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Short communication

Determination of lactate dehydrogenase in human erythrocytes by capillary electrophoresis with electrochemical detection

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Abstract

Capillary electrophoresis (CE) was employed to analyze lactate dehydrogenase (LDH) in human erythrocytes using an amperometric detector with a carbon fiber micro-disk bundle electrode. LDH activity was measured by determining the amount of NADH generated by LDH through a enzyme-catalyzed reaction between NAD^+ and lithium lactate. The factors influencing the enzyme-catalyzed reaction, separation and detection were examined and optimized. The following conditions were suitable for the determination of LDH: running buffer, 5.0×10^{-2} mol/l Tris–HCl (pH 7.5); separation voltage, 20.0 kV; detection potential, 1.00 V (versus saturated calomel electrode (SCE)). The conditions of enzyme-catalyzed reaction were: reaction buffer, 5.0×10^{-2} mol/l Tris–HCl (pH 9.3); substrates, 5.0×10^{-2} mol/l lithium lactate and 5.0×10^{-3} mol/l NAD^+; reaction time, 10 min. The concentration limit of detection (LOD) of the method was 0.017 U/ml at a signal-to-noise (S/N) ratio of 3, which corresponded to 1.10×10^{-10} mol/l, and the mass LOD was 2×10^{-20} mol. The linear dynamic range was 0.039–4.65 U/ml for the injection voltage of 5.0 kV and injection time of 10 s. The relative standard deviation (R.S.D.) was 0.85% for the migration time and 1.8% for the electrophoretic peak area. The method was applied to determine LDH in human erythrocytes. The recovery of the method was between 98 and 101%.
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1. Introduction

Lactate dehydrogenase (LDH) is a key enzyme in the glycolytic pathway at the dividing point between the anaerobic (formation of lactate) and aerobic metabolism of pycuvate through the tricarboxylic acid cycle. It is very valuable in diagnosing different kinds of diseases, such as liver disease, myocardial infarction, etc. [1]. There was a report of detection of LDH by electrophoresis fluorometric method [2]. A procedure was described for determination of serum LDH activity by use of Bindschedler's Green [3]. Capillary electrophoresis (CE) with laser induce fluorescence (LIF) detection has been used to detect LDH in erythrocyte [4] and leukemia [5], even single LDH molecules [6]. Capillary electrophoresis with electrochemical (EC) detection has already been applied to study intracellular components in different kinds of cells [7–20]. However, the compounds in cells studied so far are those with relatively small molecular weight (MW). Most of other components like enzyme which have thousands even millions MW are still inaccessible with CE–EC detection.

LDH is electroinactive, so it cannot be directly detected by amperometric detection. LDH can catalytically produce NADH from nicotinamide adenine dinucleotide (NAD^+) in the presence of lactate, and NADH can be determined by CE–EC detection. Therefore, the LDH activity can be indirectly measured by determining NADH. Although Yang et al. reported the electrophoretic behavior of LDH using CE–EC detection [21], no quantitative analysis was performed. In this work, we describe a method for quantification of LDH activity in human erythrocytes with CE–EC detection.

2. Experimental

2.1. Linear sweep voltammetry

An electrochemical analyzer (Model CHI 802, CH Instrument, Austin, TX, USA) coupled with a computer was

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used. It was used in connection with a cell, using potentiostatic control of the electrode potential by means of a three-electrode system, which consisted of a carbon fiber bundle electrode as the working electrode, a Pt wire as the auxiliary electrode and a saturated calomel electrode (SCE) as the reference electrode. The reference electrode was connected to the analyte via a salt bridge filled with the same supporting electrolyte as in the cell.

2.2. Instrumentation

The CE separation system used in this work was similar to the one described previously [22]. Briefly, a fused-silica capillary (10 μm i.d., 375 μm o.d., 35 cm in length), from Yongnian Optical Conductive Fiber Plant (Yongnian, China) was placed between two buffer reservoirs. A high voltage was applied at the injection end, while the reservoir containing the electrochemical detection cell was held at ground potential. Separation was carried out at 20 kV provided by a high-voltage power supply (Model 9323-HVPS, Beijing Institute of New Technology, Beijing, China). The EC detection at a constant potential was performed using the electrochemical analyzer (Model CHI 802, CH Instrument, Austin, TX, USA). The detection cell was housed in a Faraday cage in order to minimize interference from noise of external sources. Electrochemical detection was carried out with a three-electrode system. It consisted of a carbon fiber micro-disk bundle electrode (about 30 carbon fibers with 6 μm diameter) as the working electrode, a SCE as the reference electrode and a coiled Pt wire (0.5 mm diameter, 4 cm in length) placed at the bottom of the cell as the auxiliary electrode. The alignment between the working electrode and the capillary outlet was carried out with the help of a microscope and a small mirror by adjusting a three-dimensional adjuster. The carbon fiber micro-disk bundle electrodes used here were described previously [23].

