Chapter 5

On-line assay of ortho- acetyl- L-carnitine

5.1. Introduction

L-carnitine (3-hydroxy-4-N-trimethyl aminobutyrate) (Car) was discovered in beef [1], and it has been proved to play an important role in fatty acid metabolism, e.g., the transport of fatty acid into mitochondria and the modulation of intramitochondrial ratio [2]. O-Acetyl-L-carnitine (Acar) in connection with Car, also attracts a keen interest, being regarded as a transient substance of fatty acid metabolism in mitochondria, and is also found to have some important pharmacological properties [3-4]. The amount of serum Acar has close relation to chronic fatigue syndrome (CFS). Accordingly; it is possible to make a diagnosis of CFS by measurement of serum Acar [5]. The relationship between the amount of serum Acar and exhaustive exercise or starvation was also reported [6]. More interest has been focused on the biological and medical significance of Acar itself in many fields [7-10] besides CFS.

Many analytical methods have been reported to analyse ortho-acetyl-L-carnitine as metabolite of the pharmaceutical formulations, and in biological fluids by using chromatographic techniques, e.g., HPLC [11,12], and capillary zone electrophoresis [13], as well as spectrometric methods [14]. 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.

In this chapter two amperometric biosensors based on different enzymes are described for the assay of ortho-acetyl-L-carnitine. Physical immobilization techniques were used for the design of those sensors [15,16]. Due to the reliability of its response characteristics, the biosensors were incorporated in the conduits of sequential injection analysis (SIA) system and used for on-line determination of ortho-acetyl-L-carnitine form the pharmaceutical formulation and clinical analysis.

5.2. Experimental section

5.2.1. Reagents and materials

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). L-carnitine hydrochloride from Equine muscle and D-carnitine hydrochloride was 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, horseradish peroxidase (HRP)(EC 1.11.1.7 Type I from Horseradish) solution, solutions of L- (D)-carnitine (10^{-4} mol/L) and O-acetyl-L-carnitine (10^{-4} mol/L), respectively, were also prepared in de-ionized water.

5.2.2. Amperometric biosensors design

Two plastic tips were filled with plane carbon paste leaving an empty space of 3-4 mm in the top part 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 was wetted with de-ionized water and then polished with an alumina paper (polished strips 30144-011, Orion) before use. The biosensors were stored dry at 4^0C,when not in use.

Physical immobilization mono- and bienzyme electrodes were constructed. All the enzyme solutions used for the biosensors design were prepared in 0.1 mol/L phosphate buffer of pH=7.

5.2.2.1. Monoenzyme amperometric biosensors

Paraffin oil and graphite powder were mixed in a ratio 1:4 (w/w) to form a graphite paste. 100 µL from the solution (1mg/enzyme/mL): L-AAOD was added to the carbon paste to obtain the monoenzyme electrode.

5.2.2.2. Bienzyme amperometric biosensors

1 mg of HRP was dissolved in 50 µL of L-AAOD solution (0.25 mg/mL); the mixture was incorporated in the carbon paste (100 mg graphite powder and 40 µL paraffin oil) to obtain the bienzyme electrode.

5.2.3. Apparatus

A 663 VA Stand (Metrohm, Herisau, Switzerland) in combination with the PSTAT 20 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. The biosensors were incorporated into the conduits of SIA system shown in Figure 5.1A below.

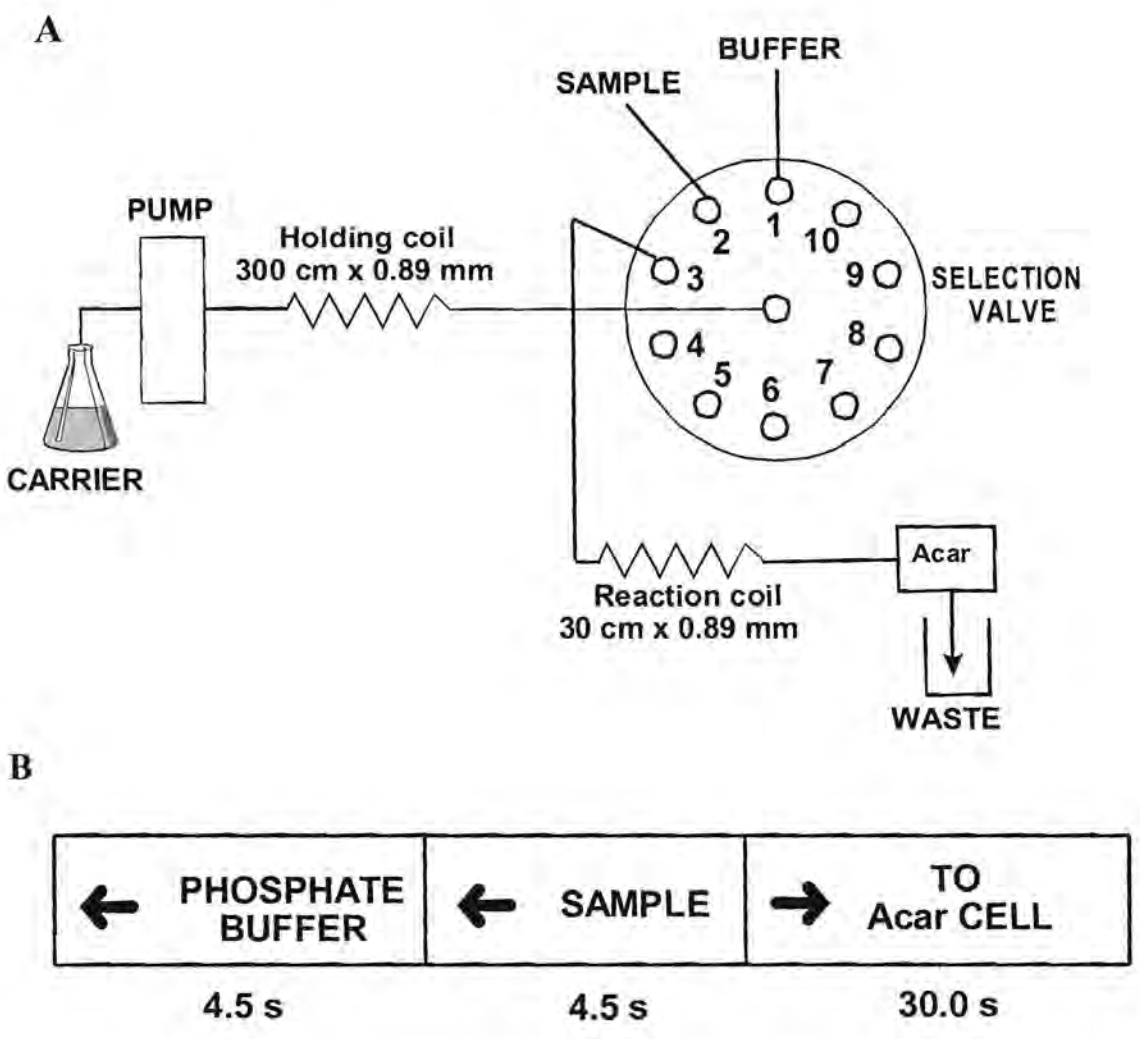

Figure 5.1. SIA system used for the determination of Acar (A) Schematic flow diagram (B) sequence of sample, phosphate buffer, and electrochemical cells for Acar.

The SIA system constructed from a Gilson Minipuls peristaltic pump and a 10-port electrically actuated selection valve (Model ECSD10P, Valco Instruments, Houston, TX). Tygon tubing (0.76 mm i.d. for holding coil and 0.89 mm i.d for mixing coil) 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 75 samples per hour. The device operating sequence is shown in Table 5.1.The device control was achieved using a PC30-B interface board (Eagle Electric, Cape Town, South Africa). The FlowTEK [17] software package (obtained from MINTEK) for computer-aided flow analysis was used throughout for device control. An optimum flow rate of 3.61 mL/min was used to propel the solutions, the timing and flow direction is shown in Figure 5.1B. It follows from this that in the SIA system, the sample and buffer consumption is only 270 µL of each, which is very economical.

Table5. 1. 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		Ortho acetyl L-carnitine cell	-select ortho-acetyl- L-carnitine cell line (valve position 3)
18	Forward		-pump stack of zones to ortho-acetyl-L-carnitine cell
48	Off		-pump stops

5.2.4. Recommended procedure: 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 ortho- acetyl- L-carnitine solution. The intensity of current measured was plotted versus the concentration of ortho- acetyl- L-carnitine. The unknown concentrations of ortho- acetyl- L-carnitine were determined from the calibration graphs.

5.3 Results and discussion

5.3.1. Electrode response

Response characteristics of the amperometric biosensors were measured at different potential in order to determine the best working potential (higher sensitivity, lower limit of detection, shorter response time, etc.) for the determination of ortho-acetyl-L- carnitine (Table 5.2) without SIA and (Table 5.3) with SIA system. 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) were obtained when the measurement was performed at 250mV using L-AAOD with and without SIA system.

