FLOW-INJECTION ENZYMATIC DETERMINATION OF L-LACTATE WITH AMPEROMETRIC DETECTION

Marek TROJANOWICZ * Wojciech MATUSZEWSKI
and Tadeusz KRAWCZYŃSKI VEL KRAWCZYK

Department of Chemistry, University of Warsaw, Pastaura 1,
02-093 Warsaw, Poland

ABSTRACT

Flow-injection system was develpoed for lactate determination in blood serum, in which amperometric detection with a large volume wall-jet cell and a flow-through microreactor with immobilized lactate oxidase were employed. In the optimized system with a dialyser a linear response was obtained up to 40 mM lactate with detection limit 0.4 μM for 20 μl injected sample volume.

Introduction

L-lactate is one of the most important metabolites for which a reliable method of determination is required in food analysis, biotechnology and especially in clinical chemistry. In clinical analysis lactate level is an indication of respiration failures, whereas in sport medicine the blood lactate level is used for estimating the intensity of exercise.

In the design of biosensors or enzymatic measuring systems for the determination of lactate most often lactate oxidase (LOD) and lactate dehydrogenase is utilized, but also cytochrome b_2. In recently reported designs of electrochemical biosensors very much attention is paid to multimembrane systems enabling the effective elimination of electrochemical interferences [1] and to the arrangements, where besides enzyme also appropriate mediator is immobilized in order to improve the sensitivity of biosensor response and to extend the linear calibration range [2,3].

An increasing interest is observed also in the literature in design of measuring flow systems for continuous monitoring of lactate with the use of integrated biosensors, mainly electrochemical ones, or measuring systems with enzymatic reactors and suitable flow detector. The amperometric biosensors with immobilized LOD were applied to continuous monitoring of lactate in the system with continuously withdrawing blood from the patient, 10-fold diluted and

Paper dedicated to the memory of the late Professor Nobuhiko Ishibashi

ultrafiltrated [4] and in a similar system without ultrafiltration, where whole blood prior the measurement was diluted with heparine containing saline [5].

In order to decrease the sample consumption in modern systems for lactate monitoring the flow-injection concept of performing flow analysis is utilized either with chemoluminescence detection of hydrogen peroxide produced in the presence of LOD [6,7] or with electrochemical detections [8,9]. In the latter ones with the use of a Clark oxygen electrode a consumption of oxygen in the reaction of lactate catalyzed by LOD was monitored [8] or puryvate and hydrogen peroxide formed in the same reaction was determined polarographically [9]. The mentioned flow-injection methods were applied to determination of lactic acid in fermentation process of that substrate [7], to determination of lactate in wine [8] and to determination of lactate in deproteinized and diluted human serum samples [9].

The aim of this study was to develop a method of flow-injection analysis for the determination of lactate in the system with LOD immobilized in flow-through microreactor and with anodic detection of hydrogen peroxide formed as the product of lactate enzymatic oxidation.

EXPERIMENTAL

Apparatus

Amperometric measurements were carried out using a polarograph type PLP 225C from Zalmed (Warsaw, Poland) as potentiostat connected to a strip chart recorder Potentiograph E436 from Metrohm (Herisau, Switzerland). Flow-injection manifolds were assambled using an Ismatec (Zürich, Switzerland) multichannel peristaltic pump type MP13-GJ4 and a Rheodyne 5020 low-pressure rotary valve (Cotati, CA, USA) for sample injection. 80 mm long enzyme reactor was made of 3.0 mm i.d. glass tubing filled with controlled pore glass (CPG) with immobilized lactate oxidase.

A thin layer detector with platinum working disk electrode of 0.3 and 1.0 mm diameter was the same as used earlier for discrete non-flow measurements of glucose in undiluted whole blood [10]. Prior the measurements the surface of Pt disk electrode was polished with three different grades of alumina (1, 0.5 and 0.03 μm), washed with distilled water and methanol and then 10 min cleaned electrochemically in hot alkaline phosphate bath by alternated polarization between +5 and -5 V. Lactate measurements were carried out at +0.65 V vs. Ag/AgCl reference electrode.

