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Short communication

On-line microdialysis assay of L-lactate and pyruvate in vitro and in vivo by a flow-injection system with a dual enzyme electrode

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Abstract

A flow-injection biosensor system with an on-line microdialysis sampling is proposed for the simultaneous assay of L-lactate and pyruvate in serum and rat brain. The dialysate collected in the sample loop by perfusing Ringer's solution through the microdialysis probe is automatically injected into the flow-injection line with a dual enzyme electrode arranged in parallel for the flow direction. The dual enzyme electrode is constructed by hybridizing a poly(1,2-diaminobenzene) film to two sensing parts, which respond selectively to L-lactate and pyruvate, respectively, without any cross-reactivity. Both the sensing parts respond linearly to the concentrations of both analytes between 0.01 and 5 mM, without any interference from oxidizable species and low-molecular weight proteins present in the dialysate. The proposed flow-injection analysis (FIA) method can be successfully applied to the simultaneous in vitro and in vivo assays of both analytes in serum and rat brain, respectively. The system can be automatically processed at an analytical speed of 19 dialysates h^{-1} over a period of 5 h.

Keywords: Flow-injection analysis (FIA); Dual enzyme electrode; Microdialysis sampling; In vivo analysis; L-Lactate; Pyruvate

1. Introduction

Glucose is a main nutrient in the brain, while L-lactate and pyruvate are formed from glucose in an actively glycolyzing system under anaerobic and aerobic conditions, respectively, and are of interest in metabolic disorders, including cerebral ischemia and lactate acidosis in diabetica [1]. Therefore, the simultaneous on-line monitoring of L-lactate and pyruvate concentrations in the brain tissue and blood stream would be of great benefit for studies on glucose metabolism. For such an in vivo analysis, microdialysis is a useful sampling technique [2–4] and has successfully been used in pharmacological and biological studies of biomolecules in dialysate. In many cases, dialysate collected by the microdialysis probe at a given time interval of 5–30 min is assayed usually by liquid chromatography [5,6], but the time resolution of the assay is not suitable for real-time monitoring. In recent years, in vivo biosensor systems combining such a microdialysis sampling have been developed for the continuous monitoring of concentration changes of biomolecules in blood [7], subcutaneous tissue [8], and brain [9]. For such researches, some miniaturized enzyme-based amperometric biosensors have been proposed as specific detectors for the in vivo monitoring of glucose [8,10–12], L-glutamate [13–15], acetylcholine [16], and L-lactate [8,17]. A similar attempt was achieved by using an enzyme reactor as a recognition element of a particular analyte in dialysate, e.g. glucose [18,19] and L-glutamate [19–22]. However, these methods with microdialysis sampling have generally been used for the determination of a single analyte. In addition, most of these methods can not calibrate continuously the sensor sensitivity during prolonged monitoring.

This article describes an in vivo flow-injection biosensor system with an on-line microdialysis sampling which makes possible the simultaneous monitoring of L-lactate and pyruvate in serum in vitro and in rat brain in vivo. The analytical system is based on the combination of a microdialysis sampling system and a flow-injection system with a dual enzyme electrode. The dual enzyme electrodes have been applied to the simultaneous measurement of two analytes [23–27], but it is not yet applied to the in vivo analysis with a microdialysis sampling. The dual enzyme electrode prepared in this work responded selectively to each of L-lactate and pyruvate

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without any cross-reactivity between the two sensing parts. As a result, it was found that the proposed biosensor system could be successfully applied to the simultaneous assay of both analytes in serum in vitro and in rat brain in vivo.

2. Experimental

2.1. Reagents

Lactate oxidase (LOD: EC 1.1.3.2, 39 U mg^{-1} of solid from *Pediococcus* sp.), pyruvate oxidase (POD: EC 1.2.3.3, 6.0 U mg^{-1} of solid from Bacterial), L-lactic acid, and sodium pyruvate were obtained from Sigma (St. Louis, MO). Ascorbic acid, uric acid, cysteine, dopamine, gelatin, bovine serum albumin (BSA), 1,2-diaminobenzene, glutaraldehyde (20% solution), and control serum were obtained from Wako (Osaka). These chemicals were used as received. The phosphate buffers used as a carrier solution were prepared from sodium dihydrogenphosphate. The Ringer's solution used as a perfusion medium was prepared from NaCl (147 mM), KCl (4 mM), $CaCl_2$ (1.2 mM), and $MgCl_2$ (1.0 mM). Distilled water purified using a Millipore Milli-Q system (Nippon Millipore, Tokyo) was used throughout.