Table 5. 2. Response characteristics for the amperometric biosensors designed for ortho-acetyl-L-carnitine without SIA system

Enzyme(s) used for the design of the biosensor	E (mV)	Linear conc. Range	Detection limit	Equation of calibration*	Correlation coefficient (r)
L-AAOD	250	6-4000 fmol/L	4 fmol/L	[1,a] I= 117.47 +2.22C	0.9998
	650	2-100 pmol/L	0.8 pmol/L	[2,b] I=0.164 +0.362C	0.9999
L-AAOD +HRP	650	0.08-0.8 pmol/L	0.04 pmol/L	[2,b] I=0.08 +9.86C	0.9947

* I is the intensity of current in [1]nA [2]μA and C is the concentration of ortho acetyl L-carnitine in [a]fmol/L and [b]pmol/L.

Table 5. 3. Response characteristics for the amperometric biosensors designed for ortho-acetyl-L-carnitine with SIA system

Enzyme(s) used for the design of the biosensor	E (mV)	Linear conc. Range	Detection limit	Equation of calibration *	Correlation coefficient (r)
L-AAOD	250	4-60 fmol/L	2 fmol/L	[1,a] H= 2.49 +21.13C	0.9999
	650	2-40 pmol/L	0.6 pmol/L	[1,b] H=11.30+43.28C	0.9999
L-AAOD +HRP	650	0.4-1 pmol/L	0.06 pmol/L	[2,b] H=0.21 +9.53C	0.9998

* H is the peak height in [1]nA [2]μA and C is the concentration of ortho-acetyl-L-carnitine in [a]fmol/L and [b]pmol/L.

5.3.2. Selectivity of the amperometric biosensors

The selectivity of all biosensors was checked by both separate and mixed solution method over L- and D-carnitine possible interfering species. Amperometric selectivity coefficients were determined following the method proposed by Wang [18]. In the evaluation the concentration of the possible interferent, was selected to be ten times higher that of ortho-acetyl-L-carnitine and the result of mixed solution method shown in Table 5.4. From the $pK_{i,j}^{amp}$ value all the amperometric selectivity coefficients were found to be lower than 10^{-3}. This means that these compounds do not interfere, and that it is possible to determine the ortho-acetyl-L-carnitine in biological samples.

Table 5. 4. Selectivity of amperometric biosensors designed for the assay of ortho-acetyl-L-carnitine with and without SIA system

Enzyme(s) used for the design of the biosensor	Interference Species	E (mV)	$pK_{i,j}^{amp}$	
			without SIA*	with SIA*
L-AAOD	L-carnitine	250	2.70	2.58
	D-carnitine		2.51	2.20
	L-carnitine	650	2.43	2.17
	D-carnitine		2.72	2.70
L-AAOD +HRP	L-carnitine	650	2.01	3.19
	D-carnitine		3.10	3.30

*n=10

5.4. Conclusion

The constructions of biosensors are simple and reproducible. The reliability of the analytical information is assured by the RSD values obtained in recovery tests. The proposed amperometric biosensors/SIA system is suitable for on-line purity tests of ortho- acetyl- L-carnitine as raw material at a rate of 75 samples per hour when used as detector in SIA system. The main advantage of the proposed method over the other methods described for ortho- acetyl- L-carnitine assay is the possibility of its determination directly without any prior separation with a high precision, rapidity, low consumption of sample and buffer, and utilization of a not expensive carrier, NaCl solution 0.1 mol/L.

5.5. References

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Chapter 6

On-line simultaneous determination of creatine and creatinine

6.1. Introduction

Creatinine is a final product of creatine metabolism in mammals. The measurement of creatine and creatinine in blood is important in clinical analyses, because creatinine is important in monitoring of kidney function while creatine is a valuable index of muscle damage. The physiologically normal concentration range for creatinine and creatine is below 0.14 mmol/L in serum, but during kidney disfunction or muscle disorder their concentrations may raise up to value higher than 1 mmol/L [1]. Also, creatine has recently great popularity as ergogenic aid. This popularity is based on the fact that creatine is converted to phosphocreatine in muscle in a reversible reaction with adenosine triphosphate (ATP). When muscle contractions deplete the immediate supply of ATP, the phosphocreatine can rephosphorylate adenosine diphosphate (ADP) to replenish the supply of ATP. Creatinine is the means by which creatine and phosphocreatine are excreted from the body and this occurs at rates of 0.016 and 0.03 per day for creatine and phosphocreatine, respectively. The amount of creatinine in the urine is proportional to the amount of creatine and creatine phosphate present in the body and hence also to the muscle mass [2].

The following techniques were proposed for the assay of creatine and creatinine: HPLC [3,4], mass spectroscopy [5], IR spectroscopy [6], capillary zone electrophoresis [7,8].

The emphasis of this chapter is to determine simultaneously the amount of creatine and creatinine using amperometric biosensors without and with a SIA system. The amperometric biosensors are based on creatininase, creatinase and sarcosine oxidase. The enzyme creatininase hydrolyses creatinine to yield creatine. Creatinase hydrolyses creatine first to sarcosine. Sarcosine oxidase is acting on sarcosine, forming formaldehyde, glycine and the hydrogen peroxide - the product that will be measured by the amperometric transducer [9].

6.2 Experimental section

6.2.1. Reagents and materials

Graphite powder, 1-2 μ, synthetic was purchased from Aldrich (Milwaukee, WI, USA). Paraffin oil was purchased from Fluka (Buchus, Switzerland); phosphate buffer (pH=7.6) was purchased from Merck (Darmstadt, Germany). De-ionized water from a Modulab system (continental water systems, San Antonio, TX, USA) was used for all solution preparations. Creatinase (CI) from Flavobacterium was obtained from Fluka, Sarcosine oxidase (SO) from Arthrobacterium species was obtained from Aldrich, and creatininase (CA) from Flavobacterium species was obtained from Sigma. Creatine and creatinine were purchased from Aldrich. Solutions of creatine and creatinine (10^{-4} mol/L) were prepared in de-ionized water. Enermax Creatine capsule (500 mg creatine monohydrate/capsule) were obtained from Nutrent (Sandton, South Africa) and

Roboforce effervescent creatine with ribose (5 g creatine/27.5 g powder) was purchased from EAS (Golden, CO, USA).

6.2.2. Amperometric biosensors design

Bi- and trienzyme electrodes were constructed. All the enzyme solutions used for the biosensors design were prepared in 0.1 mol/L phosphate buffer pH=7.6.

Two plastic tips were filled with plane carbon paste leaving an empty space of 3-4 mm in the top part 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.

6.2.2.1.Bienzyme electrode for assay of creatine

2 µL of CI solution (0.2 µg SO/25 µL phosphate buffer pH=7.6) was mixed with 2 µL of SO solution (0.2 µg SO/µL phosphate buffer pH=7.6). The mixture was incorporated in the carbon paste (25 mg graphite powder and 10 µL paraffin oil), to obtain the bienzyme electrode.

6.2.2.2.Trienzyme electrode for assay of creatinine

Paraffin oil and graphite powder was mixed in the ratio 1:4 (w/w) to form a carbon paste. 2 µL of CI solution (0.2 µg CI/25 µL phosphate pH=7.6) was first mixed with 2 µL of SO

solution (0.2 µg SO/25 µL phosphate buffer pH=7.6) and then with 1 µL of CA solution (0.2 µg CA/25 µL phosphate buffer pH=7.6). The resulting solution was added to the carbon paste.

6.2.3. Apparatus

A 663 VA stand (Metrohm, Herisau, Switzerland) in connection with a PGSTAT 20 and software (Eco Chemie version 4.8) was used for all chronoamperometric measurements. A Pt electrode and an Ag/AgCl electrode served as counter and reference electrodes in the cell. A Multiplexer module SCNR16 was connected to the PGSTAT 20 in order to be able to make the simultaneous analysis of creatine and creatinine.

6.2.4. Recommended procedures

6.2.4.1 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 =7.6 and different aliquots of creatine and creatinine solutions. The intensity of current measured was plotted versus the concentration of creatine and creatinine. The unknown concentration of creatine was determined directly from the calibration graph of the bienzyme electrode. Because the trienzyme electrode can determine the full amount of creatine and creatinine in the solution, the concentration of creatinine was determined by making the difference between the concentration obtained from the calibration graph of the trienzyme electrode and the concentration of creatine determined using the bienzyme electrode.

