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ORIGINAL ARTICLE

Research on a New Micro-Volume Fluorescence Capillary Biosensor Assay for Sequentially Quantifying Pyruvate and Lactate

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Abstract It was studied that making conditions of a micro-volume fluorescence capillary biosensor for determining pyruvate (PA) and lactate (LA). The biosensor made under the optimized conditions could be used for sequential quantifications of LA in the range 0.10–1.2 mM and PA in 4–120 μM, and its recovery for PA and LA was in a satisfactory range 97–106% for human serum samples, with detection limits of 0.023 mM for LA (RSD < 1.89%, n = 11) and 0.87 μM for PA (RSD < 1.70%, n = 11). The new assay possessed these advantages that the LDH immobilizing on capillary realized the reuse of expensive enzyme in fluorospectrophotometry, and the consumption of serum samples or chemical reagents decreased to 9 μL in per assay, and the analytes no needed to preseparation, and it also are accurate and reliable. Consequently, the fluorescence capillary biosensor should have a good prospect in assaying PA and LA or LA/PA ratios for clinical medicines or biology field. The optimization conditions and parameters obtained in this study have also a certain guiding significance for the development of biochip based on glass substrate.

Keywords Fluorescence capillary analysis · Immobilized enzyme · Biosensor · Pyruvate · Lactate · Lactate dehydrogenase

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Introduction

Lactic acid (LA) is the final product of anaerobic glycolysis in the human body and can produce in all organizations. Its normal value in human serum is in 0.5–2 mM [1]. Pyruvic acid (PA) is the ultimate product in glycolysis process, and also is intermediate product in sugar, protein and fat metabolisms [2]. PA can enter the Krebs cycle to aerobic metabolism, or be reduced into LA [3]. Its normal value is 40–100 μM in human serum [1]. Under cases of severe acute hypoxia, acute renal or liver failures, preliminary mitochondrial toxicity [4, 5], LA/PA ratios in serum is more reliable than information of single LA or PA concentrations for clinical diagnosis [6, 7]. The ratio value's normal range is between 10 and 20. When this value is greater than 30, it is considered abnormal in healthy conditions [8]. Brain's LA and PA levels as well as LA/PA ratio have been advocated for estimating the severity of stroke [9], cerebral ischemia, hypoxia and reperfusion [10], and also used as a tool for prognostication of the outcome. Besides, some researchers had investigated correlation of LA and PA from full-term, pre-term infants and patients with suspected lactic academia [11], and with Menkes disease [12]. Therefore, sequential determination of LA and PA concentrations in micro-volume serums, body fluids, or the cell lysis solution is of high importance in medicine and for clinical diagnosis.

Various methods have been reported for PA and LA quantitations, such as the high-performance liquid chromatography coupled with ultraviolet [10–14] and fluorimetric detections [15], liquid chromatography-tandem mass spectrometry [11], gas chromatography with mass spectrometry [16], flow-injection dual-enzyme electrode [17], capillary electrophoresis coupled with ultraviolet [18] and fluorescence spectrometry [19] and enzymatic assays with fluorescence [20–22] or absorbance measurements [23, 24]. However, these test methods for testing PA and LA will need larger-volume

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samples and require laborious sample pretreatment. Thus, clinical analysts and biologists hope to have a better micro-volume method for determining the PA and LA concentrations in micro-volume biosamples.

So far, the most common assay for PA or LA is to utilize this reversible reaction under catalytic action of lactate dehydrogenase (LDH): lactate + $NAD^+ \longleftrightarrow^{LDH}$ pyruvate + NADH + H^+. When pH is in range 6.0–7.5, the reaction favours the conversion of PA to LA, and when pH is more than 7.5, it favours the conversion of LA to PA [25]. Based on this reaction, an assay determining LA and PA in whole blood was early examined by Olsen [20], in which enzyme-catalyzed reactions involved changes in the concentration of reduced coenzyme (NADH), and major improvements in the assay were to reduce sample dosage from 4 mL to 0.2 mL per assay. For further reducing the sample dosage, a fluorimetry was once described [22], but its actual application in assaying did not reported up to now.