2.2. Construction of a dual enzyme electrode

A BAS cross-flow electrochemical flow-cell was used for the surface modification of the electrode. The electrode assembly consisted of a dual electrode with two platinum disks (3 mM in diameter) as a working electrode, a silver–silver chloride reference electrode, and a stainless steel as an auxiliary electrode. Prior to the enzyme coating, the surface of the platinum disks was polished with 1 μm diamond particles (BAS) and then with 0.05 μm alumina particles (BAS), rinsed with distilled water, and then coated with the poly(1,2-diaminobenzene) film by the electropolymerization of 1,2-diaminobenzene according to the previously described procedures [21,22]. Both the platinum disks were then modified by cross-linking LOD or POD and gelatin using glutaraldehyde. The method was similar to that described previously [28] and was as follows. The LOD (0.5 mg; 20 U) or POD (0.5 mg; 3 U) and 10 μl of 5% (w/v) aqueous gelatin were added to 20 μl of 0.05 M sodium phosphate buffer (pH 6.5). A 4 μl portion of a 1% (v/v) solution of glutaraldehyde was added to each solution and the combinations mixed well. A 5 μl aliquot of the resulting solutions was carefully spread out onto each of two poly(1,2-diaminobenzene) film-coated platinum disks. The membranes were allowed to form for half a day at room temperature, open to the air. In this way, each of two platinum disks of the dual electrode was modified with the LOD or POD membrane cross-linked with gelatin by glutaraldehyde. Then the dual electrode was assembled into the electrochemical flow-cell and washed with 0.1 M glycine buffer (pH 7.5) to remove the excess of enzymes and the residual aldehyde groups onto the enzyme membranes. The completed electrode was stored in phosphate buffer (0.1 M, pH 6.0) at 4–5 °C when not in use.

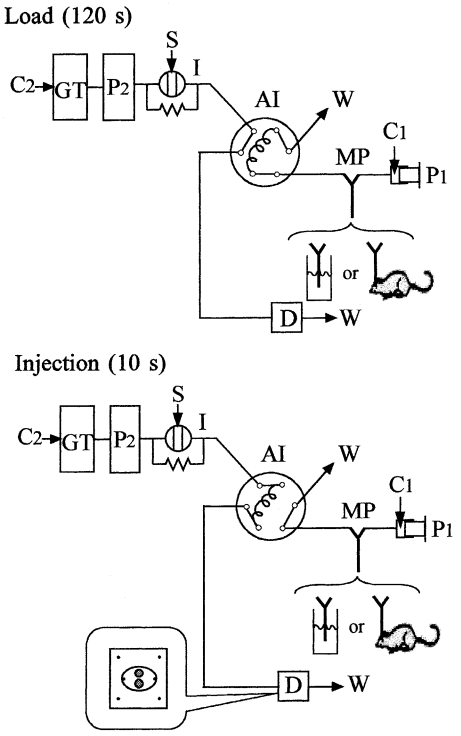

Fig. 1. Two stages (load and injection) of a microdialysis FIA system used for the simultaneous monitoring of L-lactate and pyruvate concentrations in vitro and in vivo. GT, gastorr; P_1, syringe pump; P_2, double plunger μl pump; MP, microdialysis probe; AI, six-way autoinjector with a sample loop of 2.5 μl; I, injector with a sample loop of 5 μl; D, dual enzyme electrode; C_1, perfusion solution (Ringer's solution); C_2, carrier buffer (0.1 M, pH 6.0, sodium phosphate buffer containing 147 mM NaCl); S, standard solution containing 0.1 mM each of L-lactate and pyruvate to calibrate the detection system; W, waste. Switching of the valve (AI) was repeated automatically at a regular time interval (120 s for load and 10 s for injection, respectively).

2.3. Microdialysis FIA system and procedures

The microdialysis FIA system used in this work is outlined in Fig. 1. A flow-through microdialysis probe (BDP-I-8-03) was a gift from Eicom (Kyoto). The design was almost identical to that described before [21,22]. A two-channel microsyringe pump (Eicom EP-60) was used for the delivery of perfusate (Ringer's solution). A microdialysis probe was connected to one port of a six-way autoinjector (Eicom EAS-20) with a 50-cm length of microline tubing (0.1 mm i.d.). The FIA set up was made up of an Eicom GASTORR, an Eicom double plunger μl pump, an injector (Rheodyne 7125) with a sample loop of 5 μl, a six-way autoinjector with a sample loop of 2.5 μl, an electrochemical flow-cell with a dual enzyme electrode, a Fuso HECS-966 multichannel potentiostat, and a multipen recorder (Nippon Denshi Kagaku U-638). The dual enzyme electrode was arranged in parallel

for the carrier flow direction and connected to the outlet of the autoinjector with an 30 cm length of (0.25 mm i.d.). A constant potential (+0.5 V versus Ag/AgCl) was applied to the two sensing parts of the dual enzyme electrode, respectively.