6.2.4.2.Uniformity content test for creatine capsules and powder

Ten Creatine capsule (500 mg Creatine/capsule) were individually placed in ten 100 mL volumetric flasks, and dissolved in de-ionized water. 0.0182 g of creatine powder was dissolved in 100 mL calibrated flask and then diluted to the mark with de-ionized water. Different aliquots from the solutions prepared were added to phosphate buffer (pH=7.6) in the electrochemical cell. Direct amperometry was used to determine the unknown concentration of creatine and creatinine in pharmaceutical formulations.

6.2.4.3.Determination of creatine and creatinine in serum samples

Different aliquots of serum samples were diluted with buffer solution. Direct amperometry was involved to determine the content on creatine and creatinine in serum samples.

6.3. Results and discussion

6.3.1. 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, wide linear concentration range, etc.) for the simultaneous detection of creatine and creatinine (Table 6.1). The electrode response was highly stable and reproducible over one week. The best response characteristics (large concentration range, lower limit of detection and highest sensitivity) for assay of creatine were obtained at 240 mV when the bienzyme electrode was used and the best response characteristics were obtained for creatinine when measurements were done at 650 mV using the trienzyme electrode. The

response obtained for all biosensors revealed good stability and reproducibility for tests performed every day over one week (RSD<0.1%).

Table 6.1. Response characteristics for the amperometric biosensors designed for creatine and creatinine

Type of Biosensor	E (mV)	Linear conc. Range	Detection limit	t_R (s)	Equation of calibration*	r
Bienzyme	240	4-200 pmol/L	2 pmol/L	30	$^{1,a}I=60.19+12.67C$	0.9984
	650	2-20 nmol/L	1 nmol/L	120	$^{2,b}I=0.26+2.86C$	0.9998
Trienzyme	420	4-40 nmol/L	2 nmol/L	60	$^{2,b}I=0.11+0.04C$	0.9981
	650	4-100 nmol/L	2 nmol/L	30	$^{2,b}I=0.345+0.746C$	0.9999

*H is the peak height in ^{1}nA and ^{2}μA, and C is the concentration of creatine and creatinine respectively in ^{a}pmol/L and ^{b}nmol/L.

6.3.2. Selectivity of the amperometric biosensors

The selectivity of both biosensors was checked using both the mixed and separate solutions methods. Amperometric selectivity coefficients were determined following the method proposed by Wang [10], for the same potentials used for the determination of the response characteristics of the proposed amperometric biosensors. In the evaluation, the concentration of the creatinine was selected to be ten times higher than that of creatine. The values of the amperometric selectivity coefficients ($pK_{i,j}^{amp}$) (obtained using mixed solution method) for the bienzyme biosensor over creatinine are: 2.09 when the measurements were done at 240 mV, and 2.75 when the measurements were done at 650 mV. The $pK_{i,j}^{amp}$ values show that the bienzyme electrode has got the best selectivity for creatine assay, when measurements are performed at 650 mV.

The selectivity of both electrodes was tested also over glycine, methionine, ribose and taurine. For all of these substances, the values of the amperometric selectivity coefficients

are less than $1x10^{-4}$. These results proved the selectivity of the proposed electrodes over these substances that can be found in the pharmaceutical formulations of creatine.

6.3.3. Analytical Applications

The amperometric biosensors proved useful for determination of the creatine and creatinine in pharmaceutical products and serum samples. In order to prove the accuracy of the assay of creatine in the presence of creatinine, synthetic samples containing creatine and creatinine in different ratios were prepared. The results obtained (Table 6.2) demonstrated the suitability of the proposed amperometric biosensors for testing the purity of creatine due to the good recovery values obtained for the assay of creatine in the presence of creatinine. No significant differences in the recovery tests were recorded for creatine: creatinine ratios between 1:9 and 1:99.9.

Table 6.2. Determination of creatine in the presence of creatinine using the bienzyme electrode

E (mV)	**Recovery Creatine, (%)***				
	Creatine: Creatinine				
	2:1	1:1	1:2	1:4	1:9
240	99.65 ± 0.03	99.60 ± 0.02	99.68± 0.02	99.63± 0.03	99.60 ± 0.01
650	99.20 ± 0.01	99.25 ± 0.02	99.23 ± 0.01	99.30± 0.02	99.28 ± 0.01

* n=10

The results obtained for the uniformity content tests are presented in Table 6.3 and 6.4 for Enermax Creatine capsule (500mg creatine monohydrate/capsule) and Roboforce effervescent creatine with ribose (5g creatine/27.5g powder), respectively. The uniformity content tests show that the tested pharmaceutical formulations contain as main component the creatine and only small amounts of the creatinine. The recovery values for

creatine are within the labeled amount of 90-110%, with RSD values less than 6.00% required by the USP XXV [11].

Table 6.3. Determination of creatine and creatinine in Enermax Creatine capsules (500 mg creatine/capsule)

Type of biosensor	E (mV)	Recovery of Creatine, (%)*	Recovery of Creatinine, (%)*
Bienzyme	240	93.14 ±0.12	-
	650	93.05 ± 0.10	-
Trienzyme	420	-	3.98 ± 0.12
	650	-	3.82 ± 0.14

* n=10

Table 6.4. Determination of creatine and creatinine in Roboforce effervescent creatine (5 g/27.5 g of powder)

Type of biosensor	E (mV)	Recovery of Creatine, (%)*	Recovery of Creatinine, (%)*
Bienzyme	240	95.95 ±0.18	-
	650	96.02 ±0.16	-
Trienzyme	420	-	0.69 ±0.06
	650	-	0.72 ±0.07

* n=10

Creatine and creatinine were simultaneous assay also from serum samples using a standard method [12] as well as the proposed biosensors (Table 6.5). Good correlation between the results was obtained using both standard and new developed method.

Table 6.5 Determination of creatine and creatinine from serum samples

Standard method [12] (μ mol/L)			Proposed method (μ mol/L)	
Number	**Creatine**	**Creatinine**	**Creatine**	**Creatinine**
1	20	14	19.75	13.90
2	65	60	64.20	59.59
3	7.6	80	7.3	79.40
4	16	18	15.82	17.92

All values are average of ten determinations. RSD (%) values are lower than 0.1%.

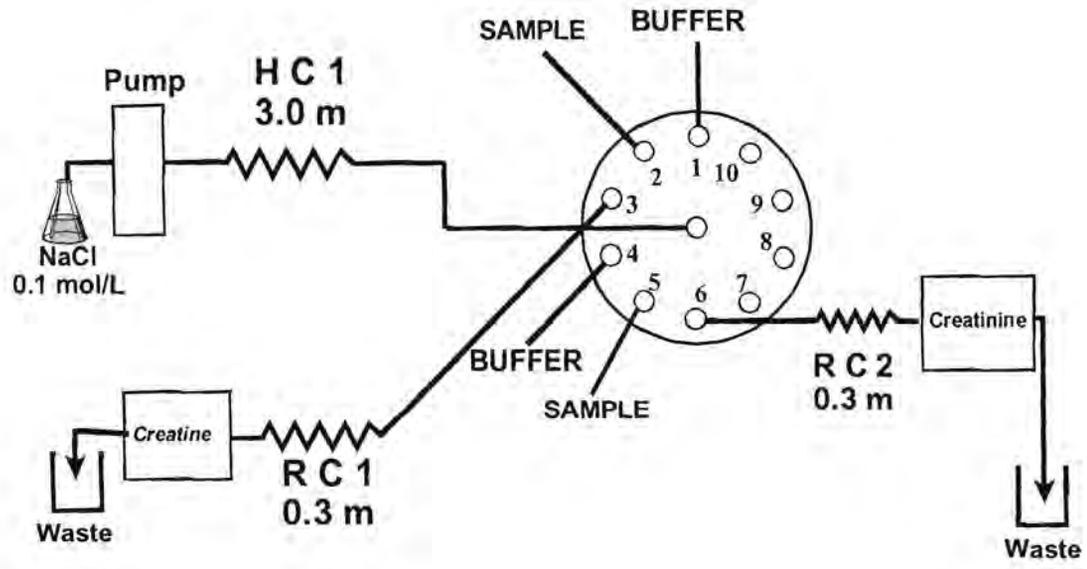
The amperometric biosensors electrodes described have excellent features in pharmaceutical and clinical analyses. The construction of the electrodes is simple, fast and reproducible and it is also assuring reliable response characteristics for the proposed amperometric biosensors. The selection of the working potential in the assay of the compound 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 selectivity.

6.4. Sequential injection analysis system for on-line simultaneous determination of creatine and creatinine

6.4.1 Sequential injection system

The biosensors were incorporated into the conduits of SIA system (Figure 6.1A) constructed from a Gilson Minipuls peristaltic pump and a 10-port electrically actuated selection valve (Model ECSD10P, Valco Instruments, Houston, TX). 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.); 0.1mol/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 6.6. The device control was achieved using a PC30-B interface board (Eagle Electric, Cape Town, South Africa). The FlowTEK [13] software package (obtained from MINTEK) for computer-aided flow analysis was used though out for device control.