We once reported a new assay called as the fluorescence capillary analysis (FCA) [26], which consists of a medical glass capillary (ID: 0.50–0.90 mm, length: 3–5 cm) and a special capillary holder as well as a common fluorospectrophotometry. The capillary in the FCA is not only a sample container, but also is a reactor container, in which catalysts and probes and even reagents may be immobilized to realize multi-objective applications and development of disposable analytical kits. The capillary (≤ 18 μL) replacing a routine quartz-cuvette (1–4 mL) for fluorescence assay can markedly cut down the sample or reagent consumptions, and can save about 99.5% of assay costs in comparison with general fluorospectrophotometry or spectrophotometry. And this benefit also brings the great reduce of biotic and chemical wastes and is very beneficial to our environment. Consequently, utilizing the FCA, some researches have been carried out, for example, determining LA in yogurts [27], glucose in bloods [28], DNAs marked by Cy-5 reagent [29] and Goldview [30], etc.

The previous immobilized-emzyme capillary biosensor [27] was lower in sensitivity and also cannot be used for determining pyruvate, so the purpose of the research is to further improve the capillary biosensor's sensitivity and to realize sequentially determining PA and LA in micro-volume samples.

Experimental

Reagent and Instruments

LDH (EC 1.1.1.27, 53 unit/mg) was purchased from Amersco (Solon, US). β-NADH, β-NAD^+ and PA were from Sanland Co. (Amoy, China). LA was purchased from Maoye Chemical Co. (Chongqing, China). Tris reagent, hexane and glutaraldehyde (Grade 1, 50% solution) were from Kelong Chemical Co. (Chengdu, China). 3-aminopropyl triethoxysilane (JH-A110) was purchased from Jianghan Chemical Co. (Jingzhou, China).

All fluorescence measurements were made under the pass-band widths at 3 nm for the excitation wavelength (λ_{ex}) and 15 nm for emission wavelength (λ_{em}), with a RF-5301PC fluorophotometer from Shimadzu Co. (Tokyo, Japan). A self-made capillary holder was mounted on the fluorospectrophotometry [26]. Medical glass capillaries (i.d.: 0.9 mm, o.d.: 1.1 mm) were purchased from Drummond Scientific Co. (Broomall, USA). These capillaries were cleaned by the PBS buffer before using. A dry-block thermostat from Aosheng Instrument Co. (Hangzhou, China) was used for heating the capillaries. Initial conditions of the research were from the reference [27].

Preparation of Reagent Solutions

Buffer I: 6.055 g of Tris was dissolved in about 400 mL water, and its acidity was adjusted to pH 9.0 by using 4.0 M HCl, and final volume of this solution was completed to 500 mL with water.

Buffer II: 500 mL of Na_2HPO_4 (0.1 M) was taken and then its acidity was adjusted to pH 7.5 with 0.1 M KH_2HPO_4.

5.0 kU/L LDH: LDH solution (5.0 kU/mL) was exactly taken 100 μL in a 10-mL calibrated flask, and diluted to the mark with the buffer I.

10 mM NAD^+: 6.60 mg of NAD^+ was dissolved in water and diluted to 1.0 mL with the buffer I.

2.0 M NADH: 1.50 mg of NADH was dissolved in water and diluted to 1.0 mL with the buffer II.

50 mM LA: 12.5 mg of LA (85–90%) was dissolved in 25-mL buffer II, and then was stored in a refrigerator at 4 °C.

50 mM PA: This solution was prepared by dissolving 0.1375 g of PA in 25-mL buffer II.

Ultra-purified water (conductivity: 0.065 μS/cm) was used for preparation of above solutions.