For the determination of lactate in serum samples a home-made dialyzer of perspex was used with 80 mm length of effective dialyzing channel of 2.0 mm diameter, in which a nylon membrane NYTRAN type NY13N of pore size 0.45 μm from Schleicher and Schuell (Dassel, Germany) was placed.

Reagents

Lactate oxidase (LOD) from *Pedicoccus species* and lactic acid was purchased from Sigma (St.Louis, MO, USA). 100 units of LOD was immobilized on 200 mg of aminopropylated controlled pore glass of 700 A from Cormay (Warsaw, Poland) according to the procedure reported earlier [11]. Lyophilized control serum Serachem was obtained from Fisher Scientific (Orangenburg, NY, USA).

RESULTS AND DISCUSSION

Optimization of flow-injection system

The magnitude and dynamic characteristics of analytical signal in flow-injection measurements with enzymatic conversion and amperometric detection of hydrogen peroxide produced depends on numerous chemical and physical factors and geometry of the flow measuring setup. Several of them only were optimized during this study as many of them were already widely discussed in the literature. Without further optimization for the anodic detection of hydrogen peroxide a platinum disk electrode polarized at +0.65 V vs. Ag/AgCl was used and all determinations were carried out in phosphate buffer pH 6.5.

For a given size of enzymatic reactor and activity of immobilized enzyme and at given geometry of flow-through detector and the size of working electrode, the magnitude of signal from the substrate depends on the concentration of substrate solution, volume of injected sample and flow-rate of solutions transported in the manifold. The effect of those factors was investigated in the system schematically shown in Fig.1 using Pt disk electrode of 0.3 mm diameter as working electrode.

The effect of total flow-rate in the detector on the peak height for injections of 200 μl 0.8 mM lactate solution is shown in Fig.2. The monotoneous increase of signal magnitude with flow-rate in the examined range indicates a predominating role of the transport rate of hydrogen peroxide to the detector surface and not the enzymatic conversion rate. Below 2.0 ml/min total flow-rate the broadening of peaks increases significantly.

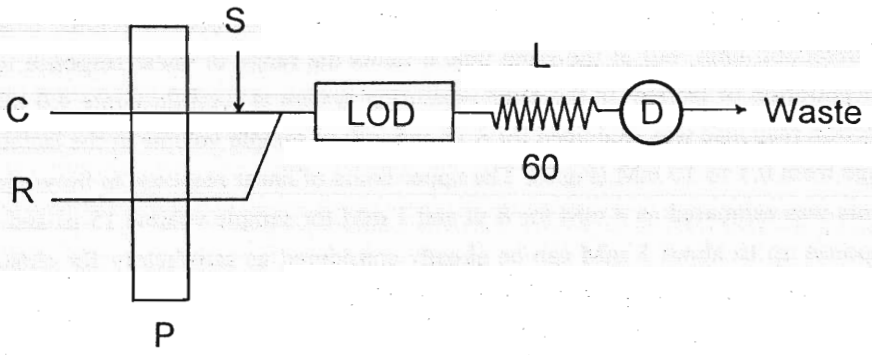

Fig.1. Schematic diagram of the flow-injection system used for enzymatic amperometry of lactate. C - carrier stream of distilled water, R - stream of 0.1 M phosphate buffer of pH 6.50, P - peristaltic pump, S - sample injection point, LOD - enzyme reactor with immobilized lactate oxidase, L - 60 cm mixing delay coil, D - flow-through detector.

The magnitude of flow-injection signal and especially a concentration range of linear relationship between the peak height and the lactate concentration in the injected samples depends significantly on the sample volume used. In clinical samples average level of lactate is 2 mM, while samples analyzed in sport medicine, in food analysis and biotechnological monitoring may