A



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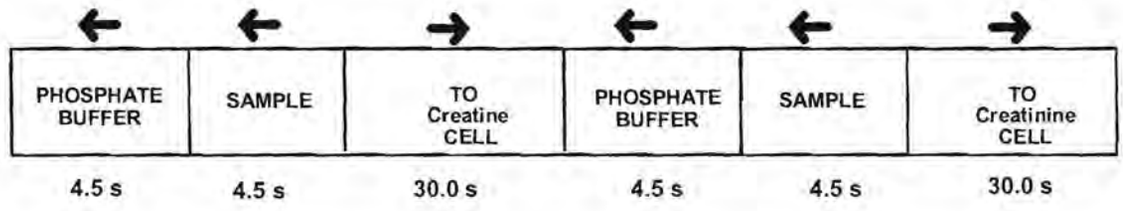

Figure 6.1. SIA system used for the simultaneous determination of creatine and creatinine Schematic flow diagram (B) sequence of sample, buffer, and electrochemical cells for creatine and creatinine.

An optimum flow rate of 3.61 mL/min was used to propel the solutions, the timing and flow direction is shown in Figure 6.1B. It follows from this that in the SIA system, the sample and buffer consumption is only 270 μL each per measurement of creatine and creatinine, which is very economical.

Table 6.6. 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		Creatine cell	-select creatine cell line (valve position 3)
18	Forward		-pump stack of zones to creatine 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		Creatinine cell	-select creatinine cell line (valve position 6)
63	Forward		-pump stack of zones to creatinine cell
93	Off	Home	-pump stops, return valve to starting position (valve position 1)

6.4.2. Response characteristics of the amperometric biosensors in SIA system

The response characteristics of the electrodes were measured at different potential in order to determine the best working potential (higher sensitivity, lower limit of detection, wide linear concentration range, etc.) for the simultaneous detection of creatine and creatinine (Table 6.7). 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 compounds. The response obtained for all biosensors revealed good stability and reproducibility for tests performed over one week.

Table 6.7. Response characteristics for the amperometric biosensors designed for creatine and creatinine when the biosensors are used as detectors in SIA system

Type of biosensor	E (mV)	Linear conc. range	Detection limit	Equation of calibration*	Correlation coefficient (r)
Bienzyme	240	0.4-60 pmol/L	0.2 pmol/L	[1,a] H=26.71 + 26.12C	0.9995
	650	4-10 nmol/L	0.8 nmol/L	[2.b] H=0.67 +0.995C	0.9998
Trienzyme	420	6-400 nmol/L	0.4 nmol/L	[1,b] H=0.664 +0.629C	0.9999
	650	4-60 nmol/L	0.6 nmol/L	[1,b] H=8.24 +5.98C	0.9994

*H is the peak height in [1]nA and [2]μA, and C is the concentration of creatine and creatinine respectively in [a]pmol/L and [b]nmol/L.

6.4.3. Selectivity of the amperometric biosensors in SIA system

The selectivity of both biosensors was checked using both the mixed and separate solutions methods. Amperometric selectivity coefficients were determined following the method proposed by Wang [10], for the same potentials used for the determination of the response characteristics of the proposed amperometric biosensors. In the evaluation, the concentration of the creatinine was selected to be ten times higher than that of creatine. The values of the amperometric selectivity coefficients ($pK_{i,j}^{amp}$) (obtained using mixed solution method) for the bienzyme biosensor over creatinine are: 2.07 when the measurements were done at 240 mV, and 3.05 when the measurements were done at 650 mV. The $pK_{i,j}^{amp}$ values show that the bienzyme electrode has got the best selectivity for creatine assay, when measurements are performed at 650 mV.

The selectivity of both electrodes was tested also over glycine, methionine, ribose and taurine. For all of these substances, the values of the amperometric selectivity coefficients are less than $1x10^{-5}$. These results proved the selectivity of the proposed electrodes over these substances that can be found in the pharmaceutical formulations of creatine.

6.4.4. Analytical applications of the SIA/amperometric biosensors system

The flow systems obtained by incorporation of the amperometric biosensors in the SIA conduits, proved to be useful for the simultaneous assay of creatine and creatinine. The results obtained for the recovery of creatine in the presence of creatinine (Table 6.8) demonstrated the accuracy of the proposed flow system for on-line simultaneous determination of creatine and creatinine. No differences were recorded in recovery (%) of the creatine when the ratio between creatine and creatinine increased from 1:9 to 1:99.

Table 6.8. Determination of creatine in the presence of creatinine using SIA/amperometric

E (mV)	Recovery Creatine, (%)* Creatine: Creatinine				
	2:1	1:1	1:2	1:4	1:9
240	99.80 ± 0.02	99.81 ± 0.02	99.79± 0.01	99.82± 0.02	99.80 ± 0.01
650	99.68 ± 0.01	99.70 ± 0.01	99.69 ± 0.02	99.72± 0.01	99.71 ± 0.01

* n=10

In the uniformity content tests of Enermax Creatine capsules and Roboforce effervescent creatine, the height of the peaks were 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 compound, and furthermore, it's content in the corresponding pharmaceutical formulation. The results

obtained for the uniformity content tests (Tables 6.9 and 6.10) show that the tested pharmaceutical formulations contain small amounts of creatinine. The recoveries of creatine, is within the ranges required by the labeled amount of 90-110%, with RSD values less than 6.00% required by the USP XXV [11].

Table 6.9. Recovery of creatine and creatinine in Enermax Creatine capsules (500 mg creatine/capsule) using SIA amperometric biosensors system

Type of biosensor	E (mV)	Recovery of Creatine, %	Recovery of Creatinine, (%)*
Bienzyme	240	93.10 ±0.05	-
	650	93.02 ± 0.06	-
Trienzyme	420	-	4.01± 0.12
	650	-	3.98± 0.16

* n=10

Table 6.10. Determination of creatine and creatinine in Roboforce effervescent creatine (5 g creatine/27.5 g of powder) using SIA amperometric biosensors system

Type of biosensor	E (mV)	Recovery of Creatine, (%)*	Recovery of Creatinine, (%)*
Bienzyme	240	96.49 ±0.07	-
	650	96.53 ±0.09	-
Trienzyme	420	-	0.72 ±0.02
	650	-	0.90±0.03

*n=10

Creatine and creatinine were simultaneous assay also from serum samples using a standard method [12] as well as the proposed SIA/amperometric biosensors system (Table 6.11). Good correlation between the results was obtained using both standard and new developed method.

Table 6.11. Determination of creatine and creatinine from serum samples using SIA amperometric biosensors system

Standard method [12] (μ mol/L)			Proposed method (μ mol/L)	
Number	**Creatine**	**Creatinine**	**Creatine**	**Creatinine**
1	19	20	18.99	19.05
2	18	50	17.43	49.30
3	6.9	73	6.72	72.99
4	12	215	11.19	214.18

All values are average of ten determinations. RSD % values are lower than 0.1%.

6.5. Conclusion

The main advantage of the proposed system are: simplicity of construction and operation involved for biosensors as well as for the sequential injection analysis system; 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.

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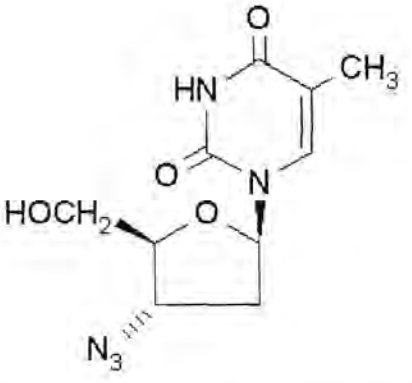
Chapter 7

On-line assay of azidothymidine

7.1. Introduction

Azidothymidine (zidovudine, AZT [1]) (Scheme 1) is a thymidine analogue antiretroviral drug active against human immunodeficiency virus (HIV). AZT acts through the inhibition of nucleoside/nucleotide reverse transcriptase and when administered orally and intravenously it proved effective in reducing the incidence of opportunitic infections and neoplasms in acquired immunodeficiency syndrome (AIDS) and AIDS-related complex patients. AZT also increases helper T lymphocyte numbers and improves survival rates and quality of life [1-3]. Although several antiviral agents have been developed, AZT remains the mainstay in the treatment of patients infected with HIV [1,4].



Scheme 1. The structure of azidothymidine

Among electrochemical immunosensors, amperometric immunosensors represent the best combination of sensitivity and selectivity; since amperometric transducers ensure the highest sensitivity and the immunoreaction ensure the best selectivity [5-7]. The reliability of immunosensors construction is influencing the reliability of the analytical information and it will also contribute to the validation of the immunosensors for pharmaceutical analysis [8]. Accordingly, a physical immobilization of the antibody into carbon paste is preferred for the design of the amperometric immunosensor [5].