Confirmation of Fluorescence Reaction System

In presence of LDH, PA reacts with the fluorescent NADH to create LA and the non-fluorescent NAD^+ at pH 7.5, so the PA content can be quantified by detecting the fluorescent decrease of NADH; if the acidity in the reaction system is adjusted to pH 9.0, the reaction direction changes to reverse, namely, the LA can react with NAD^+ to create NADH and PA, so, utilizing the reversed reaction can assay the LA content. Because the acidity is crucially important to the reaction direction, reciprocal conversion between PA and LA was herein confirmed in different acidic environment.

It was known that detecting the fluorescent intensity (F) of NADH is an important tache in the research, namely, only the F value and the NADH concentration (c_{NADH}) was in the

linear correlativity, it can be used for quantification of PA or LA contents. Therefore, the correlativity was first investigated under different λ_{ex} and λ_{em}. The capillary beforehand suctioned a 400 mg/L NADH standard and it was scanned in 300–600 nm range at room temperature. Obtained λ_{ex} and λ_{em} of the NADH were 345 nm and 460 nm, respectively. Subsequently, a series of NADH standards were detected at the λ_{ex} and λ_{em}, and relative data between F and c_{NADH} were obtained. The data indicated that there was the best linear relativity between F and c_{NADH} in 0–1200 μM ($F = 399.5c_{NADH} + 0.068$, $r = 0.999$).

In order to evaluate the feasibility of the reaction system assaying LA at pH 9.0, the solutions of LA, NAD^+, LDH, LA/NAD^+, LA/LDH and LA/LDH/NAD^+ were respectively sucked into different capillaries to detect their fluorescence intensities in wavelength interval of 280–580 nm, and obtained results were as shown in Fig. 1a.

Similarly, to evaluate the practicability of the system for assaying PA at pH 6.0, the solutions of PA, NADH, LDH, PA/NADH, PA/LDH, LDH/NADH and PA/LDH/NADH were respectively sucked into the different capillaries, and then the solution in each capillary was scanned respectively in the same range, obtained data was shown in Fig. 1b.

From Fig. 1a, it can be see that the excitation and emission spectra curves of LA, NAD^+, LDH, LA/NAD^+ and LA/LDH solutions were basically overlapped each other and their fluorescence intensities were weaker, in which the LDH/NAD^+ mixture was slightly different, but it's fluorescence intensity was far less than one of the LA/LDH/NAD^+ mixture.

From Fig. 1b, we can know that the spectra curves of PA, LDH and PA/LDH solutions were basically overlapped and there are no the maximum fluorescence emission peak. Spectrum curves of NADH, PA/NADH and LDH/NADH solutions overlapped each other and there was the strongest fluorescent peak at 460 nm, this is because they all contain the NADH; but the spectrum curve of the PA/LDH/NADH solution was lower than one of NADH, PA/NADH and LDH/NADH solutions, this is because the NADH in the PA/LDH/NADH solution took place the fluorescence quenching.

In assessing the feasibility of the reaction system assaying LA at pH 8.0, we confirmed that under catalysis of LDH, LA indeed took place the chemical reaction with non-fluorescent NAD^+ to form fluorescent NADH. In confirming the reaction system assaying PA at pH 6.0, PA indeed resulted in the fluorescence quenching. So the phenomenon of the fluorescent enhancement and quenching was adopted in next research on capillary biosensors for the LA and PA assays.