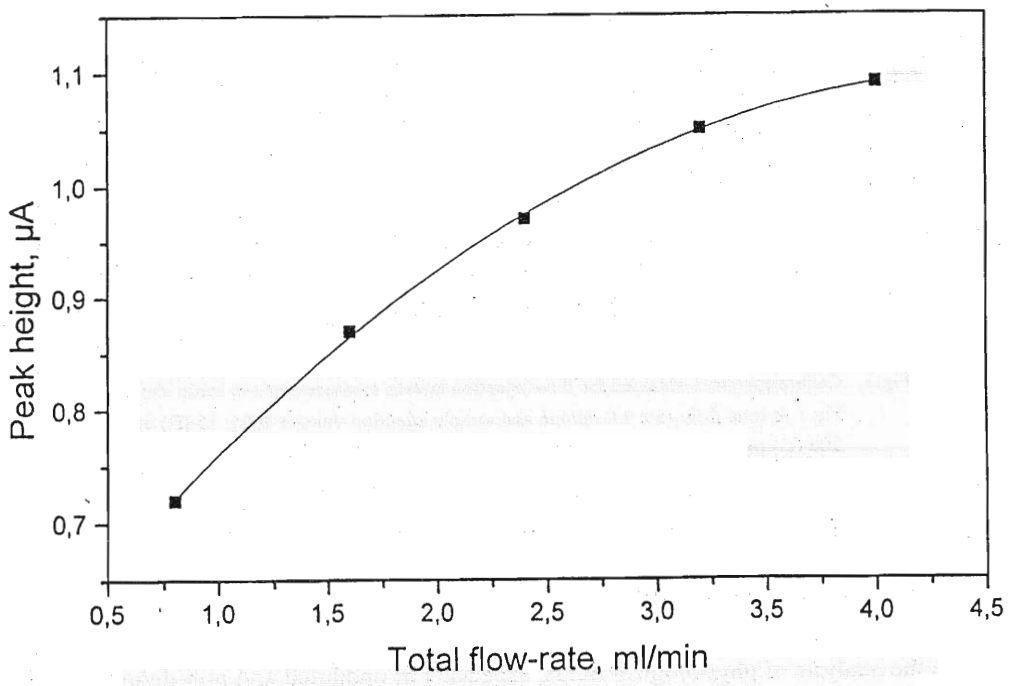

Fig.2. Effect of total flow-rate through detector on flow-injection response for lactate determination obtained for injections of 200 μl 0.8 mM lactate solution in the setup shown in Fig.1.

contain even up to 20 mM lactate. The increase of sample injection volume enables to improve the detection limit, but at the same time it limits the range of linear response to relatively low concentration of lactate. In the same measuring system at total flow-rate 4.0 ml/min, the flow-injection response was examined for 8,15 and 200 µl sample volume in the lactate concentration range from 0.1 to 10 mM (Fig.3). The upper limits of linear response in flow-injection measurements was estimated as 4 mM for 8 µl and 1 mM for sample volume 15 µl and larger. A linear response up to about 5 mM can be already considered as satisfactory for clinical applications, but it is insufficient for sport medicine and food analysis.

For the largest sample volume used, the detection limit considered as a concentration giving a signal equal to triple value of the amplitude of base-line noise, was estimated as 0.4 µM lactate.