Separation (e.g., CZE [9,10], HPLC [11-17]), immunoassay (e.g., RIA [18-20], ELISA [20,21]) and polarographic [22] techniques as well as UV/Vis spectrometry [20], and mass spectrometry [20], were described for the assay of AZT.

In this chapter an amperometric immunosensor based on the physical immobilisation of anti-AZT in a carbon paste is proposed for the assay of AZT. Due to the reliability of its response characteristics, the immunosensor was incorporated in the conduits of a sequential injection analysis (SIA) system and used for on-line determination of AZT from the pharmaceutical formulation.

7.2. Experimental section

7.2.1. Reagents and materials

The immunological system composed from azidothymidine and anti-azidothymidine was purchased from Sigma as a kit (St. Louis, MO, USA). APO-Zidovudine capsules were obtained from APOTEX, Inc, Ville St, Laurent, Que, Canada. Graphite powder with a particle size of 50 μm was obtained from Merck (Darmstadt, Germany). Paraffin oil was

obtained from Fluka (Buchs, Switzerland). All other reagents were of the highest analytical grade. All the solutions were prepared using doubly distilled water.

7.2.2. Amperometric immunosensor design

The antiserum was diluted to a working dilution of 1:30 in 0.01 mol/L phosphate buffered saline, pH = 7.4, containing 0.1% sodium azide. The graphite powder was heated at 700°C for 15 s in a Muffle furnace and cooled to ambient temperature in a dessicator. The paraffin oil and graphite powder were mixed in a ratio of 1:4 (w/w) and then it was added to the diluted anti-AZT to obtain a final composition of 0.9% (w/w) in anti-AZT. The carbon paste (graphite powder and paraffin oil) was filled into a plastic pipette tip leaving about 3 to 4 mm empty in the top to be filled with the chemical modified carbon paste that contained anti-AZT. The diameter of the immunosensor was 3 mm. The electric contact was done by inserting a silver wire in the carbon paste. Before each use, the surface of the electrode was wetted with double distilled water and then polished with an alumina paper (polishing strips 30144-001, Orion). When not in use, the amperometric immunosensor was stored in a dry state at 5°C.

7.2.3. Apparatus

A 663 VA Stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 20 and a software version 4.8 was used for all amperometric measurements. A platinum electrode and a Ag/AgCl (0.1 mol/L KCl) electrode served as counter and reference electrodes in the cell.

The electrodes were incorporated into the conduits of a SIA system shown in Figure 7.1A. It was constructed from: a Gilson Minipuls peristaltic pump and a 10-port electrically actuated selection valve (Model ECSD10P, Valco Instruments, Houston, Texas).

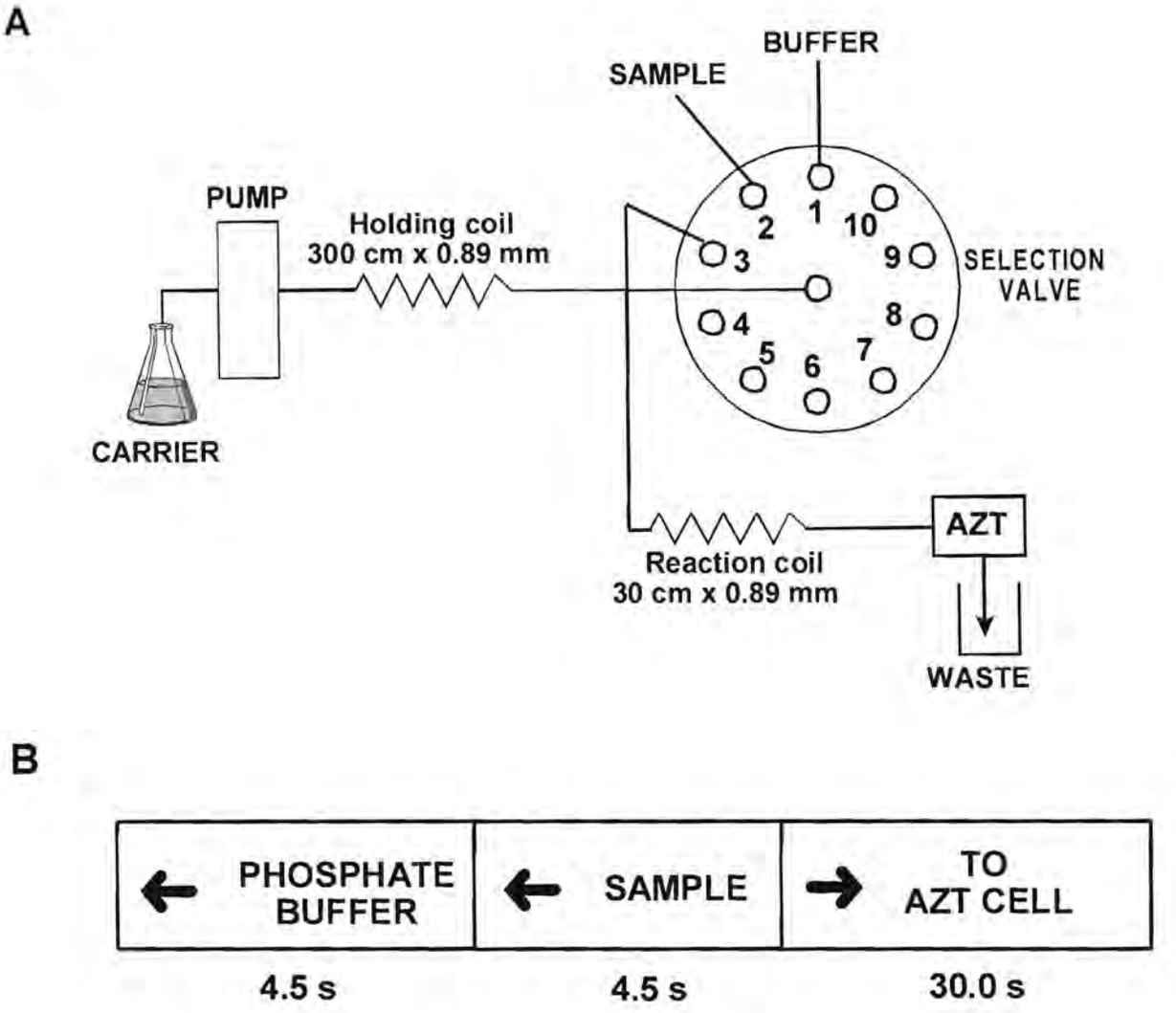


Figure 7.1. SIA system used for the determination of AZT (A) Schematic flow diagram (B) sequence of sample, phosphate buffer, and electrochemical cells for AZT.

Tygon tubing (0.89 mm i.d. for the holding coil and 0.76 mm for the reaction coil) was used to construct the manifold; coils were wound around suitable lengths of glass tubing (15 mm o.d.). A 0.1 mol/L NaCl solution was used as carrier. The capacity of the system is about 75 samples per hour. The device operating sequence is shown in Table 7.1. The device control was achieved using a PC30-B interface board (Eagle Electric, Cape Town, South Africa). The FlowTEK [23,24] software package (MINTEK, Randburg, South Africa) for computer-aided flow analysis was used throughout for device control.

Table 7.1 Device sequence for one cycle of the SIA system

Time (s)	Pump	Valve	Description
0	Off	Buffer	-pump stop, select buffer stream (valve position 1)
5	Reverse	Buffer	-draw up buffer solution
9.5	Off		-pump stop
10.5		Sample	-select sample stream (valve position 2)
11.5	Reverse	Sample	-draw up sample solution
16	Off		-pump stop
17		AZT Cell	-select AZT cell line (valve position 3)
18	Forward		-pump stack of zones to AZT cell
48	Off		-pump stop

An optimum flow rate of 3.61 mL/ min was used to propel the solutions. The timing and flow direction is shown in Figure 7.1B. The sample and buffer consumption is only 270 µL of sample, which is very economical.

7.2.4. Recommended procedures

7.2.4.1.Direct amperometry

The technique used for the direct amperometric assay was chronoamperometry; the potential applied was +435 mV vs. Ag/AgCl. The working temperature was 25°C. The sensor was dipped into a thermostated cell (25°C) containing 10 mL of phosphate buffered saline, pH = 7.4, containing 0.1% sodium azide. Different aliquots of stock AZT solution ($c = 10^{-3}$ mol/L) were added to generate a series of concentration steps.

7.2.4.2.Content uniformity test for APO-Zidovudine capsules

Ten Zidovudine capsules (100 mg AZT/capsule) are individually placed in ten 100 mL calibrated flasks, and solved in the phosphate buffer. The apparatus cell was filled with the prepared solution and the current developed was measured. The unknown concentration was determined from the calibration graph. In the proposed immunosensor/SIA system peak heights were measured and interpolated into the calibration graph.