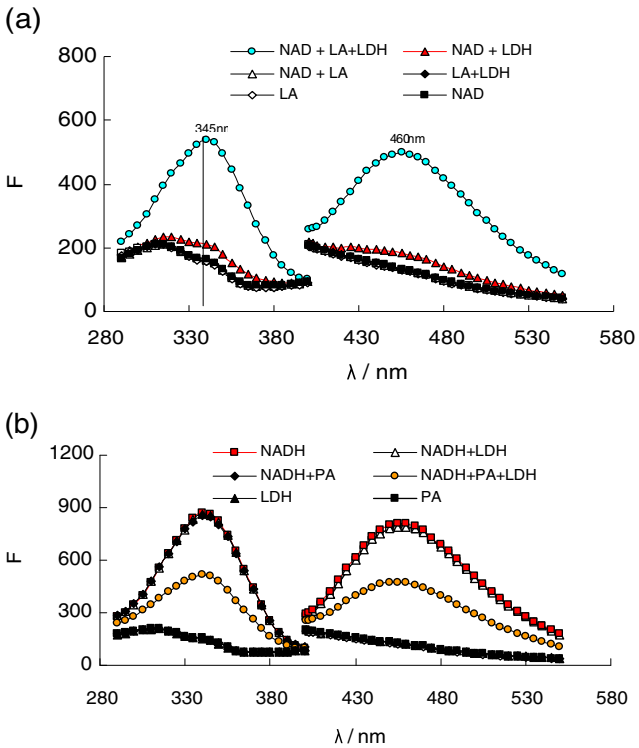

Fig. 1 Scanning spectra for fluorescent enhancement of lactate/NAD^+/LDH (**a**) and fluorescent quenching of pyruvate/NADH/LDH (**b**) reaction systems

Making Procedure of Capillary Biosensor

The previously made capillary biosensor was lower in the sensitivity (slope of the calibration was only 0.40 F L^{-1} $mmol^{-1}$ for lactate) and cannot be used for sequentially determining PA and LA [27], so, to further improve the fluorescent sensitivity of the capillary biosensor, the optimization was conducted again in the LDH modification method.

In order to facilitate the modification effect, in pretreating course of the glass capillaries, a new amination step was added, in which the capillaries sucked the 3-aminopropyl triethoxysilane (JH-A110) solution to covalently combine with Si-OH of capillary's inner-surface. And then in −0.04 MPa vacuum condition, the capillaries were marinated in 2.5% (*w*/*v*) of glutaraldehyde solution for 1 h to cross-link, where a Schiff-base reaction takes place between groups of amidogen and aldehyde. In last step, capillaries sucked the LDH solution for 12 h at 4 °C to immobilize. In this way, the fluorescence capillary biosensors have been made. If not in use, the biosensors were stored in the buffer II to retain activity of the modified LDH.

Procedures for Sequential Assay PA and LA Standards

The PA Standard Quantification Fig. 2 is an illustration on the method. A 100-μL of the PA standard (c_{SPA}) and a 100-μL NADH were beforehand mixed in a 1-mL test tube to form a mixture reaction solution, and then a portion of the mixture was immediately sucked into a biosensor. The biosensor was inserted in the top hollow of the capillary holder, which was installed in the fluorophotometer's dark sample chamber, and