Microdialysis probe was immersed in a standard solution or control serum containing L-lactate and pyruvate for an in vitro assay, while implanted into the frontal lobe of a rat brain according to the conventional procedure [29] for an in vivo assay. In the first stage (load mode in Fig. 1; for 120 s) of the operation, the dialysate from the probe was delivered to the sample loop of a six-way autoinjector by perfusing Ringer's solution at a flow rate of 2 μl min^{-1}. Simultaneously, a standard solution containing L-lactate and pyruvate (0.1 mM each) was occasionally injected in the FIA line for checking the drifts in the sensor sensitivity. The optimized carrier buffer was a 0.1 M, pH 6.0, sodium phosphate buffer containing 147 mM NaCl and pumped at 0.2 ml min^{-1}. In the second stage (injection mode in Fig. 1; for 10 s), the dialysate collected in the sample loop was injected into the carrier stream by switching the valve. Switching of the valve was repeated automatically at a regular time interval. In this manner both analytes in the dialysates were detected simultaneously and repeatedly at a downstream dual enzyme electrode.

3. Results and discussion

The cross-linking technique of enzyme and BSA using glutaraldehyde has frequently been used as a convenient method for the construction of biosensors with a high sensitivity and a rapid response [30–32]. In the beginning of this work, each of two platinum disks of the dual electrode was modified with the LOD or POD membrane cross-linked with BSA using glutaraldehyde. However, the lactate sensing part did not respond at all to L-lactate and the pyruvate sensing part was poor in the operational stability, as shown in Fig. 2A. In contrast, when the dual electrode was modified with the enzyme membrane cross-linked with gelatine instead of BSA, both sensing parts responded sensitively to L-lactate and pyruvate, respectively, and their operational stabilities were fairly good compared to those of enzyme membrane electrode cross-linked with BSA (see Fig. 2).

In the preliminary experiment, two sensing parts of the dual enzyme electrode were arranged in series for the carrier flow direction and 0.1 M sodium phosphate buffer (pH 6.0) was pumped at a flow rate of 0.2 ml min^{-1}. The injection of a mixed solution of L-lactate and pyruvate into the flow line gave two FIA signals, because it resulted in the production of electroactive hydrogen peroxide as a product by enzymatic reaction occurring during diffusion through enzyme membrane on each sensing part. However, some fractions of the hydrogen peroxide generated enzymatically at the upstream electrode were detected at the downstream electrode. In comparison, when the dual enzyme electrode was arranged in parallel for the flow direction, the L-lactate and pyruvate

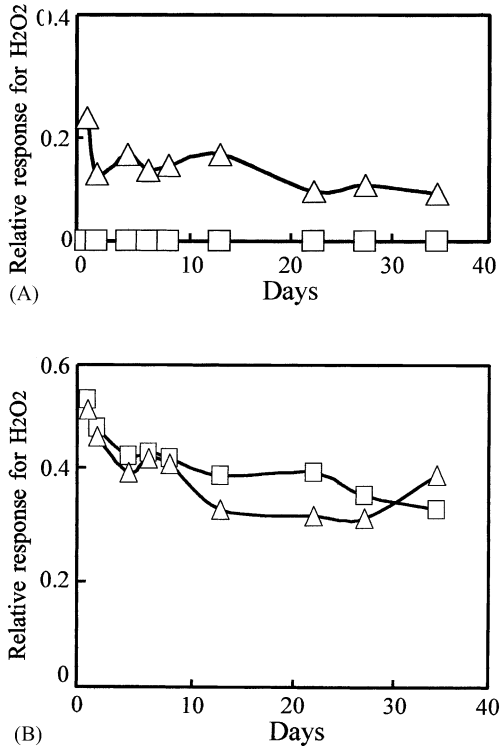

Fig. 2. The comparison of the stability of the dual enzyme electrode prepared by cross-linking enzyme and bovine serum albumin (A), or enzyme and gelatine (B) using glutaraldehyde. The responses of L-lactate (□) and pyruvate (△) for H_2O_2 response were plotted over an operational period of 35 days.

sensing parts responded selectively to L-lactate and pyruvate, respectively, and there was no apparent cross-reactivity between the two sensing parts (see Fig. 3). From this result, the in parallel arrangement was found to be more suitable for the simultaneous detection of L-lactate and pyruvate.