7.3. Results and discussion

7.3.1. Electrode response

The electrode response was determined using a chronoamperometric technique (E = +435 mV vs Ag/AgCl). The calibration equations obtained for the amperometric immunosensor when used without and with the SIA system are as follows:

without SIA: $I = 3.8 + 0.1\ c$; $r = 0.9986$

With SIA: $H = -0.12 + 7.38\ c$; $r = 0.9998$

where I (<I> = nA) is the intensity of the current, H (<H> = nA) is the peak height, and c (<c> = nmol/L) is the concentration of AZT. All the values are the average of the measurements performed during one week (10 measurements for each concentration were made every day). The RSD (%) values for the intensity of current and peak height, respectively, in one day were lower than 1%, and between the first and seventh day, lower than 2%. Accordingly, the response obtained for the immunosensor revealed a good stability and reproducibility for one week over the tests performed.

The limits of detection for the amperometric immunosensor are: 20 nmol/L and 10 nmol/L when the sensor is used without and with SIA system, respectively, with the corresponding

working concentration range between 40 and 200 nmol/L and between 80 and 2000 nmol/L, respectively. The response time of the amperometric immunosensor is 20s.

7.3.2. Analytical applications

The immunosensor proved to be useful for the purity tests of AZT using the chronoamperometric (E = +435 mV vs Ag/AgCl electrode) technique without and with SIA system. An average recovery of 99.98 ± 0.03% (n=10) and of 99.98 ± 0.01% (n=10) were recorded for the immunosensor when used without and with a SIA system, respectively.

The results obtained for the uniformity content test are presented in Table 7. 2. AZT can be reliably assayed from the APO-Zidovudine capsules with an average recovery of 99.74 ± 0.13% (n=30) and of 99.74 ± 0.10% (n=10) were recorded for the immunosensor when used without and with a SIA system, respectively. The results are in good agreement, and within the range given in The United States Pharmacopoeia XXIV [25]: 90 to 110% AZT in a capsule.

Table7.2. Uniformity content test of APO-Zidovudine capsules using immunosensor without and with SIA system

Recovery, AZT (%)*	
Without SIA	**With SIA**
99.53 ± 0.05%	99.57 ± 0.02%
99.97 ± 0.04%	99.95 ± 0.02%
99.72 ± 0.06%	99.71 ± 0.01%

*n=30

7.4. Conclusion

The construction of immunosensor is simple and reproducible. The reliability of the analytical information is assured by the RSD values obtained in the recovery tests. The proposed amperometric immunosensor/SIA system is suitable for on-line purity tests of AZT as raw material and from its pharmaceutical formulations, at a rate of approximately 75 samples per hour when used as detector in SIA system. The main advantage of the proposed method over the other methods described for AZT assay is the possibility of its determination directly without any prior separation with a high precision, rapidity, low consumption of sample and buffer, and utilization of a not expensive carrier, NaCl solution 0.1 mol/L.

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Chapter 8

Diamond paste based electrodes for the assay of creatine, creatinine and azidothymidine

8.1. Simultaneous assay of creatine and creatinine

8.1.1. Introduction

Creatine is a naturally occurring metabolite in the body. Some is derived from dietary sources, but endogenous synthesis covers individual requirements. Creatine deficiency occurs only in subjects with genetic disorders. Oral supplementation at supraphysiological does seems to slightly improve the performance of some types of muscle exercise, but only those lasting less than 30 seconds; even this small effect is inconsistent. Creatine is a powerful energy conversion molecule that plays an important role in creating muscle energy by converting adenosine triphosphate into adenosine diphosphate allowing for more intense activity [1]. Creatinine, as the final product of creatine metabolism, is a widely used clinical parameter for evaluation of renal function and muscle damage [2]. Its serum concentration and its urinary excretion are not affected by short-term dietary changes increasing but if the normal value of creatinine in the body increase result in severe renal insufficiency.

The following techniques are proposed for the assay of creatine and creatinine: HPLC [3-5,] mass spectroscopy [6], NMR [7,8], and capillary zone electrophoresis [9,10].

Enzymatic biosensors, allowing direct measurements of creatine and creatinine in sample, promising economy of time and saving of costs in pharmaceutical and clinical analysis by enabling on-site analysis of samples. The technique is based on active enzymes immobilization on matrix inducing electrochemical detectable changes.

In this chapter, a new design for amperometic biosensors based on monocrystalline diamond paste is proposed. The advances in single-crystal diamond have enabled the development of a wide range of monocrystalline diamond products to meet the exacting requirements of many electrochemical applications [11]. Also, the monocrystalline diamond has got good holes and electrons, mobility [12]. The enzymes used for the biosensors design were creatininase (CA), and/or creatinase (CI) and sarcosine oxidase (SO). The three enzyme sequence catalyses the conversion of creatinine via creatine and sarcosine to glycine, formaldehyde and hydrogen peroxide as depicted in the following reaction sequence [2]:

$$creatinine + H_2O \xleftrightarrow{CA} creatine$$
$$creatine + H_2O \xleftrightarrow{CI} sar\cos ine + urea$$
$$sar\cos ine + H_2O + O_2 \xleftrightarrow{SO} formaldehyde + glycine + H_2O_2$$

Amperometric detection of enzymatically generated hydrogen peroxide gives direct access to the original creatinine and creatine concentration. For more information about creatine and creatinine see paragraph 6.1.

8.1.2. Experimental section

8.1.2.1. Reagents and materials

Natural diamond powder with particle size ca.50 μ was purchased from Aldrich. Paraffin oil was purchased from Fluka (Buchus, Switzerland); phosphate buffer (pH=7.6) was purchased from Merck (Darmstadt, Germany). De-ionized water from a Modulab system (continental water systems, San Antonio, TX, USA) was used for all solution preparations. Creatinase (CI) from Flavobacterium was obtained from Fluka, Sarcosine oxidase (SO) from Arthrobacterium species was obtained from Aldrich, and creatininase (CA) from Flavobacterium species was obtained from Sigma. Creatine and creatinine were purchased from Aldrich. Solutions of creatine and creatinine (10^{-4} mol/L) were prepared in de-ionized water. Enermax Creatine capsule (500mg creatine monohydrate/capsule) were obtained from Nutrent (Sandton, South Africa) and Roboforce effervescent creatine with ribose (5g creatine/27.5g powder) was purchased from EAS (Golden, CO, USA).

8.1.2.2. Amperometric biosensors design

Bi- and trienzyme electrodes were constructed. All the enzyme solutions used for the design of the biosensors were prepared in a 0.1 mol/L phosphate buffer pH=7.6.

Two plastic tips were filled with plane diamond paste leaving an empty space of 3-4 mm in the top part filled with diamond 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 diamond paste. The biosensors tips were gently rubbed on fine abrasive paper to produce a flat surface. The surface of the biosensors

were wetted with de-ionized water and then polished with an alumina paper (polished strips 30144-001, Orion) before use. The biosensors were stored dry at 4^0C, when not in use.

8.1.2.2.1. Bienzyme electrode for assay of creatine

2 µL of CI solution (0.2 µg CI/25 µL phosphate buffer pH 7.6) was mixed with 2 µL of SO solution (0.2 µg SO/25 µL phosphate buffer pH 7.6). The mixture was incorporated in the diamond paste (25 mg diamond powder and 5 µL paraffin oil), to obtain the bienzyme electrode.

8.1.2.2.2. Trienzyme electrode for assay of creatinine

5 µL paraffin oil and 25 mg diamond powder were mixed to form a diamond paste. 2 µL of CI solution (0.2 µg CI/25 µL phosphate buffer pH 7.6) was first mixed with 2 µL of SO solution (0.2 µg SO/25 µL phosphate buffer pH 7.6) and then with 1 µL of CA solution (0.2 µg CA/25 µL phosphate buffer pH 7.6). The resulting solution was added to the diamond paste.

8.1.2.3. Apparatus

A 663 VA stand (Metrohm, Herisau, Switzerland) in connection with a PGSTAT 20, a Multiplexer module SCNR16 and a software (Eco Chemie version 4.8) was used for all chronoamperometric measurements. A Pt electrode and an Ag/AgCl electrode served as counter and reference electrodes in the cell.

8.1.2.4. Recommended procedures

8.1.2.4.1. 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 =7.6 and different aliquots of creatine and creatinine solutions. The intensity of current measured was plotted versus the concentration of creatine and creatinine. The unknown concentration of creatine was determined directly from the calibration graph of the bienzyme electrode. Because the trienzyme electrode can determine the full amount of creatine and creatinine in the solution, the concentration of creatinine was determined by making the difference between the concentration obtained from the calibration graph of the trienzyme electrode and the concentration of creatine determined using the bienzyme electrode.

