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Short communication

Simultaneous determination of L- and D-carnitine using a sequential injection analysis/amperometric biosensors system

Raluca-Ioana Stefan [a,*], Rahel Girmai Bokretsion [a], Jacobus F. van Staden [a], Hassan Y. Aboul-Enein [b]

[a] *Department of Chemistry, University of Pretoria, Pretoria 0002, South Africa*
[b] *Pharmaceutical Analysis and Drug Development Laboratory, Biological and Medical Research Department (MBC-03-65), King Faisal Specialist Hospital and Research Centre, P.O. Box 3354, Riyadh 11211, Saudi Arabia*

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Abstract

A sequential injection analysis (SIA) system is described for the simultaneous determination of L-and D-carnitine using amperometric biosensors as detectors. The SIA system was used, because of its high precision, accuracy and low sample and buffer consumption. The biosensors were designed using physical and chemical immobilization of L-amino acid oxidase and horseradish peroxidase (HRP) for the assay of L- carnitine, and D-amino acid oxidase and HRP for the assay of D-carnitine. The linear concentration ranges are in the pmol/l to nmol/l magnitude order, with very low limits of detection. The biosensors/SIA system was used reliably for on-line process control of the enantiopurity of carnitine with a frequency of 34 samples per hour.

Keywords: L(D)-Carnitine; Amperometric biosensors; L(D)-Amino acid oxidase; Horseradish peroxidase; Sequential injection analysis

1. Introduction

Enantiopurity tests of biologically active substances became increasingly important for chiral drugs, because in most cases only one of the enantiomers possesses the desired pharmacological effects while the other one can be toxic, less active, or can have unwanted side effects or even act as an antagonist to the pharmacologically active enantiomer [1]. Up to now, most utilized methods for the simultaneous assay of enantiomers are based on chromatography. However, chromatographic techniques do not always provide enough precision for this type of analysis, they are expensive and not easy to use in routine analysis [2].

Sequential injection analysis (SIA) opened the possibility for simultaneous assay using electrochemical sensors as detectors with good accuracy [3,4]. SIA parameters can be optimized for enantiomer's assay. The main advantage for enantiopurity tests is the possibility of on-line simultaneous detection of enantiomers in the technological process. The unique problem is to

* Corresponding author. Tel.: +27-12-420-2510; fax: +27-12-362-5297.
E-mail address: raluca.stefan@chem.up.ac.za (R.-I. Stefan).

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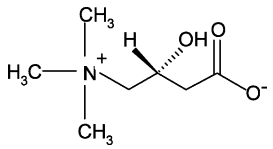
find the best enantioselective sensors for the assay of enantiomers. Accordingly, the ratio between enantiomers and their concentration magnitude order should also be taken into account.

L-Carnitine, (*R*)-3-carboxy-2-hydroxy-*N*,*N*,*N*-trimethyl-1-propaminium hydroxide inner salt (Scheme 1), is a naturally occurring substance, essential for fatty acid oxidation and energy production in human body. Without L-carnitine, long chain fatty acids cannot be transported from the cellular cytoplasm into the mitochondria, resulting in the loss of energy and toxic accumulations of free fatty acids [5]. However, other functions are recognized, such as interconversion in the mechanisms of regulation of cetogenese and termogenese [6]. Also L-carnitine is used in the therapy of primary and secondary deficiency, and in various other diseases such as dislipoproteinemia [7]. D-carnitine has different pharmacokinetic and pharmacodynamic behavior.

Many analytical methods have been reported to analyze carnitine in pharmaceutical formulations and in biological fluids using chromatographic techniques, e.g. HPLC [8], and capillary zone electrophoresis [9–12], fluorimetry [13], and spectrometry [14].

The use of biosensors in SIA systems for the assay of single enantiomers was successful, but only the simultaneous assay of enantiomers using a sequential system will be able to compete with the chromatographic techniques. The emphasis of this paper is on a SIA system designed for the simultaneous assay of the L- and D-carnitine. The electrochemical sensors used as detectors are amperometric biosensors.



Scheme 1. L-Carnitine.

2. Experimental

2.1. Reagents and materials

Graphite powder, 1–2 μm was supplied by Aldrich (Milwaukee, WI, USA). Paraffin oil was obtained from Fluka (Buchus, Switzerland). Phosphate buffer (pH 7.00, prepared using the sodium salts of phosphoric acid) 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-Aldrich)) 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).

2.2. Amperometric biosensors design

Four 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 biosensor tips were gently rubbed on fine abrasive paper to produce a flat surface. The surfaces of the biosensors were wetted with de-ionized water and then polished with an alumina paper (polished strips 30144-011, Orion Research Incorporated, Boston, MA, USA) before use. The biosensors were stored dry at 4 °C, when not in use.

Physical immobilization was used for the design of mono- and bienzyme electrodes. All the enzyme

solutions used for the biosensors design were prepared in 0.1 mol/l phosphate buffer of pH 7.

2.2.1. 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 graphite paste. 100 μl from the solution (1 mg of enzyme/ml) of L-AAOD or D-AAOD, were, respectively, added to two separate portions of carbon paste.

2.2.2. Bienzyme amperometric biosensors

Two mixtures of enzymes were used for the design of amperometric biosensors: (1) 1 mg of HRP was dissolved in 50 μl of L-AAOD solution (0.25 mg/ml); (2) 1 mg 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.

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 the counter and reference electrodes in the cell.

2.4. Sequential injection analysis system

The biosensors were incorporated into the conduits of a SIA system (Fig. 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.); 0.1 mol/l NaCl was used as carrier. The capacity of the system is about 34 samples per hour. 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 the 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 Fig. 1B. The sample and buffer consumption is only 270 μl of each per measurement of L- and D-enantiomer, which is very economical.