At an applied potential of 0.5 V versus Ag/AgCl, however, two sensing parts coated with enzyme–gelatin membrane alone gave fairly large responses to the electroactive interferents such as ascorbate, urate, cysteine, and dopamine. In contrast, the hybridization of poly(1,2-diaminobenzene) film to each enzyme membrane was effective to block the access of such electroactive compounds to the platinum electrode surface, as already demonstrated by several authors [30–34]; the response currents for ascorbate, urate, cysteine, and dopamine were decreased up to negligibly small values, though the response currents to L-lactate and pyruvate were decreased to 50–52% of their previous values (see Table 1). In addition, the polymer-hybridized enzyme electrodes effectively excluded these electroactive species below 2 mM. However, this electropolymerized film lost its permselective characteristics after the repetitive use (ca. 30 samples per day) for 20 days.In the FIA system shown in Fig. 1, Ringer's solution was pumped to the sample loop of a six-way autoinjector through a dialysis probe with a microsyringe pump, while the carrier solution was also pumped with a double plunger μl pump. The sodium phosphate buffers (0.1 M) at

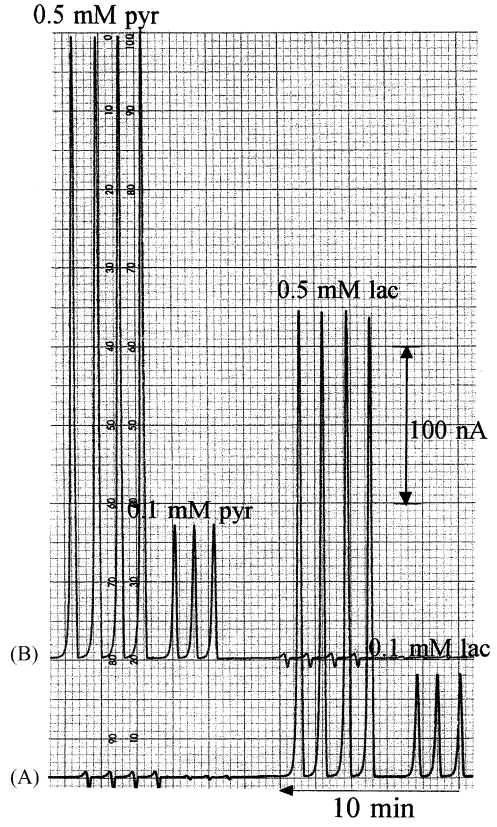

Fig. 3. Flow injection signals obtained by alternate injections of 0.1 and 0.5 mM solutions of L-lactate (lac) and 0.1 and 0.5 mM solutions of pyruvate (pyr) at the dual enzyme electrodes arranged in parallel for the carrier flow direction: (A) FIA signals at lactate sensing part; (B) FIA signals at pyruvate sensing part. Carrier solution: 0.1 M sodium phosphate buffer (pH 6.0) pumped at a flow rate of 0.2 ml min^{-1}. Applied potential: +0.5 V vs. Ag/AgCl.

various pH values were tested as the carrier solution. The signal currents for L-lactate and pyruvate were gradually increased with increasing pH and decreased at pH values above 6.0, respectively. Therefore, 0.1 M sodium phosphate buffer at pH 6.0 was selected as the optimum carrier buffer.

Table 1
Effect of poly(1,2-diaminobenzene) film hybridized to enzyme-sensing parts of dual enzyme electrode

Substrate (0.5 mM each)	Pyruvate sensing part[a] (nA)		L-Lactate sensing part[a] (nA)	
	Without film	With film	Without film	With film
Ascorbic acid	828	<1	752	<1
Uric acid	779	<1.8	712	<2.0
Cysteine	652	<1	598	<1
Dopamine	760	<1.6	697	<1.8
Glucose	n.d.	n.d.	n.d.	n.d.
Pyruvate	805	402	n.d.	n.d.
L-Lactate	n.d.	n.d.	719	374

[a] Comparison of response (peak current) obtained by dual enzyme electrode without and with poly(1,2-diaminobenzene) film; n.d., not detected.

Furthermore, 147 mM NaCl was added to the carrier buffer to adjust the ion strength, because the dialysate from the probe was Ringer's solution containing a high concentration of salts.