8.1.2.4.2. Uniformity content test

Ten Creatine capsules (500 mg Creatine/capsule) were individually placed in ten 100 mL volumetric flasks, and dissolved in de-ionized water. 0.0182 g of Roboforce effervescent creatine with ribose (5 g creatine/27.5 g powder) were dissolved in 100 mL calibrated flask and then diluted to the mark with de-ionized water. Different aliquots from the solutions prepared were added to phosphate buffer (pH=7.6) in the electrochemical cell. Direct amperometry was used to determine the unknown concentration of creatine and creatinine in pharmaceutical formulations.

8.1.2.4.3. Determination of creatine and creatinine in serum samples

Different aliquots of serum samples were diluted with buffer solution. Direct amperometry was involved to determine the content on creatine and creatinine in serum samples.

8.1.3. Results and discussion

8.1.3.1.Response characteristics of the amperometric biosensors

The response characteristics of the amperometric biosensors are shown in Table 8.1. For both biosensors, the working concentration ranges are in the fmol/L magnitude order with very low limits of detection. The properties of the diamond - used as matrix in biosensors design (low background current, high signal/noise ratio, lack of adsorption) are the explanation for such behaviour of the proposed biosensor. The response characteristics obtained for the biosensors revealed good stability and reproducibility for tests performed over one week, when used every day for the measurements.

Table 8.1. Response characteristics for the amperometric biosensors designed for creatine and creatinine

Type of biosensor	E (mV)	Linear conc. range (fmol/)	Detection limit (fmol/L)	t_R (s)	Equation of calibration*	r
Bienzyme	200	$4 \times 10^{-4} - 1 \times 10^{-3}$	2×10^{-4}	180	$I=5.68 \times 10^{-4} + 21.23C$	0.9992
Trienzyme	400	$2 \times 10^{-4} - 2 \times 10^{-3}$	1×10^{-4}	60	$I=9.67 \times 10^{-4} + 54.60C$	0.9986

*I is the intensity of current in mA, and C is the concentration of creatine and creatinine in fmol/L.

8.1.3.2. Selectivity of the amperometric biosensors

The selectivity of both biosensors was checked using both the mixed and separate solutions methods. 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 creatinine was selected to be ten times higher than that of creatine. The value of the amperometric selectivity coefficient ($pK_{i,j}^{amp}$) (obtained using the mixed solution method) for the bienzyme biosensor over creatinine was 2.65. This $pK_{i,j}^{amp}$ value shows that the creatinine does not interfere in the assay of creatine.

The selectivity of both electrodes was tested also over glycine, methionine, ribose and taurine. For all of these substances, the values of the amperometric selectivity coefficients are less than $1x10^{-5}$. These results proved the selectivity of the proposed electrodes over these substances that can be found in the pharmaceutical formulations of creatine.

8.1.3.3. Analytical applications

The proposed amperometric biosensors were used with good results for the determination of creatine and creatinine as raw materials, in pharmaceutical products and serum. The advantage of the proposed method is high sensitivity and selectivity and no need of separation of creatine and creatinine from pharmaceutical products or biological fluids.

In order to prove the accuracy of the assay of creatine in the presence of creatinine, synthetic samples containing creatine and creatinine in different ratios were prepared. The results obtained for the recovery of creatine in the presence of creatinine (Table 8.2) demonstrated the suitability of the proposed amperometric biosensor for testing the purity

of creatine. No significant differences in the recovery tests were recorded for creatine: creatinine ratios between 1:9 and 1:99.9.

Table 8.2. Determination of creatine in the presence of creatinine

Recovery of creatine, (%)*				
Creatine: Creatinine				
2:1	1:1	1:2	1:4	1:9
99.89 ± 0.01	99.90 ± 0.01	99.87± 0.02	99.89± 0.02	98.91 ± 0.01

*n=10

The uniformity content tests show that the tested pharmaceutical formulations: Enermax Creatine capsules (500mg creatine monohydrate/capsule) and Roboforce effervescent creatine with ribose (5g creatine/27.5g powder) contain as main component creatine (average recovery, 93.12±0.10% and 96.12±0.12%, respectively) and only small amounts of creatinine (impurity resulted in the synthesis of creatine) (average recovery, 3.87±0.18% and 0.70±0.05%, respectively). The recovery values for creatine are within the labelled amount of 90-110%, with RSD values less than 6.00% required by the USP XXV [14].

Creatine and creatinine were simultaneous assay also from serum samples using a standard method [15] as well as the proposed biosensors (Table 8.3). Good correlation between the results was obtained using both standard and new developed method.

Table 8.3. Recovery of creatine and creatinine in serum samples

Standard method [15](µmol/L)			Proposed (µmol/L)	
Number	**Creatine**	**Creatinine**	**Creatine**	**Creatinine**
1	19	13	18.92	12.68
2	70	54	69.54	53.02
3	6.9	79	6.84	78.98
4	14	154	13.90	153.82

All values are average of ten determinations. RSD % values are lower than 0.1%.

The amperometric biosensors described have excellent features in the assay of creatine and creatinine in pharmaceutical compounds and biological fluids. Due to the high biocompatibility of the diamond, the electrodes will be able to be use, after miniaturization, for *in vivo* simultaneous assay of creatine and creatinine. The construction of the electrodes is simple, fast and reproducible and it is also assuring reliable response characteristics for the proposed amperometric biosensors.

8.2. Differential pulse voltammetry for determination of creatine and creatinine

8.2.1. Differential pulse voltammetry

The technique used for the direct voltammetric assay was differential pulse voltammetry with the applied potential pulse amplitude of 25mV vs. Ag/AgCl. The diamond paste electrode together with the reference and auxiliary electrodes were dipped into a cell containing phosphate buffer (pH=8.0 for the assay of creatine, and pH=7.0 for the assay of creatinine) and sodium pyrophosphate as supporting electrolyte in a ratio of 3.5:1, as well as the synthetic mixture between creatine and creatinine. All solutions were deoxygenated for 5 min before the measurements with N_2. The peak height measured at 500 mV and 580 mV vs Ag/AgCl for creatinine and creatine, respectively, was plotted versus their concentration. The unknown concentrations of creatine and creatinine were determined from the corresponding calibration graphs.

8.2.2. Apparatus

A 663 VA Stand (Metrohm, Herisau, Switzerland) in combination with a μAutolab and software (Ecochemie version 4.8) were used for all differential pulse voltammetry measurements. A platinum electrode and an Ag/AgCl (0.1 mol/L KCl) electrode served as the counter and reference electrodes in the cell.

8.2.3. Electrode design

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

8.2.4. Uniformity content test

Ten Enermax Creatine capsules (500mg creatine monohydrate/capsule) were individually placed in ten 100 mL volumetric flasks, and dissolved in de-ionized water. 0.0182 g of Roboforce effervescent creatine with ribose (5 g creatine/27.5 g powder) were dissolved in 100 mL calibrated flask and then diluted to the mark with de-ionized water. Different aliquots from the solutions prepared were added to phosphate buffer (pH=7.0 or pH=8.0) in the electrochemical cell. Differential pulse voltammetry was used to determine the unknown concentrations of creatine and creatinine in pharmaceutical formulations.

8.2.5. Determination of creatine and creatinine in serum samples

Different aliquots of serum samples were diluted with buffer solution. Differential pulse voltammetry was involved to determine the content on creatine and creatinine in serum samples.

8.2.6. Electrode response

The electrode response was determined using differential pusle voltamettry (DPV) technique. The calibration equations obtained for creatine and creatinine were as follows:

Creatine: $H = 6.025 + 0.108\ c$; $r = 0.9563$

Creatinine: $H = 0.214 + 0.01\ c$; $r = 0.9969$

where H is the peak height (<H>=μA), c is the concentration of creatine, and creatinine, respectively (<c>= pmol/L for creatine <c>=nmol/L for creatinine), and r is the correlation coefficient.

For the assay of creatine, the linear concentration range is between 1 and 500 pmol/L with a limit of detection of 0.1 pmol/L, while for the assay of creatinine, the linear concentration range is between 0.01 and 100 nmol/L with a limit of detection of 1 pmol/L. The signal to background ratio is very high when compared to classical glassy carbon (GC) and carbon paste electrodes. The reproducibility of peak current was excellent (RSD% values recorded were less than 0.1%), when the measurements were done everyday for a period of 6 months.

8.2.7. Selectivity of the diamond paste based electrode

The selectivity of the electrode was checked using the mixed solution method. The amperometric selectivity coefficients were determined using the equation proposed by Wang [13]. The ratio between the concentrations (mol/L) of the main analyte and interferent was 1:10. The $pK_{i,j}^{amp}$ values of the selectivity coefficients were 2.60 when the main analyte was creatine and the supposed interferent creatinine and 3.34 when the main analyte was creatinine and the supposed interferent creatine. These values indicates that creatine and creatinine can be determined simultaneous from the solutions using the same electrode, but at different pH values.