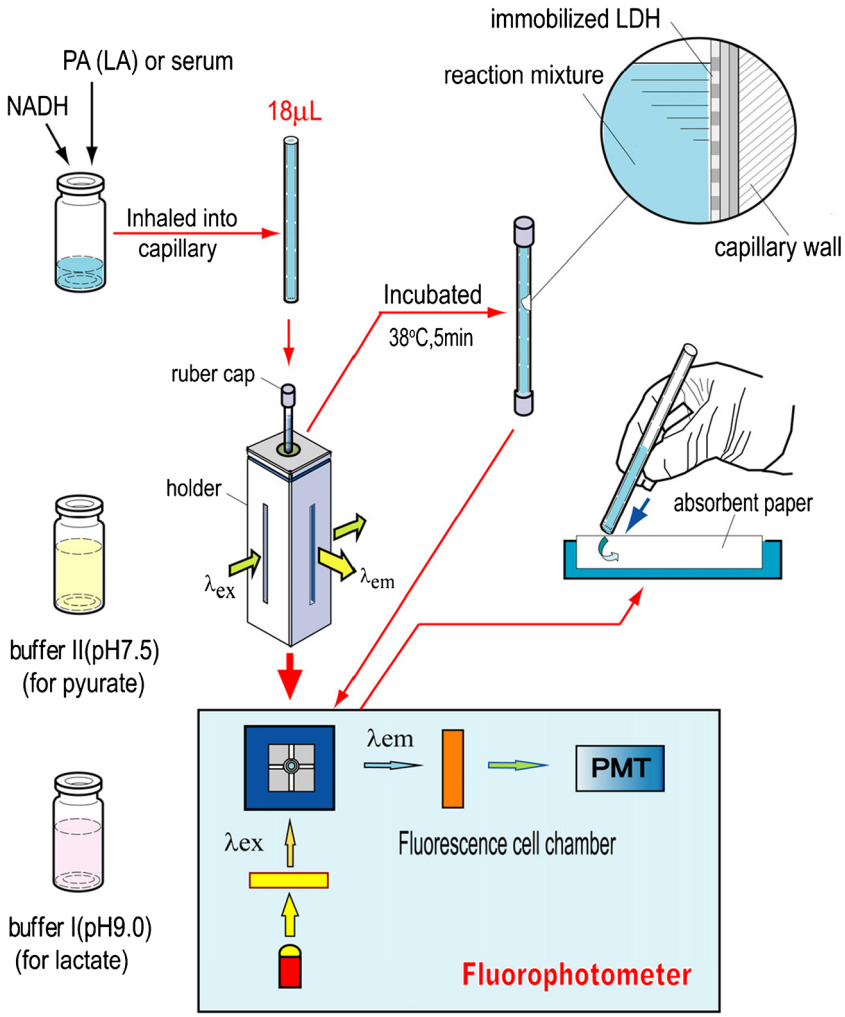

Fig. 2 Procedure sketch of an fluorescence capillary pyruvate/ lactate biosensor for sequentially quantifying pyruvate and lactate

detected to obtain a fluorescent blank (F_1). And then, the biosensor was taken out from the holder, and its one end was enveloped with a rubber cap and placed in the dry-block thermostat for 5 min at 38 °C to conduct the LDH's catalysis reaction. A fluorescent ending value (F_2) of the biosensor was again detected to obtain a fluorescent difference value ($\Delta F_{SPA} = F_1 - F_2$). Finally, the PA concentration was quantified by calculating the ΔF_{SPA}.

The LA Standard Quantification A 100-μL LA standard (c_{SLA}) and a 100-μL NAD^+ solution were beforehand mixed in another 1-mL test tube, and then a portion of the mixture was sucked into the biosensor which has been cleaned with the buffer I (pH 9.0) and the biosensor was detected to obtain another fluorescent blank (F_1'). After the LDH's catalysis reaction, the biosensor containing the mixture was again detected to obtain another fluorescent ending value (F_2') of the biosensor and a ΔF_{SLA}. Finally the LA concentration was determined by calculating the ΔF_{SLA}.

Results and Discussion

Acid and Alkali Pretreatments of Capillary

In order to form the PA/LA fluorescence biosensor, the optimization was carried out for the pretreatment condition of the glass capillary. Some capillaries having the same fluorescent blank were respectively put into two beakers, in which contained 0.1 M NaOH or 0.1 M HNO_3 solutions, to heat for 1 h at circa 60 °C. And then the desiccation, the amination, the aldehydation and the modification of LDH (50 kU/L) were conducted to these capillaries, and two kinds of capillary biosensors were made.

Subsequently, fluorescence quenching extents (ΔF) of the PA/NADH mixture solution inhaled in the capillary biosensors were detected by using a 0.6 mM PA standard as testing sample. Obtained results were shown in Fig. 3a. It can be seen that, the ΔF values from capillary biosensors pretreated by the NaOH solutions were higher than one of biosensors pretreated by the acid solutions, so the NaOH solution was selected to pretreat the glass capillary before the capillary amination.

