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Determination of pyruvic acid by using enzymic fluorescence capillary analysis

Yuan-Yuan Zhao [a], Xiu-Feng Gao [b], Yong-Sheng Li [a,*], Xiang Ju [a], Jia Zhang [a], Jia Zheng [a]

[a] School of Chemical Engineering, Sichuan University, Chengdu 610065, China
[b] West China School of Preclinical and Forensic Medicine, Sichuan University, Chengdu 610041, China

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ABSTRACT

A new method (P-LE–FCA) for the determination of pyruvic acid was proposed based on liquid enzyme method (LE) and fluorescence capillary analysis (FCA). The optimum experimental conditions were as follows: the excitation and emission wavelengths were 350 and 460 nm, respectively; the reaction time and temperature were 20 min and 38 °C, respectively; the pH of phosphate buffer solution was 7.5; the concentrations of nicotinamide adenine dinucleotide and lactate dehydrogenase were 1.0 mmol L^{-1} and 5.0 kU L^{-1}, respectively. The linear range of this method was 0.2–1.2 mmol L^{-1} ($\Delta F = 327.13C - 10.018$, $r = 0.9942$). Its detection limit was 0.012 mmol L^{-1}. And its relative standard deviation was 0.86%. Only 18 μL of total reaction solution is enough for the detection. P-LE–FCA has some merits such as lower cost, simple operation procedure and micro determination. It has been used for the determination of pyruvic acid content in human urine samples.

1. Introduction

Pyruvic acid is a biologically important molecule involved in a variety of biochemical reactions in plants and animals, either as a substrate, product or intermediate. Recently, because pyruvic acid has excellent results in losing weight, it has become a dietary supplement [1]. Pyruvic acid can improve exercise endurance capacity [2], effectively reduce cholesterol [3] and serve as a potent antioxidant [4]. Therefore, the measurement of its concentration can give valuable information to the progress of specific biochemical reactions. In medical field, pyruvic acid serves as an aid for diagnosing ischemia [5], diabetic acidosis [6], alcoholism and hypovitaminosis and can also be used to optimize respiratory monitoring. In food industry, assaying this compound provides an indication of bacterial contamination of various media, such as dairy products.

Several techniques for the determination of pyruvic acid in various samples have been reported over the past few years, such as fluorescence spectra [8–10], chemiluminescence (CL) [11], ultraviolet spectrophotometry [12,13], enzyme electrode [14–16], voltamperometric analysis [17], colorimetric method [18], flow injection analysis (FIA) [19,20], high performance liquid chromatography (HPLC) [21–25], gas chromatography (GC) [26] and ion chromatography [7].

In the history of routine fluorometry for determining pyruvic acid, Olsen [8] first used it for the determination of 0.2 mL blood sample. Their procedure was based on an enzyme-catalyzed reaction system involving concentration changes of reduced nicotinamide adenine dinucleotide (NADH) which was a fluorescence substance. Linear correlation between pyruvic acid concentration and fluorescence intensity was established. Later, Ozand et al. [9] has developed a micro-autoanalytic system for the enzymic fluorometric determination of pyruvate. As many as 60–80 duplicate samples can be analyzed simultaneously in 8 h. The reproducibility, efficiency and versatility of this autoanalytic system will warrant its wide application in metabolic studies. Compared with the manual method developed by Olsen, this method has realized automation and quickened analytical speed. Xue and Yeung [10] used fluorescein as the fluorophore for indirect detection of pyruvate and other anions, and researched on the concentration variety of pyruvate in the intracellular fluid of erythrocytes.

Yoo et al. [18] has conducted many research on the determination of pyruvic acid. First they utilized colorimetric method to determine pyruvic acid in onions. Dinitrophenylhydrazine (DNPH) was added into onion sample solutions as color reagent, and the reaction mixture was heated for 10 min at 40 °C. Then, NaOH was added into the mixture and its absorbance was detected at 420 nm. Subsequently, they developed an automated system [19] based on FIA for pyruvic acid analysis. The system included two plunger pumps, an autosampler, a column heater, a spectrophotometric detector and an integrator. Onion juice sample was filtered through a nylon filter (microfilter) and injected into DNPH solution with an autosampler. Detection was made by an UV–vis detector and peak area was recorded by the integrator. This automated system showed highly significant correlations with the colorimetric

* Corresponding author. Tel.: +86 28 85417299.
E-mail address: lysgxf2005@yahoo.com.cn (Y.-S. Li).

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method. In 2001, they used HPLC [21] to determine the pyruvic acid. It took only 30 min to run a sample. This HPLC method was employed instead of DNPH method, the possible interference from the related compounds could be eliminated.

In a word, each method as mentioned above has its advantages and disadvantages. GC and CL are not suitable for field determination. HPLC is applied increasingly, but eluting of sample residue in separation column still remains a problem in many instances. Whereas, the enzyme method has been proved that it has good selectivity and specificity and is a promising approach if enzyme price can changed to be cheap.

Fluorescence capillary analysis (FCA) [27] is a new method developed by the authors, based on common spectrofluorimetry and medical capillary (ID: 0.50–0.90 mm, length: 3–5 cm). The FCA can realize micro-dosage of expensive enzymes and chemical reagents, and promote the micromation of traditional fluorescence analysis instruments [28]. The FCA replaces the routine fluorescence cell (1 mL) with the capillary ($\leq$18 μL) and can save about 99% enzymes or reagents. Based on FCA, we have successfully developed an immobilized enzyme capillary bioreactor for the determination of alcohol in distilled spirit [29–31], and an immobilized probe biosensor marked by using Cy-5 reagent [32] for the determination of DNA, etc. [28]. Subsequently, Ho et al. [33] also have made an immobilized enzyme capillary bioreactor with a longer capillary (ID: 0.53 mm, length: 85 cm) and used for a FIA amperometric analysis system to determine blood glucose.

