted with de-ionized water and the height of the peak was recorded using the proposed SIA/amperometric biosensors system. The results were interpolated into the calibration graph of the corresponding

electrode, in order to determine the concentration of each enantiomer, and furthermore, it's content in the corresponding pharmaceutical formulation.

Table 3.16. Determination of L- and D-Mtx in Methotrexate tablets (2.5 mg Mtx/tablet) using SIA/amperometric biosensors system

Enzyme(s) used for the design of the biosensor	E (mV)	Recovery of L-Mtx, (%)**	Recovery of D-Mtx, (%)**
Glox	150	95.20 ± 1.69	-
	650	95.26 ± 1.58	-
L-AAOD	650	95.39 ± 1.75	-
L-AAOD +HRP	650	95.31 ± 1.60	-
L-AAOD +Glox	360	95.36 ± 1.61	-
	650	95.15 ± 1.70	-
L-AAOD +Glox +HRP	240	95.20 ± 1.75	-
	650	95.20 ± 1.69	-
Glox +HRP*	650	95.43 ± 1.44	-
D-AAOD	650	-	3.92 ± 1.87
D-AAOD +HRP*	650	-	3.91 ± 1.83

*Chemical immobilization
**n=10

Table 3.17. Determination of L- and D-Mtx in Methotrexate injection (25 mg Mtx/mL Injection) using SIA/amperometric biosensors system

Enzyme(s) used for the design of the biosensor	E (mV)	Recovery of L-Mtx, (%)**	Recovery of D-Mtx, (%)**
Glox	150	98.56 ± 0.04	-
	650	99.02 ± 0.02	-
L-AAOD	650	98.94 ± 0.01	-
L-AAOD +HRP	650	98.75 ± 0.01	-
L-AAOD +Glox	360	98.92 ± 0.03	-
	650	99.00 ± 0.01	-
L-AAOD +Glox +HRP	240	98.97 ± 0.03	-
	650	99.02 ± 0.01	-
Glox +HRP*	650	98.76 ± 0.02	-
D-AAOD	650	-	0.82 ± 0.01
D-AAOD+HRP*	650	-	0.92 ± 0.02

*Chemical immobilization
**n=10

The results obtained for the uniformity content tests for Methotrexate tablets and injections (Tables 3.16 and 3.17) show that the tested pharmaceutical formulations contain small amounts of D-Mtx. The recoveries of the main enantiomer, L-Mtx is

within the ranges required by the USP XXV: 90-110%, with RSD values less than 2.00% [19].

3.5. Conclusion

The main advantages of the proposed system are: simplicity of construction and operation involved for biosensors as well as for the sequential injection analysis system; possibility of on-line monitoring of enantiomers during the synthesis of methotrexate, high reliability of analytical information, rapidity and, low cost of analysis. The high precision of the SIA over the manual method is due to the fact that all measurements are done after the same interval of time and the surface of the biosensors are continuously brushed by the sodium chloride or phosphate buffer carrier streams.

3.6. References

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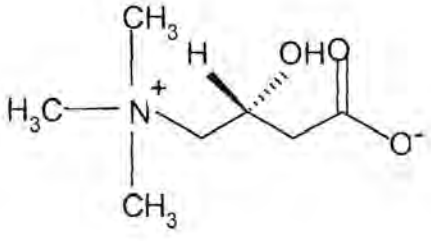
Chapter 4

On-line enantioselective analysis of carnitine

4.1. Introduction

L-carnitine, (R)-3-carboxy-2-hydroxy-N,N,N-trimethyl-1-propaminium hydroxide inner salt (scheme 1), is naturally occurring substance, essential for fatty acid oxidation and energy production in human body.



Scheme 1. The structure of L-carnitine

Without L-carnitine, long chain fatty acids cannot be transported from the cellular cytoplasm into the mitochondria, resulting in loss of energy and toxic accumulations of free fatty acids [1]. However, other functions are recognized, such as interconversion in the mechanisms of regulation of cetogenese and termogenese [2]. Also L-carnitine is used in the therapy of primary and secondary deficiency, and in various other diseases such as dislipoproteinemia [3]. The enantiomer D-carnitine has different pharmacokinetic and pharmacodynamic behaviour.