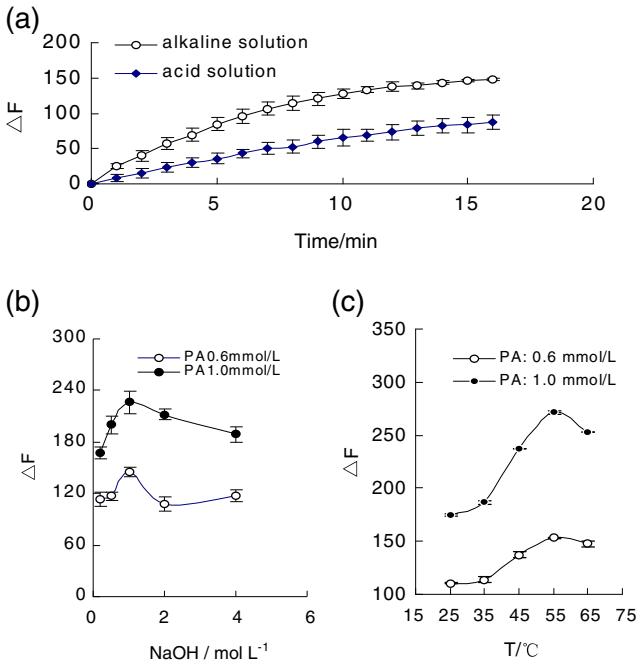

Fig. 3 Effects of sort and concentration as well as temperature for pretreatment reagents in capillary biosensor construction

Concentration and Temperature of NaOH Solution in Capillary Pretreatment

Under premise of fixing other experimental conditions, the effect of NaOH concentration for pretreating the capillary on the ΔF of NADH in the PA/NADH mixture was examined with the capillary biosensor.

Some capillaries were immersed respectively in 0.2, 0.50, 1.0, 2.0, 4.0 M NaOH and heated for 1 h at circa 60 °C to conduct pretreating. The NaOH-treated capillaries carried out the amination, the aldehydation and the LDH modification to form the biosensors. And then the ΔF of the biosensors inhaling PA standards were detected. Examination results in Fig. 3b revealed that fluorescence quenching extents had an increasing trend when the NaOH concentration was lower than 1 M, and a decreasing trend when the concentration was above 1 M. The latter producing reasons might be that a few NaOH powder formed in the drying process was deposited on the capillary inwall in higher NaOH concentration pretreating, which affected the bonding between the amidogen reagent and oxhydryl of the capillary inwall. Hence, the NaOH concentration was only chosen 1.0 M for the capillary pretreatment.

Furthermore, fixing other test conditions, temperature effect of the NaOH solution was also investigated, in which some capillaries were immersed respectively in 25, 35, 45, 55 and 65 °C of 1.0 M NaOH solution and heated for 1 h. Fig. 3c indicated that fluorescence quenching extents were elevated with the increase in the alkali solution temperature, and there was the maximum ΔF at 60 °C. Therefore, the temperature for pretreating the capillary was selected at 60 °C.

Concentration and Time as well as Temperature for Capillary Amination

The effect of the amination agent concentration (JH-A110) was examined in the biosensor construction process. Under optimized conditions above-mentioned, the capillaries pretreated by the NaOH solution were aminated for 6 h with different concentrations of JH-A110 (solvent was *n*-hexane) at room temperature. It is worthy to note that the amination reaction must be in anhydrous condition to avoid the hydrolysis of the amination agent. After the amination, the capillaries continued to actualize the aldehydation and the LDH modification to make capillary biosensors.

After the capillary biosensor was inhaling the PA/NADH mixture, its fluorescent quenching extent was measured. In this way, the effect of the amination reagent concentration on the performance of the manufactured capillary biosensor was indirectly estimated. From Fig. 4a, we found that the ΔF was monotonically increased in 1–4% (*v*/v) range and contrarily decreased over 4.0% with the increase of the amination reagent concentration, so, the JH-A110 concentration was selected 4% (*v*/v) for the capillary amination.

After that, other experimental conditions were ibid., the effect of elapsed time for the capillary amination on the performance of the finished capillary biosensor was evaluated. Fig. 4b showed that with prolonging the amination time for the capillary, the ΔF from the PA/NADH mixture within the finished capillary biosensor gradually increased and reached a constant state after 6 h. So, the amination time needed in the capillary was chosen for 6 h.