The selectivity of the diamond paste electrode was tested also over glycine, methionine, ribose and taurine. For all of these substances, the values of the amperometric selectivity coefficients are less than $1x10^{-4}$. These results proved the selectivity of the proposed electrodes over these substances that can be found in the pharmaceutical formulations of creatine.

8.2.8. Analytical applications

The differential pulse voltammtey proved useful for determination of the creatine and creatinine as raw materials as well as in pharmaceutical formulations and serum samples. The uniformity content tests show that the tested pharmaceutical formulations: Enermax Creatine capsule (500mg creatine monohydrate/capsule) and Roboforce effervescent creatine with ribose (5g creatine/27.5g powder) contain as main component the creatine (average recoveries, 95.65±0.19% and 92.46±0.21%, respectively) and only small

amounts of creatinine (average recoveries, 3.25±0.16% and 0.75±0.09%, respectively). The recovery values for creatine are within the labelled amount of 90-110%, with RSD values less than 6.00% required by the USP XXV [14].

Creatine and creatinine were simultaneous assayed also from serum samples using a standard method [15] as well as the proposed diamond paste based electrode (Table 8.4). Good correlation between the results was obtained using both standard and new developed method.

Table 8.4. Recovery of creatine and creatinine from serum samples

Standard method [15] (μ mol/L)			Proposed method (μ mol/L)	
Number	**Creatine**	**Creatinine**	**Creatine**	**Creatinine**
1	20	20	19.87	19.81
2	80	80	79.62	79.04
3	8.9	83	8.84	82.34
4	20	420	19.88	416.43

All values are the average of ten determinations. RSD(%) values are lower than 0.1%.

The electrode described has excellent features in the simultaneous assay of creatine and creatinine in pharmaceutical products and biological fluids. Furthermore, the utilization of diamond as electrode material is making possible the utilization of the proposed electrode for *in vivo* simultaneous assay of creatine and creatinine. The construction of the electrode is simple, fast and reproducible and it is also assuring reliable response characteristics for the proposed electrode.

8.3. Diamond paste based immunosensors for the determination of azidothymidine

Azidothymidine (zidovudine, AZT) is a thymidine analogue antiretroviral drug active against the human immunodeficiency virus (HIV). For more information about azidothymidine see paragraph 7.1.

Among electrochemical immunosensors, amperometric immunosensors represent the best combination of sensitivity and selectivity, hence amperometric transducers ensure the highest sensitivity and the immunoreaction ensures the best selectivity [16-18]. The reliability of immunosensors construction is influencing the reliability of the analytical information and it will also contribute to the validation of the immunosensors for pharmaceutical analysis [19]. Accordingly, a physical immobilization of the antibody into diamond paste is preferred for the design of the amperometric immunosensor [16].

8.3.1. Experimental section

8.3.1.1. Reagents and materials

The immunological system composed from azidothymidine was obtained from Sigma (St.Louis, MO, USA). APO-Zidovudine capsules were obtained from APOTEX, Inc, Ville St, Laurent, Que, Canada. Diamond powder with a particle size ca .50μ was obtained from Aldrich. Paraffin oil was obtained from Fluka (Buchs, Switzerland). All other reagents were of the highest analytical grade. All the solutions were prepared using de-ionized water.

8.3.1.2. Apparatus

A 663 VA Stand (Metrohm, Herisau, Switzerland) connected to a μAutolab and software version 4.8 was used for all amperometric measurements. A platinum electrode and a Ag/AgCl (0.1 mol/L KCl) electrode served as counter and reference electrodes in the cell.

8.3.1.3. Amperometric immunosensor design

The antiserum was diluted to working dilution of 1:30 in 0.01 mol/L phosphate buffer saline, pH=7.4, containing 0.1 % sodium azide. The paraffin oil and diamond powder were mixed in a ratio of 1:4 (w/w) and then it was added to the diluted anti-AZT to obtain a final composition of 0.9% (w/w) in anti-AZT. The diamond paste (diamond powder and paraffin oil) was filled into a plastic tip leaving about 3 to 4 mm empty in the top to be filled with the chemical modified diamond paste that contains anti-AZT. The diameter of the immunosensor was 3mm. The electric contact was done by inserting a silver wire in the diamond paste. Before each use, the surface of the electrode was wetted with double distilled water and then polished with an alumina paper (polishing strips 30144-001, Orion). When not in use, the amperometric immunosensor was stored in a dry state at 4^0C.

8.3.1.4. Recommended procedures

8.3.1.4.1. Direct amperometry

The technique used for the direct amperometric assay was chronoamperometry; the potential applied was +240 mV vs. Ag/AgCl. The working temperature was 25^0C .The sensor was dipped into a thermostatic cell (25^0C) containing 10 mL of phosphate buffered

saline, pH= 7.4 containing 0.1% sodium azide. Different aliquots of stock AZT solution ($c=10^{-4}$ mol/L) were added to generate a series of concentration steps.

8.3.1.4.2.Uniformity content test for APO-Zidovudine capsules

Ten Zidovudine capsules (100 mg AZT/capsule) are individually placed in ten 100 mL calibrated flasks, and solved in the phosphate buffer. The apparatus cell was filled with the prepared solution and the current developed was measured. The unknown concentration was determined from the calibration graph.

8.3.2. Response characteristics of the amperometric immunosensors

The electrode response was determined using the chronoamperometric technique (E= +240mV vs. Ag/AgCl). The calibration equations obtained for the amperometric immunosensor is as follows:

$$I=3.05+42.7c;\ r=0.9999$$

where I (<I> =μA) is the intensity of the current and c (<c>= fmol/L) is the concentration of AZT.

The limit of detection for the amperometric immunosensor is $2x10^{-4}$ fmol/L with working concentration range between $4x10^{-4}$ and $6x10^{-2}$ fmol/L. The response time of the amperometric immunosensor is 60s. The response obtained for the immunosensor revealed a good stability and reproducibility for one week over the tests performed.

8.3.3. Analytical applications

The immunosensor proved to be useful for the purity tests of AZT using the chronoamperometric (E= +240mV vs. Ag/AgCl electrode) technique. An average recovery of 99.96±0.03% (n=10) was recorded for the assay of AZT raw material.

AZT can be reliably assayed from the APO-Zidovudine capsules with an average recovery of 98.71±0.21% (n=30). The results are in good agreement, and within the range given in the United State Pharmacopoeia XXIV [20]: 90 to 110% AZT per capsule.

8.4. Conclusion

The amprometric biosensors electrodes described have excellent features in the assay of creatine and creatinine in pharmaceutical compounds and biological fluids. Due to the high biocompatibility of the diamond, the electrodes will be able to be use, after miniaturization, for *in vivo* simultaneous assay of creatine and creatinine. The construction of the electrodes is simple, fast and reproducible and it is also assuring reliable response characteristics for the proposed amperometric biosensors. The construction of immunosensor is simple and reproducible. The reliability of the analytical information is assured by the RSD value obtained in the recovery tests. The proposed amperometric immunosensor is suitable for the assay of AZT raw material as well as from its pharmaceutical formulations. The main advantage of the proposed method over the other methods described for AZT assay is the possibility of its determination directly without any prior separation with a high precision, rapidity, low consumption of sample and buffer.

8.5. References

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Chapter 9

Conclusions

There is an increase demand for rapid analysis and miniaturization in industrial and process analytical control. SIA is a technique that has a tremendous potential for on-line processes measurements. The development of this technique was aimed to provide pharmaceutical industries with reliable, precise and cost-effective instrumentation of performing analysis.

The importance of this work was to develop a biosensors and immunosensors capable for on-line determinations. The system must have a high sample throughput with minimum reagent and sample consumption. Sequential injection analysis was coupled to the amperometric detectors with the aim of meeting the objectives mentioned above. The method confirmed its feasibility for use in process analysis because it is fully computerized with minimum reagent and sample consumption and high sample frequency. The main advantages of the proposed system are: simplicity of construction and operation involved for biosensors and immunosensors as well as for the sequential injection analysis system; possibility of on-line simultaneous monitoring of enantiomers during the synthesis of methotrexate, carnitine, ortho-acetyl-L-carnitine as well as of azidothymide, creatine and creatinine, and high reliability of analytical information,

rapidity and, low cost of analysis. The high precision of the SIA over the manual methods is due to the fact that all measurements are done after the same interval of time and the surface of the biosensors and immunosensors are continuously brushed by the sodium chloride or phosphate buffer carrier streams.

The amprometric biosensors and immunosensors described have excellent features in the assay of enantiomers in pharmaceutical compounds and biological fluids. For the case of diamond paste due to the high biocompatibility, the electrodes will be able to be use, after miniaturization, for *in vivo* simultaneous assay of creatine and creatinine. The construction of the electrodes is simple, fast and reproducible and it is also assuring reliable response characteristics for the proposed amperometric biosensors and immunosensors.