Many analytical methods have been reported to analyse carnitine in pharmaceutical formulation and in biological fluids by using chromatographic techniques (e.g., HPLC [1,4], and capillary zone electrophoresis [5-8]), fluorimetry [9], and spectrometry [10]. Among the electrochemical biosensors, amperometric biosensors represent the best combination of sensitivity and selectivity. Amperometric biosensors are capable of direct sensing without additional reagents. Thus the sensors could be used when a fast analysis or a continuous *in situ* monitoring of analyte concentration are required.

Six amperometric biosensors based on different enzymes are proposed for the assay of L-carnitine and D-carnitine. Physical and chemical immobilization techniques were used for the sensors' design. L-amino acid oxidase (L-AAOD) and D-amino acid oxidase (D-AAOD) is catalyzing only the conversion of the L-or D-enantiomer of the amino acid into their keto-acids and the hydrogen peroxide formed is measured by amperometric transducer [11]. Horseradish peroxidase (HRP) usually accompanied L-AAOD or D-AAOD in the design of bienzyme sensors, and it is used in order to improve the reduction of H_2O_2 formed from the enzymatic reaction. The reliability of biosensor construction is influencing the reliability of the analytical information and it will also contribute to the validation of biosensor for pharmaceutical analysis [12].

4.2. Experimental section

4.2.1. Equipment and reagents

Graphite powder, 1-2 μ was supplied by Aldrich (Milwaukee, WI, USA). Paraffin oil was obtained from Fluka (Buchus, Switzerland). Phosphate buffer (pH=7.00) was supplied by Merck (Darmstadt, Germany). Polyethylenimine (PEI), glutaraladehyde, and 1-ethyl-3-3(3-dimethylaminopropyl)carbodiimid (EDAC) were obtained from

Sigma. L-carnitine hydrochloride from Equine muscle and D-carnitine hydrochloride were obtained from Aldrich. De-ionized water from a Modulab system (continental water systems, San Antonio, TX, USA) was used for all solution preparations: L-amino acid oxidase (L-AAOD)(E.C.1.4.3.2. Type I crude Dried Venom from Crotalus adamanteus (Sigma)) solution, D-amino acid oxidase (D-AAOD)(E.C.1.4.3.3.Type I: from porcine kidney (Sigma)) solution, horseradish peroxidase (HRP)(EC 1.11.1.7 Type I from Horseradish) solution and solutions of L-and D-carnitine (10^{-4} mol/L), respectively. Carnilean capsules (250 mg carnitine/capsule) were obtained from Nutrent (Sandton, South Africa).

4.2.2. Amperometric biosensors design

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

4.2.2.1.Physical immobilization

4.2.2.1.1. Monoenzyme amperometric biosensors

Two electrodes, based on graphite paste, were designed as follows: paraffin oil and graphite powder were mixed in a ratio 1:4 (w/w) to form a carbon paste. 100 µL from

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

4.2.2.1.2. Bienzyme amperometric biosensors

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

4.2.2.2. Chemical immobilization

Two electrodes based on chemical modified carbon paste were prepared as follows: 0.4 g graphite powder were mixed with 400 µL (0.03 mol/L) solution of EDAC and heated to 700^0 C for 60 s in a muffle furnace. After cooling down, to ambient temperature, 10 µL PEI and 10 µL of glutaraldehyde were added. 100 µL solution containing 1 mg/mL of HRP and 0.25 mg/mL of L-AAOD were added to the pretreated graphite powder for the determination of L-carnitine, and 100 µL solution containing 4 mg/mL of HRP and 1 mg/mL of D-AAOD was added to the pretreated graphite powder for the determination of D-carnitine. Each graphite enzyme mixture was allowed to react at 4^0C for 2 h and then it was mixed with 40 µL of paraffin oil to produce the modified pastes.

4.2.3. Apparatus

A663 VA Stand (Metrohm, Herisau, Switzerland) in combination with a µAutolab and software Ecochemie (version 4.8) were used for all chronoamperometric

measurements. A Pt electrode and a Ag/AgCl electrode served as the counter and reference electrodes in the cell.

4.2.4. Recommended procedures

4.2.4.1.Direct amperometry

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

4.2.4.2. Uniformity content test for Carnilean capsules

Ten carnilean capsules (250mg carnitine/capsules) were individually placed in ten 100mL volumetric flasks, and dissolved in concentrated hydrochloric acid and diluted with de-ionized water. Different aliquots from the solution prepared were added to phosphate buffer (pH=7) in the electrochemical cell. Direct amperometry was used to determine the unknown concentration of L- and D-carnitine in pharmaceutical formulations.