2.3. Preparation of human hemolysate

Human blood ca. 5 ml with anti-coagulants collected in a 10 ml centrifuge tube was centrifuged at 1000 rpm for 5 min to separate erythrocytes. The supernatant liquid was removed. In order to wash the erythrocytes, the physiological buffer saline (PBS) with a six-fold volume was added into the centrifuge tube. After vibrating lightly, the mixture was centrifuged, and then the supernatant was removed again. This step was repeated over six times until the supernatant was clear and transparent. After the supernatant liquid was removed, the erythrocytes were obtained. Then the amount of erythrocyte was calculated using a hemocytometer (Shanghai Medical Optical Instrument Plant, Shanghai, China). Yields for two sample were 1.88×10^9 and 2.32×10^9 cell/l, respectively. The erythrocyte suspensions were lysed using 5.0×10^{-2} mol/l Tris–HCl solution (pH 7.5) with a five-fold volume.

2.4. Procedure

For linear sweep voltammetry, the carbon fiber bundle electrode was directly inserted in the experimental solution containing NADH, and a cyclic voltammogram was recorded. The electrode must be cleaned in water for 2 min with an ultrasonicator before each detection.

In CE, the capillary was flushed with 0.2 mol/l NaOH, water and the running buffer for 5 min, respectively, by means of a syringe before each run. The electrode was cleaned in water for 2 min with an ultrasonicator. Then a voltage of 20.0 kV was applied across the capillary and the detection potential of 1.00 V versus SCE was applied at the working electrode. After the electroosmotic flow reached a constant value, the electromigration injection was carried out at 5.0 kV for 10 s. Then the separation voltage was applied again and the electropherogram was recorded. In addition, the electrolyte solution at the electrochemical cell was also replaced before each run. All potential were measured versus SCE.

2.5. Reagents

L-LDH (E.C. 1.1.1.27, from rabbit muscle, 135,000 molecular weight) solution (8.6 mg/ml, 1145 U/mg) was purchased from Sigma (St. Louis, MO, USA). Tris (>99.8%), NAD^+ (>97%) and NADH (>92%) were obtained from Amresco (Solon, OH, USA). PBS consisted of 0.135 mol/l NaCl, and 0.02 mol/l NaH_2PO_4–NaOH (pH 7.4). The electrophoresis buffer was 5.0×10^{-2} mol/l Tris–HCl (pH 7.5), and the reaction solution was 5.0×10^{-2} mol/l lithium lactate and 5.0×10^{-3} mol/l NAD^+ in 5.0×10^{-2} mol/l Tris–HCl (pH 9.3). A 1.00×10^{-2} mol/l stock solution of NADH was prepared by dissolving an appropriate amount of NADH in water. Dilute solutions were obtained by serial dilution of the stock with the running buffer. All solutions were prepared with double-distilled water and stored at 4 °C.

3. Results and discussion

3.1. Detection of NADH

NADH can be detected amperometrically [21,24]. Fig. 1 shows a linear sweep voltammogram of NADH at the carbon fiber bundle electrode in 5.0×10^{-2} mol/l Tris–HCl (pH 7.5). An oxidation peak with a peak potential of 0.8 V was observed. This means that NADH can be directly measured by electrochemical detection under the present conditions. It was found that 5.0×10^{-2} mol/l Tris–HCl (pH 7.5) for the running buffer, 20.0 kV for the separation voltage, and 1.00 V versus SCE for the detection potential were suitable for detection of NADH. Under these conditions, the electropherogram of 1.00×10^{-4} mol/l NADH is shown in curve 1 (Fig. 2). The peak eluting at 7.1 min is the peak of NADH. In order to identify the peak at 3.35 min, the