In this research, further combining FCA with liquid enzyme catalysis together, we have proposed a new method called pyruvic acid liquid enzymatic fluorescence capillary analysis (P-LE–FCA) for determination of pyruvic acid.

2. Experimental

2.1. Assay principle

Reaction principle used for the determination of pyruvic acid is shown in Eq. (1). In the presence of lactate dehydrogenase (L-LDH), pyruvic acid reacts with NADH. This reaction produces lactic acid and NAD^+ without fluorescence property. When NADH is excited at 350 nm, it will emit 460 nm of fluorescence. Based on fluorescence quenching the content of the pyruvic acid can be quantified.

$$\text{pyruvic acid} + \text{NADH} + \text{H}^+ \xrightarrow{\text{L-LDH(pH 7.5)}} \text{lactic acid} + \text{NAD}^+ \quad (1)$$

2.2. Chemicals and apparatus

All reagents used were of analytical-reagent grade with no further purification. Ultra-purified water (conductivity: 0.065 μS cm^{-1}) was used throughout all experiments. Sodium pyruvate was purchased from Huali Scientific Company (Chengdu, China). 2,4-Dinitrophenylhydrazine (DNPH), H_2SO_4, Na_2HPO_4 and KH_2PO_4 were obtained from Kelong Chemical Reagent Factory (Chengdu, China). NADH and L-LDH (EC 1.1.1.27, 53 units/mg) were bought from Sigma–Aldrich Japan K.K. (Tokyo, Japan).

F-4500 spectrofluorimeter used in this experiment was from Hitachi Co. (Tokyo, Japan). An AUW120D type electric balance (measuring precision: 0.01 mg) made by Simadzi Co. (Kyoto, Japan). A capillary holder was self-made and the capillaries (ID: 0.90 mm, 18 μL) were from Huaxi Scientific Company (Chengdu, China). pH meter was purchased from Leici Instrument Factory (Shanghai, China). A 721-A type spectrophotometer was product of Shanghai Third Analyzer Co. (Shanghai, China).

2.3. Preparations of reagent solutions

2.3.1. Phosphate buffer solutions (PBS, 0.10 mol L^{-1})

0.10 mol L^{-1} of Na_2HPO_4 solution was prepared by weighing 35.81 g of Na_2HPO_4, dissolving and diluting to 1000 mL with water. 0.10 mol L^{-1} of KH_2PO_4 solution was prepared by weighing 3.40 g of KH_2PO_4, dissolving and diluting to 250 mL with water. Preparations of buffer solutions with pH 4.42, 6.18, 7.50, 8.15 and 9.33 were completed by mixing Na_2HPO_4 solution and KH_2PO_4 solution together. The pH measurements were carried out on the pH meter.

2.3.2. NADH solution (2.0 mmol L^{-1})

1.5 mg of NADH was dissolved in water and diluted to 1.0 mL with PBS (pH 7.50).

2.3.3. Sodium pyruvate solution (50.0 mmol L^{-1})

The solution was prepared by weighing 0.1375 g of sodium pyruvate in 25 mL of calibrated flask, dissolving and diluting to the mark with PBS (pH 7.50).

2.3.4. L-LDH solution (50 kU L^{-1})

One milligram of L-LDH was exactly weighed and dissolved in 1.0 mL of PBS (pH 7.50). 5 kU L^{-1} of L-LDH solution used in the research was prepared by diluting 50 kU L^{-1} of L-LDH solution with PBS (pH 7.50).

2.3.5. H_2SO_4 solution (4.5 mol L^{-1})

Fifty milliliters of concentrated sulphuric acid was slowly added into ultra-purified water. The mixture was diluted to 200 mL with water.

2.3.6. DNPH solution (4%, w/v)

Four grams of DNPH was weighed, dissolved and diluted to 100 mL with H_2SO_4 solution (4.5 mol L^{-1}).

2.3.7. Fluorescent reaction solutions

No. 1 of fluorescent reaction solution was prepared by mixing 200 μL L-LDH (50 kU L^{-1}), 100 μL NADH (20 mmol L^{-1}) and 700 μL PBS.

No. 2 of fluorescent reaction solution was prepared by mixing 100 μL NADH (20 mmol L^{-1}) and 900 μL PBS.

2.4. Pretreatment of capillary

First the inner and outer surfaces of the capillaries were washed with ethanol-water solution containing 2 mol L^{-1} of NaOH, and then flushed with ultra-purified water. At last, these capillaries were put in a thermostat for drying at 40 °C.

2.5. Determination procedure

The FCA method has two important parts: a medical capillary and a self-made capillary holder [34]. When analyzing, the holder is inserted into the light path of F-4500. After reacting for 20 min, the mixture of reactant and product are sucked into the capillary. Then, the capillary is vertically inserted into the holder. When excitation beam (λ_{ex}: 350 nm) passes through the capillary, NADH in the mixture is excited and emits fluorescence (λ_{em}: 460 nm). The fluorescence with vertical direction of excitation beam enters into monochromatic filter and reaches the detector. At the same time, the fluorescence intensity is detected. In the research, the fluorescence intensity is proportional to the content of pyruvic acid.