4.3. Results and discussion

4.3.1. Response characteristics of the amperometric biosensors

The response characteristics of the electrodes were measured at different potential in order to determining the best working potential (higher sensitivity, lower limit of detection, shorter response time, etc.) for the assay of L- and D-carnitine (Table 4.1).

The electrode response was highly stable and reproducible over one week. The best response characteristics (larger concentration range, lower limit of detection and highest sensitivity) for the assay of L-carnitine and D-carnitine were obtained at 650 mV when physically immobilization of L-AAOD and HRP based biosensor and D-AAOD and HRP based biosensor, respectively was used for the biosensor design.

Table 4.1. Response characteristics for the amperometric biosensors designed for L- and D-carnitine

Enzyme(s) used for the design of the biosensor	E (mV)	Linear conc. Range	Detection limit	t_R (s)	Equation of calibration**	Correlation coefficient (r)
L-AAOD	250	40-8000 pmol/L	20 pmol/L	180	$^{1,a}I = 1.02 + 0.15C$	0.9999
	650	2-200 fmol/L	0.004 fmol/L	30	$^{2,b}I = 0.56 + 0.33C$	0.9999
L-AAOD + HRP	450	0.02-20 pmol/L	10 fmol/L	180	$^{2,a}I = 0.85 + 3.58C$	0.9991
	650	20-4000 pmol/L	0.6 fmol/L	240	$^{1,a}I = 45.5 + 5.1C$	0.9999
	750	0.8 –60 pmol/L	80 fmol/L	180	$^{2,a}I = 1.43 + 0.91C$	0.9992
L-AAOD + HRP*	440	100-400 fmol/L	20 fmol/L	60	$^{2,a}I = 2.6 + 103.9C$	0.9998
	650	0.08-20 pmol/L	60 fmol/L	30	$^{2,a}I = 0.85 + 10.86C$	0.9999
D-AAOD	380	0.4-40 pmol/L	200 fmol/L	30	$^{2,a}I = 0.23 + 0.76C$	0.9999
	520	200-1000 fmol/L	2 fmol/L	60	$^{2,a}I = 0.68 + 54.63C$	0.9993
	650	0.4-80 pmol/L	200 fmol/L	60	$^{2,a}I = 0.35 + 0.31C$	0.9996
D-AAOD + HRP	350	10-2000 pmol/L	400 fmol/L	30	$^{1,a}I = 2.62 + 3.83C$	0.9999
	530	0.4-20 pmol/L	200 fmol/L	180	$^{2,a}I = 0.14 + 1.83C$	0.9999
	650	2-200 nmol/L	100 fmol/L	30	$^{2,c}I = 0.85 + 1.38C$	0.9999
D-AAOD + HRP*	650	10-40 nmol/L	9nmol/L	120	$^{2,c}I = 28.0 + 5.2C$	0.9950

* Chemical immobilization

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

4.3.2. Enantioselectivity of the amperometric biosensors

The enantioselectivity of all biosensors was checked by both separate and mixed solution method with respect to L- and D-carnitine. Amperometric selectivity coefficients were determined following the method proposed by Wang [13], for the same potential used for the determination of the response characteristics of the proposed amperometric biosensors. The ratio between the concentration of the main

enantiomer and the other enantiomer was 1:10. The values of the amperometric selectivity coefficients (obtained using mixed solution method) for the biosensors designed for L- and D-carnitine are shown in Table 4.2. The $pK_{i,j}^{amp}$ values show that the biosensor based on L-AAOD enzyme has got the best enantioselectivity for L-carnitine assay, when measurements are performed at 250 mV, and that the biosensor based on D-AAOD has got the best enantioselectivity for D-carnitine assay, when measurements are performed at 520 mV.