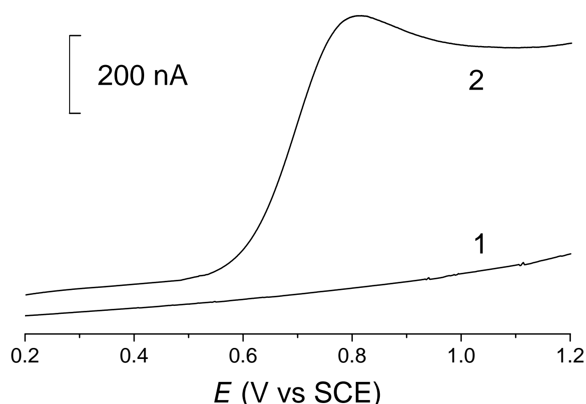


Fig. 1. Typical linear sweep voltammogram of NADH at a carbon fiber bundle electrode in (1) 5.0×10^{-2} mol/l Tris–HCl (pH 7.5) and (2) (1) + 1.00×10^{-2} mol/l NADH. Scan rate: 100 mV/s.

electropherogram was recorded in the solution containing LDH, lithium lactate and NAD^+ after the reaction for 10 min. In this case, NADH should be formed through the enzyme-catalyzed reaction. No peak appeared on the electropherogram. This means that the impurity in NADH should be responsible for the peak at 3.35 min. The response for a series of six injections of 5.00×10^{-5} mol/l NADH resulted in a relative standard deviation (R.S.D.) of 0.59% for the migration time (t_m) and 1.8% for the electrophortic peak area (q), respectively. The limit of detection (LOD) was 1.7×10^{-6} mol/l or 270 amol, calculated according to its injected volume of 0.16 nl, when the signal-to-noise (S/N) ratio was 3. A linear relationship held between the i_p, or q and concentration in the range of 5.00×10^{-6} to 1.00×10^{-3} mol/l with a correlation coefficient of 0.997 for the injection voltage of 5.0 kV and injection time of 10 s.

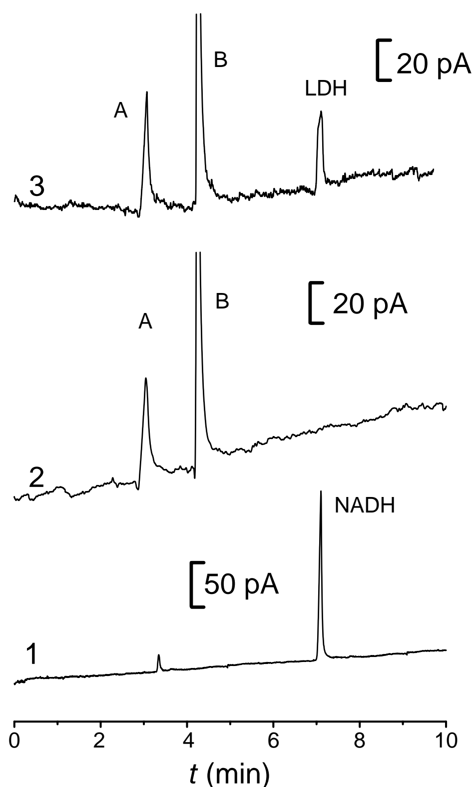
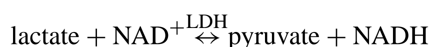


Fig. 2. Electrophorograms of (1) 1.00×10^{-4} mol/l NADH; (2) 5.0×10^{-2} mol/l Tris–HCl (pH 9.3) containing 5.0×10^{-2} mol/l lithium lactate and 5.0×10^{-3} mol/l NAD^+ and (3) (2) + 0.488 U/ml LDH. Reaction time: 10 min; running buffer: 5.0×10^{-2} mol/l Tris–HCl (pH 7.5); injection voltage: 5.0 kV; injection line: 10 s; separation voltage: 20.0 kV; detection potential: 1.00 V (vs. SCE); capillary: 10 μm i.d., 35 cm in length.

3.2. Detection of LDH

LDH can catalyze the conversion of an electroinactive substrate (NAD^+) to an electroactive product (NADH). The reaction is shown as following:



$$\text{lactate} + NAD^+ \overset{LDH}{\leftrightarrow} \text{pyruvate} + NADH$$

The product of the catalysis reaction NADH can be amperometrically measured by CE–EC detection as mentioned above. To explore the optimum experimental conditions, the pH value of reaction buffer and the concentration of the substrates were examined. It was found that 5.0×10^{-2} mol/l Tris–HCl (pH 9.3) for the reaction buffer, 5.0×10^{-2} mol/l lithium lactate and 5.0×10^{-3} mol/l NAD^+ for the substrates concentration were suitable for detection of LDH. Fig. 2 depicts the electropherograms of the reaction solution containing lithium lactate and NAD^+ without (curve 2) and with (curve 3) 0.488 U/ml LDH. There are two peaks (A and B) on curve 2. The lithium lactate should be responsible for the peak A on curve 2, because the same peak was obtained for 5.0×10^{-2} mol/l lithium lactate in the running buffer (pH 7.5). When only the reaction buffer was injected, the peak B also appeared. This means that the peak B is due to the difference between the running buffer and the reaction buffer. By comparing curve 3 with curve 1, it can be concluded that the peak at ca. 7 min should be the peak of NADH produced by LDH through the enzyme-catalyzed reaction. The response for a series of six injections of 0.488 U/ml LDH resulted in a R.S.D. of 0.85% for t_m and 0.8% for q, respectively. The peak area rather than the peak height was adopted to quantify. The concentration LOD was 0.017 U/ml or 1.10×10^{-10} mol/l (S/N = 3), which corresponded to the mass LOD of 2.7 nU or 1.77×10^{-20} mol for the injected volume of 0.16 nl. A linear relationship held between the i_p, or q detected and concentration in the range of 0.039–4.65 U/ml with a correlation coefficient of 0.9998 for the injection voltage of 5.0 kV, the injection time of 10 s and the reaction time of 10 min.

3.3. Determination of LDH in human hemolysates

The electropherograms of the hemolysate without (curve 1) and with (curve 2) lithium lactate and NAD^+ are shown in Fig. 3. It can be found that a peak, eluting at 7.10 min, appears, when both lithium lactate and NAD^+

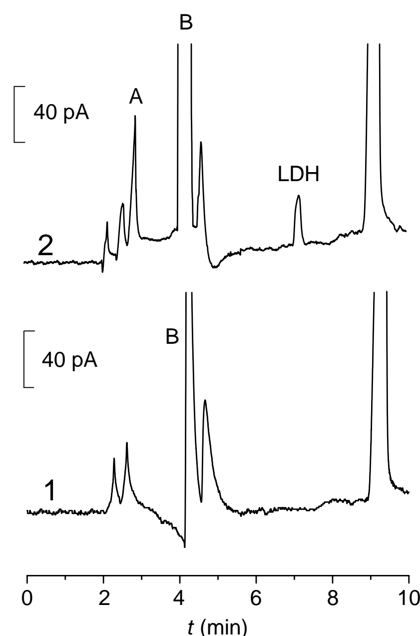


Fig. 3. Electrophorograms of hemolysate (1) without and (2) with 5.0×10^{-2} mol/l lithium lactate and 5.0×10^{-3} mol/l NAD^{+}. Other conditions are same as in Fig. 2.

are present. This peak should be corresponding to LDH based on the migration time, by comparing curve 2 with curve 3 (Fig. 2). The peak A and the peak B come from lactate and the difference between the sample solution and the running buffer as mentioned above. Other peaks in both electropherograms are blank peaks from the compounds in the hemolysate. They were not identified.

The electrophoretic peak area detected can be used to quantify LDH activity in the hemolysate. The activity concentrations of LDH in the two hemolysates obtained by the standard calibration method are 0.399 and 0.504 U/ml, respectively. To prove the reliability of the method, a certain amount of standard LDH was added to the two hemolysates and then the hemolysates were determined. From the detected activity concentrations in the hemolysates with and without the standard LDH, the recoveries calculated were between 98 and 101%, respectively. The results obtained from the two human hemolysates are listed in Table 1. The mean

Table 1
Results detected and recovery of LDH in human erythrocyte samples

Sample	Peak area (pC)	Mean peak area (pC)	C_{LDH} (U/ml)			Recovery (%)
			Sample	Added	Detected	
1	344, 352	348	0.399	0.488	0.877	98
2	402, 409	406	0.504	0.488	0.997	101

Conditions are same as used in Fig. 2.

LDH activities calculated in a single erythrocyte are 1.06 and 1.09 nU, respectively, according to the activity concentrations of 0.399 and 0.504 U/ml and the cell concentrations of 1.88×10^9 and 2.32×10^9 cells/ml. They are within the range of value 1.39 ± 0.7 nU (mean ± S.D.) reported in [4].

4. Conclusions

Our results show that CE with EC detection can be used to determine LDH in human erythrocytes. In this method, the electroactive NADH produced by LDH through the enzyme-catalyzed reaction between lithium lactate and NAD^{+} is detected. LDH activity can be obtained based on the electrophoretic peak area of NADH detected. A concentration LOD of 1×10^{-10} mol/l and a mass LOD of 2×10^{-20} mol can be achieved because of the amplification of the signal through the enzyme-catalyzed reaction of LDH.

Acknowledgements

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