3. Results and discussion

3.1. Response characteristics of the amperometric biosensors

The response characteristics of the biosensors were measured at different potentials 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 1). 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 1 week [relative standard deviation (R.S.D.) < 0.1%].

3.2. Selectivity of the biosensors

The selectivity of all biosensors was checked using both the mixed solutions and separate solution methods with respect to L- and D-carnitine. Amperometric selectivity coefficients were determined following the method proposed by Wang [16], 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 the described SIA system (Table 2). The pK_{amp} values show that the biosensor based on L-AAOD has got the best enantioselectivity for L-carnitine assay,

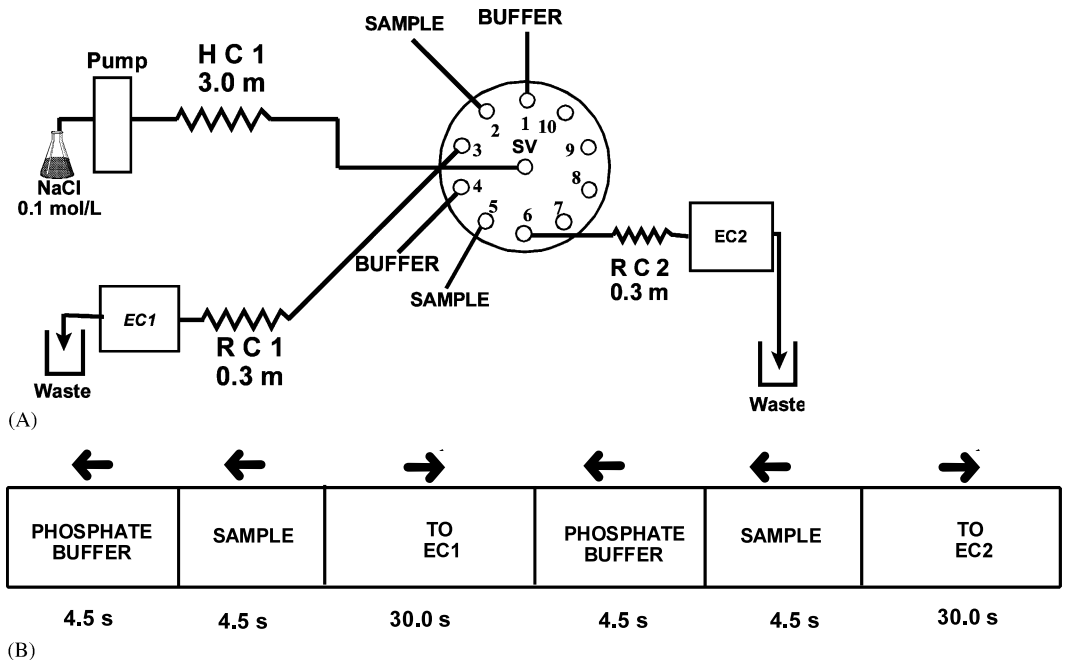


Fig. 1. SIA system used for the simultaneous determination of L- and D-carnitine (A) Schematic flow diagram (B) sequence of sample, phosphate buffer, and electrochemical cells for L-carnitine and D-carnitine. SV, selection valve; HC, holding coil; RC, reaction coil; EC1, electrochemical cell 1 used for the assay of L-carnitine; EC2, electrochemical cell 2 used for the assay of D-carnitine.

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.

3.3. Analytical applications

The results presented in Tables 3 and 4 demonstrated the suitability of the proposed SIA/amperometric biosensor system for the on-line

Table 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 concentration range	Detection limit	Equation of calibration[a]	Correlation coefficient (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

All values are the average of ten determinations (R.S.D. $< 0.1\%$).
[a] 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.

Table 2
Enantioselectivity of amperometric biosensors designed for the assay of L- and D-carnitine in a SIA system

Enzyme(s) used for the design of the biosensor	Enantiomer	E (mV)	pK_{amp}
L-AAOD	D	250	2.55
L-AAOD+HRP	D	450	2.31
	D	650	2.16
D-AAOD	L	380	2.08
	L	650	2.27
D-AAOD+HRP	L	530	3.29
	L	650	3.20

Table 3
Determination of L-carnitine in the presence of D-carnitine

Enzyme(s) used for the design of the biosensor	E (mV)	Recovery of L-carnitine (%)[a]				
		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

[a] All values are the average of ten determinations (R.S.D. < 0.04%).

Table 4
Determination of D-carnitine in the presence of L-carnitine

Enzyme(s) used for the design of the biosensor	E (mV)	Recovery of D-carnitine (%)[a]				
		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

[a] All values are the average of ten determinations (R.S.D. < 0.04%).

Table 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 (%)[a]	Recovery of D-carnitine (%)[a]
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

[a] All values are the average of ten determinations (R.S.D. < 0.9%).

enantiopurity tests of carnitine. No differences were recorded in recovery tests between 1:9 and 1:99 in the favor of each enantiomer.

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 heights of the peaks were recorded using the proposed SIA/amperometric biosensors system. The results were interpolated into calibration graphs for 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 5) 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 R.S.D. values less than 1% [17]. All the concentrations of the L- and D-carnitine fall into the linear concentration ranges of the proposed biosensors. Previous tests (Tables 3 and 4) show that these enantiomers can be accurately determined in the presence of the other. The additives used for the formulation of the carnitine pharmaceutical products do not interfere in its measurements. Accordingly, no addition method was necessary to test the suitability of the electrodes for on-line enantiopurity tests of the active substance in its pharmaceutical formulation.

4. Conclusion

The main advantages of the proposed system are: simplicity of construction and operation that is essential 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 method is due to the fact that all measurements are done during the same time interval and the surface of the biosensors are continuously washed by NaCl solution used as carrier stream.

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