Table 4.2. Enantioselectivity of amperometric biosensors designed for the assay of L- and D-carnitine

Enzyme(s) used for the design of the biosensor	**Interfering species (J)**	**E (mV)**	**$pK_{i,j}^{amp}$ ****
L-AAOD	D-carnitine	250	3.12
	D-carnitine	650	2.07
L-AAOD +HRP	D-carnitine	450	2.14
	D-carnitine	650	2.06
	D-carnitine	750	2.41
L-AAOD +HRP*	D-carnitine	440	2.16
	D-carnitine	650	2.08
D-AAOD	L-carnitine	380	2.06
	L-carnitine	520	2.24
	L-carnitine	650	2.05
D-AAOD +HRP	L-carnitine	350	2.08
	L-carnitine	530	2.09
	L-carnitine	650	2.12
D-AAOD +HRP*	L-carnitine	650	2.02

*Chemical immobilization , **n=10

4.3.3. Analytical applications

The amperometric biosensors proved useful for determination of the enantiopurity of L-carnitine raw materials and for testing the content of uniformity of carnilean capsules. The assay of L- carnitine and D-carnitine were conducted by use of different ratios between L- and D-carnitine. The results obtained (Table 4.3 and 4.4) demonstrated the suitability for the proposed amperometric biosensors for testing the

enantiopurity of carnitine raw material due to the good recovery values obtained for the assay of one of the enantiomers in the presence of the other enantiomer. No significant differences in the recovery values were recorded for the ratios between L: D or D: L enantiomers varying from 1:9 to 1:99.9.

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

Enzyme(s) used for the design of the biosensor	E (mV)	Recovery L-carnitine, (%)**				
		L: D				
		2:1	1:1	1:2	1:4	1:9
L-AAOD	250	99.40 ± 0.10	99.36 ± 0.12	99.29 ± 0.21	99.30± 0.19	99.40 ± 0.20
	650	100.00 ±0.08	99.74 ± 0.06	99.49 ± 0.07	99.71 ± 0.07	99.91 ± 0.09
L-AAOD +HRP	450	99.02 ± 0.14	99.03 ± 0.12	99.03 ± 0.14	99.04 ± 0.10	99.10 ± 0.09
	650	99.89 ± 0.10	100.00 ± 0.12	99.99 ±0.10	99.95 ± 0.12	99.93 ±0.19
	750	100.02 ± 0.10	100.01 ±0.10	99.98 ±0.14	99.72 ± 0.14	99.99 ± 0.12
L-AAOD +HRP*	440	99.25 ±0.10	99.76 ±0.15	99.66 ± 0.12	99.30 ±0.14	99.56 ±0.12
	650	99.87 ±0.09	99.25 ±0.10	99.62 ± 0.08	99.65 ± 0.09	99.65 ±0.08

*Chemical immobilization, **n=10

Table 4.4. Determination of D-carnitine in the presence of L-carnitine

Enzyme(s) used for the design of the biosensor	E (mV)	Recovery D-carnitine, (%)**				
		D: L				
		2:1	1:1	1:2	1:4	1:9
D-AAOD	380	99.80 ± 0.20	99.40 ± 0.19	99.28 ± 0.10	99.40 ± 0.12	99.68 ± 0.14
	520	99.47 ± 0.21	99.82 ± 0.19	99.47 ± 0.19	99.22 ± 0.20	99.82 ± 0.18
	650	99.08 ± 0.12	99.76 ± 0.14	99.90 ± 0.12	99.89 ±0.13	99.80 ± 0.10
D-AAOD +HRP	350	99.38 ±0.12	99.50 ± 0.10	99.13 ± 0.18	99.25 ± 0.12	99.75 ± 0.14
	530	96.11 ± 0.22	96.07 ±0.32	96.17 ± 0.24	96.32 ±0.21	96.20 ± 0.20
	650	99.48 ± 0.21	99.56 ± 0.19	99.57 ± 0.12	99.92 ± 0.15	99.91 ± 0.10
D-AAOD +HRP*	650	99.07 ± 0.18	99.51 ± 0.16	99.21 ± 0.24	99.29 ± 0.20	99.54 ± 0.18

*Chemical immobilization, **n=10

The results obtained for the uniformity content test are presented in Table 4.5 for carnilean capsules. The uniformity content test show that the tested pharmaceutical formulations contain as main component the L-carnitine and only small amounts of the D-carnitine. The recovery values for L-carnitine are within the limits requested by USP XXV: 90-110%, with RSD values less than 1%[14].