When the carrier flow-rate was increased from 0.05 to 0.5 ml min^{-1}, while keeping the flow rate of the Ringer's solution constant 2 μl min^{-1} and the sampling time by the dialysis probe of 180 s, respectively, the signal currents to both analytes gradually decreased in a similar manner, because the carrier flow-rate was related to the residence time of the sample zone on the dual enzyme electrode. The time required for the simultaneous assay of L-lactate and pyruvate, however, became excessive at lower flow rates, and so a flow rate of 0.2 ml min^{-1} was recommended. Also, when the flow rate of the Ringer's solution was 2 μl min^{-1}, the permeability into the probe was 21.5% for L-lactate and 22.5% for pyruvate, respectively. At higher flow rates of a perfusion solution, however, the permeability of both analytes into the probe decreased sharply. Under these flow conditions, the optimum time intervals of the load and injection (see Fig. 1) were 180 and 10 s, respectively, and up to 19 dialysates h^{-1} could be automatically analyzed.

Calibration graphs for both analytes were run using standard solutions of both analytes with concentrations between 0.01 and 20 mM, which were measured in triplicate under the recommended flow conditions. As a result, the linear relation between the signal currents and the concentrations was observed in the range of 0.01–5 mM for both analytes, with a linear correlation coefficient larger than 0.999. The slope and the *y*-intercept were 173 nA mM^{-1} and −0.49 nA for L-lactate, and 168 nA mM^{-1} and 0.73 nA for pyruvate. The relative standard deviations of the signal currents for seven measurements were 1.4% for L-lactate and 1.6% for pyruvate at a concentration of 1 mM.

The proposed flow-injection biosensor system with a microdialysis sampling was applied to a simultaneous assay of L-lactate and pyruvate in serum. The human control serum diluted with the Ringer's solution by a factor of three was repeatedly assayed. The analytical results gave a negligible amount for both of L-lactate and pyruvate, and agreed with the manufacture's data. Recovery studies were also carried out by adding known amounts of L-lactate and pyruvate to the serum sample diluted by a factor of three. The results showed good recoveries, ranging from 98.9 to 100.6% for both analytes (see Table 2). Also, to confirm the stability of the measurement, the signal currents for both analytes were monitored continuously after a microdialysis probe was immersed in a serum sample spiked with known amounts (0.5 mM each) of L-lactate and pyruvate. The signal currents for both analytes were almost kept constant over a period of 5 h, but after that decreased sharply. This is considered to be caused by the adsorption of proteins in the serum onto the probe. Therefore, the dialysis probe should be exchanged by a new one after 5 h, when the measurement was done over the times longer than 5 h.

Table 2
Recovery studies for the simultaneous determination of L-lactate and pyruvate in serum[a] by the present microdialysis FIA system

Added (mM)		Found (mM)		Recovery (%)	
Pyruvate	Lactate	Pyruvate	Lactate	Pyruvate	Lactate
0	0	n.d.[b]	n.d.[b]	–	–
0.49	0.49	0.493 ± 0.01	0.492 ± 0.01	100.6 ± 1.6	100.4 ± 1.5
0.95	0.95	0.948 ± 0.01	0.956 ± 0.02	99.8 ± 1.1	100.6 ± 1.7
1.82	1.82	1.82 ± 0.03	1.80 ± 0.03	100.0 ± 1.6	98.9 ± 1.7

[a] The control serum diluted with Ringer's solution by a factor of three ($n = 5$).
[b] Manufacturers data: no detectable amount for puruvate and L-lactate and 1.59 ± 0.02 mM for glucose, respectively; n.d., not detected.

Furthermore, in vivo monitoring of L-lactate and pyruvate in the rat brain was conducted by implanting a microdialysis probe into the frontal lobe of the rat. The rat was allowed to recover overnight in a large plastic box with free access to food and water. After the probe was connected to a microsyringe pump through a swivel to allow the rat free movement, both analytes in the extracellular space of rat brain were monitored simultaneously over a period of 5 h. The average amounts of L-lactate and pyruvate in the extracellular space of the rat brain were about 0.8 mM for L-lactate and less than 0.1 mM for pyruvate, respectively, although their concentrations varied much less with the elapse of time.

In conclusion, it was found from these results that the present flow-injection biosensor system with an on-line microdialysis sampling is very selective and useful for the simultaneous monitoring of L-lactate and pyruvate without any interferences from oxidizable species (such as ascorbate, urate, cysteine, and dopamine) and proteins present in serum and brain tissue. This method can be expected to be applied to the in vivo monitoring of L-lactate and pyruvate at other parts of the living cells, such as blood stream, subcutaneous tissue, and muscle, and furthermore to the simultaneous determination of both analytes in a variety of foods. Their analytical applications will be also reported subsequently.

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