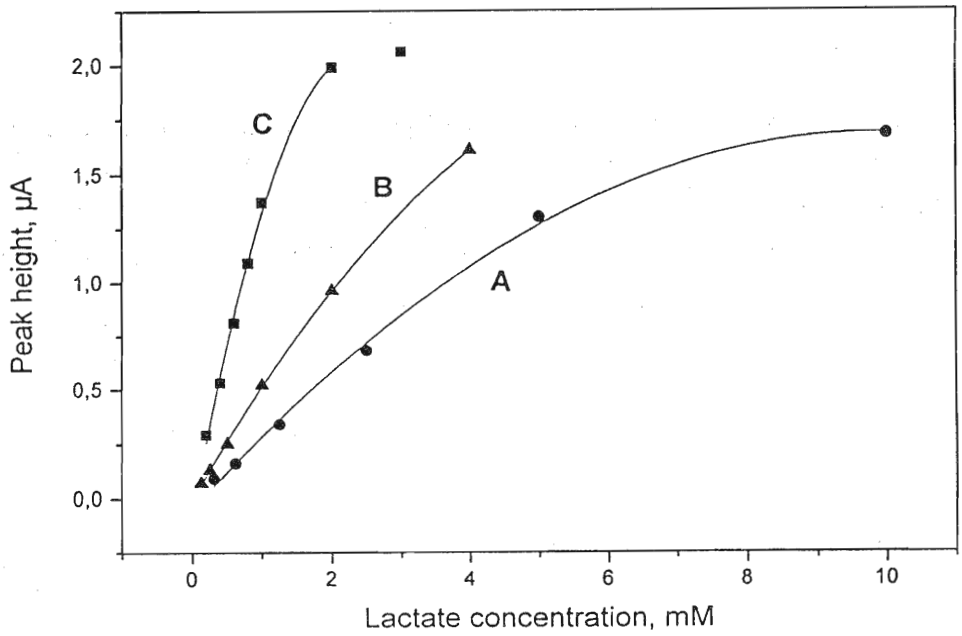

Fig.3. Calibration plots obtained for flow-injection lactate measurements in setup shown in Fig.1 at total flow-rate 4.0 ml/min and sample injection volume 8(A), 15 (B) and 200 (C) µl.

Lactate determination in blood serum

In the analysis of physiological fluids, especially in undiluted and non-deproteinized samples, a serious difficulty besides electrochemical interferences from other components of the sample, is a possibility of blocking of the access of substrate molecules to the immoblized enzyme by adsorbed proteins, lipids, red cells and other macromolecular components present in

physiological fluids. In order to avoid this obstacle, in a developed flow-injection measuring system a flow-through dialyzer was introduced for the lactate separation from the matrix. Among several membrane tested for this purpose, the best results were obtained for the nylon membrane NYTRAN of 0.45 μm porosity. Application of dialyzer in measuring system dramatically decreased the magnitude of measured signals, however, it resulted in several positive changes in the functioning of this setup

The schematic diagram of the flow-injection system used with dialyzer is shown in Fig.4. In comparison to the manifold shown in Fig.1 in a donor line an additional microcolumn with CPG (without immoblized enzyme) was added in order to create a similar flow resistance both in donor and acceptor lines. In both those lines solutions were delivered with the same flow-rate. It was found from the examination of flow-rate range from 1.4 to 3.6 ml/min, that the largest signals and independent on flow-rate are obtained for flow-rate above 2.8 ml/min and therefore this limiting value was used in each line for further measurements. An example of flow-injection signal recording for standard lactate solutions from 2 to 10 mM are shown in Fig.5. In those measurements a larger Pt working disk electrode of diameter 1 mm was used with the sample volume 20 μl. In such a configuration of measuring system a wider range of linear response was achieved up to 40 mM lactate. The calibration plot for concentration range from 2 to 40 mM was linear with correlation coefficient 0.9999 (n = 8) and with slope 1.68 nA/mM.

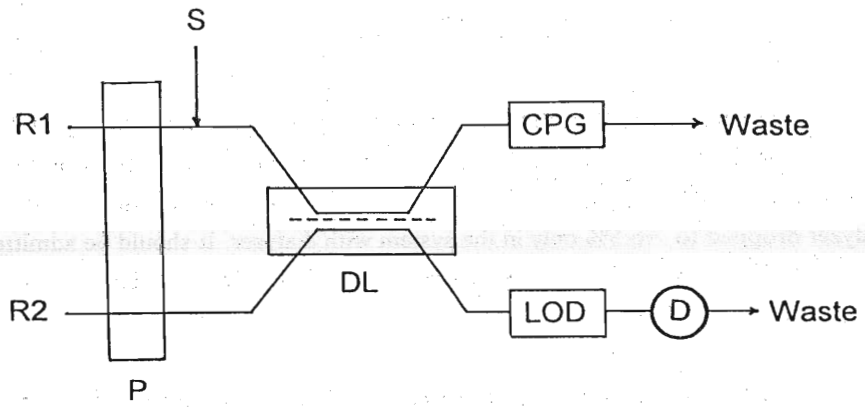

Fig.4. Schematic diagram of the flow-injection system with dialyzer for determination of lactate in blood serum. R1 - 0.1 M phosphate buffer pH 6.5, R2 - as R1 with 0.5 M NaCl, CPG - microcolumn with CPG. Other symbols as in Fig.1.