Table 4.5. Determination of L-and D-carnitine in Carnilean capsules (250 mg carnitine/capsule)

Enzyme(s) used for the design of the biosensor	**E (mV)**	**Recovery of L-carnitine, (%)****	**Recovery of D-carnitine, (%)****
L-AAOD	250	97.73 ± 0.96	-
	650	97.76 ± 0.85	-
L-AAOD +HRP	450	97.78 ± 0.83	-
	650	97.84 ± 0.88	-
	750	97.76 ± 0.89	
L-AAOD +HRP*	440	97.74 ± 0.91	-
	650	97.55 ± 0.82	-
D-AAOD	380	-	1.70 ± 0.23
	520	-	1.69 ± 0.24
	650		1.71 ±0.20
D-AAOD +HRP	350	-	1.70 ±0.21
	530	-	1.67 ± 0.26
	650	-	1.71 ± 0.24
D-AAOD +HRP*	650	-	1.72 ± 0.21

*Chemical immobilization
**All values are the average of ten determinations.

The proposed amperometric biosensors have excellent features in enantioselective analysis. The construction of the electrodes is simple, fast and reproducible and it is also assuring reliable response characteristics for the proposed amperometric biosensors. The enantioselectivity of the described biosensor made possible the performance of the enantiopurity assay of L-carnitine as raw material and from its pharmaceutical formulations. The selection of the working potential in the assay of the enantiomers of L-and D-carnitine proved to have a high effect on the performances of the amperometric biosensors, in terms of sensitivity, limit of detection, linear concentration range, response time and enantioselectivity.

4.4. Sequential injection analysis system for on-line simultaneous assay of enantiomers of carnitine

4.4.1. Sequential injection system

The biosensors were incorporated into the conduits of SIA system (Figure 4.1A) constructed from a Gilson Minipuls peristaltic pump and a 10-port electrically actuated selection valve (Model ECSD10P, Valco Instruments, Houston, TX).

