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Talanta 60 (2003) 1223–1228
Talanta
www.elsevier.com/locate/talanta

Simultaneous determination of creatine and creatinine using amperometric biosensors

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Received 16 January 2003; received in revised form 18 March 2003; accepted 19 March 2003

Abstract

In order to determine creatine and creatinine amperometric biosensors were proposed. A bienzymatic biosensor based on creatinase (CI) and sarcosine oxidase (SO) was used for the assay of creatine and a trienzymatic biosensor based on CI, SO and creatininase (CA) for the assay of creatinine. The linear concentration ranges are of pmol l^{-1} to nmol l^{-1} magnitude order, with very low limits of detection. The biosensors proved high reliability for determination of creatine and creatinine as raw material, and in the pharmaceutical formulation.

Keywords: Creatine; Creatinine; Amperometric biosensors; Creatininase; Creatinase; Sorcosine oxidase

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^{-1} in serum, but during kidney dysfunction or muscle disorder their concentrations may raise up to a value higher than 1 mmol l^{-1} [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

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0039-9140/03/$ - see front matter
doi:10.1016/S0039-9140(03)00230-3

Table 1
Response characteristics for the amperometric biosensors designed for creatine and creatinine

Type of biosensor	E (mV)	Linear concentration range	Detection limit	t_R (s)	Equation of calibration[a]	r
Bienzyme	240	4–200 pmol l^{-1}	2 pmol l^{-1}	30	$^{1,a}I = 60.19 + 12.67C$	0.9984
	650	2–20 nmol l^{-1}	1 nmol l^{-1}	120	$^{2,b}I = 0.26 + 2.86C$	0.9998
Trienzyme	420	4–40 nmol l^{-1}	2 nmol l^{-1}	60	$^{2,b}I = 0.11 + 0.04C$	0.9981
	650	4–100 nmol l^{-1}	2 nmol l^{-1}	30	$^{2,b}I = 0.345 + 0.746C$	0.9999

[a] I is the peak height in ^{1}nA and ^{2}A, and C is the concentration of creatine and creatinine, respectively in ^{a}pmol l^{-1} and ^{b}nmol l^{-1}.

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] and UV/Vis spectrometry [9].

The emphasis of this paper is to determine simultaneously the amount of creatine and creatinine using amperometric biosensors. The amperometric biosensors are based on creatininase (CA), creatinase (CI) and sarcosine oxidase (SO) physically immobilized into carbon paste. The enzyme creatininase hydrolyses creatinine to yield creatine. CI hydrolyses creatine first to sarcosine. SO is acting on sarcosine, forming formaldehyde, glycine and hydrogen peroxide—the product that will be measured by the amperometric transducer [10].

The advantages of utilization of carbon paste type of biosensors over plastic membrane [1,11], hydrogel matrix [12], and screen-printed [13] based biosensors proposed previously for the assay of creatine and/or creatinine are: simplicity and rapidity of biosensors design; reliability of the biosensors design that is involving also the reliability of the analytical information obtained; no need for a protection membrane to avoid biosensor contamination and also the interferences from substances found in the biological fluid or pharmaceutical compound matrices. These advantages were reflecting in the response characteristics, selectivity and recovery tests obtained using the proposed carbon paste based amperometric biosensors.

2. Experimental

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^{-1}) 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).

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^{-1} 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

Table 2
Determination of creatine in the presence of creatinine using the bienzyme electrode

E (mV)	Recovery creatine (%)[a]				
	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

[a] $n = 10$.

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 °C, when not in use.

2.2.1. Bienzyme electrode for assay of creatine

2 μl of CI solution (0.2 g CI/25 l phosphate buffer pH 7.6) was mixed with 2 μl of SO solution (0.2 g SO/25 l 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.

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 g CI/25 l phosphate buffer pH 7.6) was first mixed with 2 μl of SO solution (0.2 g SO/25 l phosphate buffer pH 7.6) and then with 1 μl of CA solution (0.2 g CA/25 l phosphate buffer pH 7.6). The resulting solution was added to the carbon paste.

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 SCNR16A was connected to the PGSTAT 20 in order to be able to make the simultaneous analysis of creatine and creatinine.

2.4. Recommended procedures

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

Table 3
Determination of creatine and creatinine in Enermax Creatine capsules (500 mg creatine/capsule)

Type of biosensor	E (mV)	Recovery of creatine (%)[a]	Recovery of creatinine (%)[a]
Bienzyme	240	93.14 ± 0.12	–
	650	93.05 ± 0.10	–
Trienzyme	420	–	3.98 ± 0.12
	650	–	3.82 ± 0.14

[a] $n = 10$.

Table 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, (%)[a]	Recovery of Creatinine, (%)[a]
Bienzyme	240	95.95 ± 0.18	–
	650	96.02 ± 0.16	–
Trienzyme	420	–	0.69 ± 0.06
	650	–	0.72 ± 0.07

[a] $n = 10$.

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.

2.4.2. Uniformity content tests

Ten Enermax Creatine capsule (500 mg 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 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.

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.

2.5. 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 1). The electrode response was highly stable and reproducible over 1 week. The best response characteristics (large concentration range, lower limit of detection and highest sensitivity) for the 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

Table 5
Determination of creatine and creatinine from serum samples

Standard method [12] (μmol l^{-1})			Proposed method (μmol l^{-1})	
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. R.S.D. (%) values are lower than 0.1%.

biosensors revealed good stability and reproducibility for tests performed every day over 1 week (R.S.D. $< 0.1\%$).

The sensitivities obtained for the proposed biosensors are higher than those obtained previously by using the same enzymatic systems for biosensors design [1,11–13]. The limits of detection, the response times and the R.S.D. (%) values are lower for the carbon paste based amperometric biosensors than those obtained by using other matrices for their design [1,11–13].

2.6. 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 [14], 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_{amp}) (obtained using the 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_{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, taurine, ascorbic acid, uric acid and acetaminophen. For all of these substances, the values of the amperometric selectivity coefficients are less than 1×10^{-4}. These results proved the selectivity of the proposed electrodes over these substances that can be found in the pharmaceutical formulations of creatine as well as in the biological fluids. Accordingly, it is a high improvement in the selectivity of the proposed biosensors if one compare with the selectivities obtained by using plastic membrane [1,11], hydrogel matrix [12], and screen-printed [13] based biosensors, proposed previously for the assay of creatine and/or creatinine.

2.6.1. 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 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.

The results obtained for the uniformity content tests are presented in Tables 3 and 4 for Enermax Creatine capsule (500 mg creatine monohydrate/capsule) and Roboforce effervescent creatine with ribose (5 g creatine/27.5 g 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 R.S.D. values less than 6.00% required by the USP XXV [15].

Creatine and creatinine were simultaneous assay also from serum samples using a standard method [16] as well as the proposed biosensors (Table 5). Good correlation between the results was obtained using both standard and new developed method.

3. Conclusions

The amprometric 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.

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