As pK_a values for lactic and ascorbic acids are 3.08 and 4.10, respectively [12], both species are practically completely in anionic forms at pH 6.5. Therefore it is difficult to use charged ionomer membranes to eliminate ascorbic interferences during dialysis. However, with

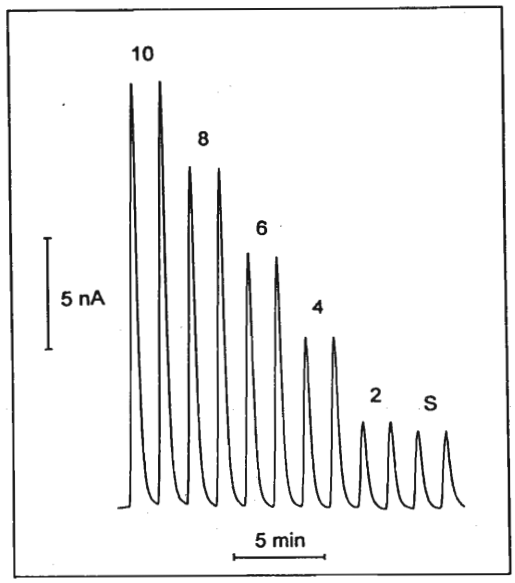

Fig.5. Flow-injection response recorded in the setup as in Fig.4 for duplicate injections of 20 µl of standard glucose solutions (concentrations shown in mM) and control serum sample (S) at flow-rate 3.6 ml/min in each line.

the use of nylon membrane in the dialyzer some improvement in selectivity of developed system towards ascorbate was obtained. For 100 mg/l ascorbic acid solution in 2 mM lactate background, a positive error 45% observed in lactate determination in the system without dialyzer dropped to +6.5% only in the system with dialyzer. It should be admitted also, that this level of ascorbate is twice as high as average in physiological level. The reason of this behaviour requires further studies.

A satisfactory results of lactate determinations were obtained in control serum sample SERACHEM from Fisher Scientific. Depending on the analyzer used, according to the manufacturer the cerified lactate contant was in the range from 1.63 to 2.14 mM, whereas from amperometric flow-injection measurements 1.79 mM concentration was obtained.

The developed measuring system is simple and microreactor with immobilized lactate oxidase has not shown any decrease of catalytic activity during at least one month of almost every day use. For one determination only 20 µl of blood serum is required and measurements can be carried out with sampling rate at least 40 samples/hr. A relative standard deviation for control serum samples was determined as 1.6% (n = 8).

Acknowledgements: This study was supported by the State Committee for Scientific Research Grant No. 4 4431 91 02.

REFERENCES

1. M.H.Faridinia, G.Palleschi, G.J.Lubrano and G.G.Guilbault, *Anal.Chim.Acta,* 278 (1993) 35.

2. G.Palleschi and A.P.F.Turner, *Anal.Chim.Acta,* 234 (1990) 459.

3. J.Kulys, W.Schuhmann and H.L.Schmidt, *Anal.Lett.*, 25 (1992) 1011.

4. M.Mascini, G.Palleschi, D.Moscone and L.Bernardi, *J.Pharm.Biomed. Anal.*, 7 (1989) 1377.

5. N.Shimojo, K.Fujino, S.Kitahashi, M.Nakao, K.Naka and K.Okuda, *Clin.Chem.*, 37 (1991) 1978.

6. K.Nikolajsen, J.Nielsen and J.Villadsen, *Anal.Chim.Acta,* 214 (1988) 137.

7. J.Nilesen, K.Nikolajsen, S.Benthin and J.Villadsen, *Anal.Chim.Acta,* 237 (1990) 165.

8. S.Yoshioka, H.Ukada, K.Matsumoto and Y.Osajima, *Electroanalysis,* 4 (1992) 545.

9. S.Hikima, K.Hasebe and T.Kakizaki, *Anal.Sci.*, 8 (1992) 165.

10. W.Matuszewski, M.Trojanowicz, A.Lewenstam, A.Moszczynska and E.Lange-Moroz, *Anal.Sci.*, Submitted for publication.

11. M.Masoom and A.Townshend, *Anal.ChimActa,* 166 (1984) 111.

12. *Handbook of Chemistry and Physics,* 73rd edition, CRC Press, Boca Raton 1992.

(Accepted 19 August, 1993)