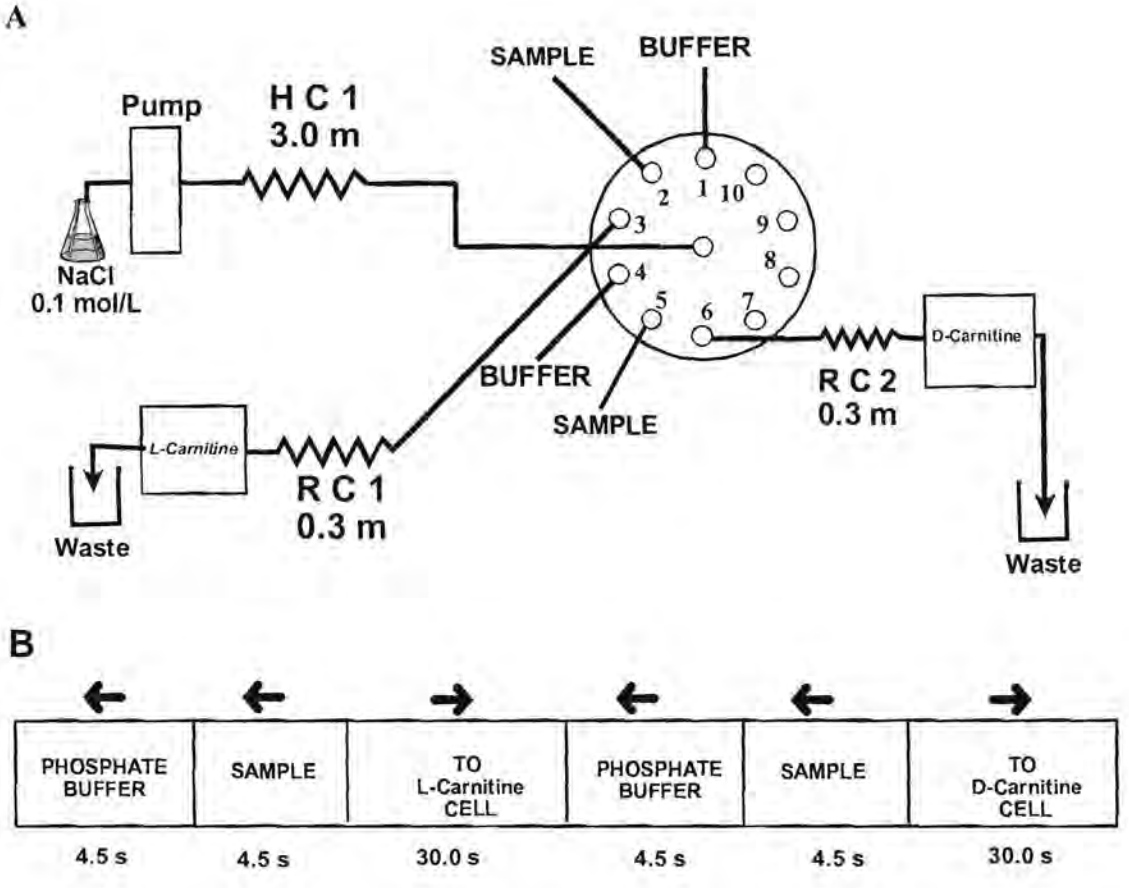


Figure 4.1. SIA system used for the simultaneous determination of L- and D-carnitine (A) Schematic flow diagram (B) sequence of sample, phosphate buffer, and electrochemical cells for L-Carnitine and D-Carnitine.

Tygon tubing (0.76 mm i.d. for both holding coils and 0.89 mm i.d for both mixing coils) was used to construct the manifold; coils were wound round suitable lengths of glass tubing (15 mm o.d.); 0.1 mol/L NaCl was used as carrier. The capacity of the

system is about 34 samples per hour. The device operating sequence is shown in Table 4.6. The device control was achieved using a PC30-B interface board (Eagle Electric, Cape Town, South Africa). The FlowTEK [15] software package (obtained from MINTEK) for computer-aided flow analysis was used though out for device control.

Table 4.6. Device sequence for one cycle of the SIA system

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

An optimum flow rate of 3.61 mL/min was used to propel the solutions, the timing and flow direction is shown in Figure 4.1B. The sample and buffer consumption is only 270 µL of each per measurement of L- and D-enantiomer, which is very economical.

4.4.2. Response characteristics of the amperometric biosensors as detectors in SIA system

The response characteristics of the biosensors were measured at different potential in order to obtain the best response characteristics (e.g., higher sensitivity, lower limit of detection, wide linear concentration range, etc.) for the simultaneous detection of L- and D-carnitine (Table 4.7). The working concentration ranges as well as the limits of detection demonstrated the suitability of the proposed amperometric biosensors for the on-line monitoring of both enantiomers. The response obtained for all biosensors revealed good stability and reproducibility for tests performed over one week (RSD<0.1%).

Table 4.7. Response characteristics for the amperometric biosensors designed for L- and D-carnitine when the biosensors are used as detectors in SIA system

Enzyme(s) used for the design of the biosensor	E (mV)	Linear conc. range	Detection limit	Equation of calibration*	Correlatio coefficien (r)
L-AAOD	250	0.6-60 nmol/L	0.2 nmol/L	[1,a] H= 16.98 +105.09C	0.9999
L-AAOD +HRP	450	0.1-1 pmol/L	2 fmol/L	[1,b] H=14.38 +394.8C	0.9997
	650	60-6000 pmol/L	10 pmol/L	[1,a] H=0.18 +82.38C	0.9975
D-AAOD	380	0.2-6 pmol/L	80 fmol/L	[1,b] H=11.52 +92.26C	0.9995
	650	0.2-8 pmol/L	40 fmol/L	[1,b] H=46.01 +257.26C	0.9994
D-AAOD +HRP	530	0.04-6 pmol/L	2 fmol/L	[1,b] H=0.409 +3376C	0.9999
	650	4-80 nmol/L	0.4 nmol/L	[1,a] H=2.23 +1.49C	0.9995

*H is the peak height in [1]nA and C is the concentration of L-and D-carnitine, respectively, in [a]nmol/L and [b]pmol/L.

4.4.3. Selectivity of the biosensors as detectors in SIA system

The selectivity of all biosensors was checked using both the mixed solutions and separate solutions methods with respect to L- and D-carnitine. Amperometric selectivity coefficients were determined following the method proposed by Wang [13], for the same potentials used for the determination of the response characteristics of the proposed amperometric biosensors. In the evaluation, the concentration of the interferent was selected to be ten times higher than that of the enantiomer of interest.

The proposed biosensors are enantioselective when used as detectors in SIA system (Table 4.8). The $pK_{i,j}^{amp}$ values show that the biosensor based on L-AAOD has got the best enantioselectivity for L-carnitine assay, when the measurement are performed at 250 mV, and that the biosensor based on D-AAOD and HRP has got the best enantioselectivity for D-carnitine assay, when measurements are performed at 530 mV.

Table 4.8. Selectivity coefficients,pK_{amp} for amperometric biosensors as detectors in SIA system

Enzyme(s) used for the design of the biosensor	**Interfering species (J)**	**E (mV)**	**$pK_{i,j}^{amp}$ ***
L-AAOD	D-carnitine	250	2.55
L-AAOD +HRP	D-carnitine	450	2.31
	D-carnitine	650	2.16
D-AAOD	L-carnitine	380	2.08
	L-carnitine	650	2.27
D-AAOD +HRP	L-carnitine	530	3.29
	L-carnitine	650	3.20

*n=10

4.4.4. Analytical applications of SIA/amperometric biosensors system

The proposed SIA/amperometric biosensors system proved to be useful for the simultaneous assay of L-and D-carnitine. The results obtained (Tables 4.9 and 4.10) demonstrated its suitability on-line purity tests of carnitine. No differences were recorded in recovery tests between 1:9 and 1:99 in the favor of each enantiomer.

Table 4.9. Recovery of L-carnitine in the presence of D-carnitine using SIA/amperometric biosensors system

Enzyme(s) used for the design of the biosensor	E (mV)	Recovery L-carnitine, (%)* L: D				
		2:1	1:1	1:2	1:4	1:9
L-AAOD	250	99.79±0.02	99.86±0.02	99.83±0.01	99.84±0.02	99.81±0.02
L-AAOD +HRP	450	99.75±0.02	99.99±0.01	99.81±0.01	99.89±0.02	99.83±0.01
	650	99.61±0.03	99.37±0.02	99.39±0.02	99.78±0.02	99.79±0.03

*n=10

Table 4.10. Recovery of D-carnitine in the presence of L-carnitine using SIA/amperometric biosensors system

Enzyme(s) used for the design of the biosensor	E (mV)	Recovery D-carnitine (%)* D: L				
		2:1	1:1	1:2	1:4	1:9
D-AAOD	380	99.26±0.02	99.18±0.01	99.20±0.02	99.24±0.02	99.28±0.01
	650	99.84±0.01	99.86±0.01	99.80±0.02	99.84±0.01	99.99±0.01
D-AAOD+HRP	530	99.92±0.01	99.90±0.02	99.96±0.01	99.94±0.01	99.95±0.02
	650	99.89±0.02	99.87±0.03	99.88±0.02	99.90±0.02	99.91±0.01

*n=10

A uniformity content test was performed for Carnilean capsules. Ten Carnilean capsules (250 mg carnitine/capsule) were individually placed in 100 mL volumetric flasks, and dissolved in concentrated hydrochloric acid. The solutions prepared from capsules were diluted with de-ionized water and the height of the peak was recorded using proposed SIA/amperometric biosensors system. The results were interpolated into calibration graph of the corresponding electrode in order to determine the concentration of each enantiomer, and furthermore, it's content in the corresponding pharmaceutical formulation. The results obtained for the uniformity content test for Carnilean capsule (Table 4.11) show that the tested pharmaceutical formulations contain small amounts of D-carnitine. The recovery values for L-carnitine are within the limits requested by USP XXV: 90-110%, with RSD values less than 1% [14].

Table 4.11. Determination of L- and D-carnitine in Carnilean capsules (250 mg Carnitine /capsule) using SIA/amperometric biosensors system

Enzyme(s) used for the design of the biosensor	E (mV)	Recovery of L-carnitine, (%)*	Recovery of D-carnitine, (%)*
L-AAOD	250	97.59±0.83	-
L-AAOD +HRP	450	97.64±0.85	-
	650	99.70±0.89	-
D-AAOD	380	-	1.65±0.25
	650	-	1.70±0.20
D-AAOD +HRP	530	-	1.68±0.19
	650	-	1.73±0.23

* n=10

4.5. Conclusion

The main advantage of the proposed system are: simplicity of construction and operation that involved for on-line monitoring of enantiomers during the synthesis of enantiomers, high reliability of analytical information, rapidity and, low cost of analysis. The high precision of the SIA over the manual method is due to the fact that all measurements are done after the same interval of time and the surface of the biosensors are continuously washed by NaCl solution used as carrier stream.

4.6 References

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