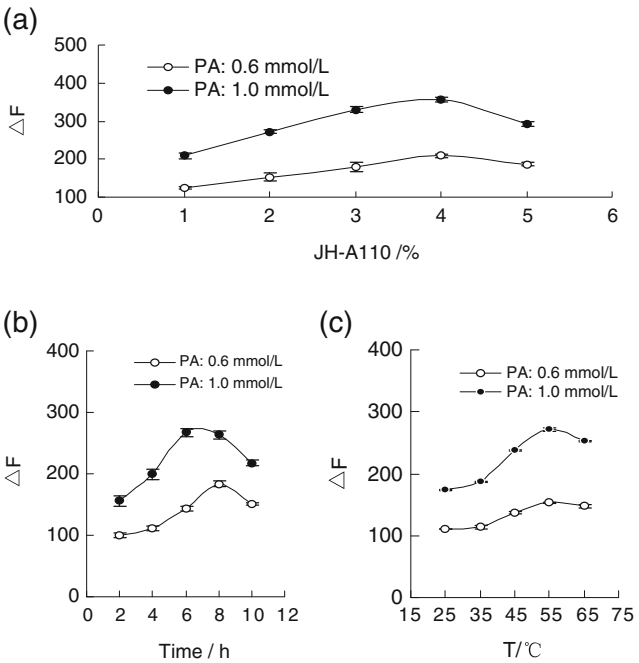

Fig. 4 Effect of concentration and time as well as temperature on amination

Subsequently, the effect of the capillary amination temperature on the biosensor performance was also examined by the same way above-mentioned. Fig. 4c was the obtained result. It can be seen that the amination temperature for the capillary heightened, the ΔF of the reaction mixture in the capillary biosensor was increased in 20–55 °C and decreased over 55 °C. Because the boiling point of *n*-hexane solvent used for dissolving the amination reagent is 68.74 °C, the selected amination temperature for the capillary want less than 55 °C to prevent evaporation of the solvent.

Concentration and Temperature as well as pH for Capillary Aldehydation

In making the capillary biosensor, glutaraldehyde was used as a cross-linking agent of LDH. However, if its dosage is not appropriate, it may lead to the LDH inactivation. Therefore, influences of the concentration and temperature as well as pH of the glutaraldehyde solution on the performance of the finished capillary biosensor were examined.

After the amination treatment, the capillary aldehydation were carried out at room temperature, in the which, we respectively used 1, 2, 2.5, 3, 4 and 5% (*v*/v) of glutaraldehyde solutions to modify the capillaries, and the obtained result was in Fig. 5a. The results showed that, when the glutaraldehyde concentration for the capillary aldehydation was in the range 1–3%, the ΔF of the PA/NADH mixture in the biosensor which modified LDH was gradually increased, and reached the maximum at 3%. Therefore, 3% of glutaraldehyde was selected to treat the aminated capillary.

Further, effects of the capillary's aldehydation time on the performance of the capillary biosensor were investigated in different time intervals. Obtained experiment results were shown in Fig. 5b. The results exhibited that the ΔF of the PA/NADH mixture in the finished capillary biosensor reached the maximum when the elapsed time for the capillary aldehydation was for 2 h. So, the time was chosen for 2 h.

Whereafter, a series of glutaraldehyde solutions in which acidities was different were prepared by using the buffer II, and the effect of the acidity on the performance of constructed capillary biosensors was investigated. From the change trend of curves in Fig. 5c, we can know that, the ΔF of the PA/NADH mixture in the capillary biosensor which modified LDH had no obvious change when the glutaraldehyde solution's acidity changed from pH 4 to pH 7, but it began to decrease when the pH value was than 7. So, the acidity needed in the capillary aldehydation was selected at pH 6.0.

Likewise, the temperature suitable for the capillary aldehydation was also optimized by using the change of the fluorescence quenching extent in the biosensor, in which there was the PA/NADH mixture, and the PA was used as the test samples. Obtained curves were shown in Fig. 5d. The curves

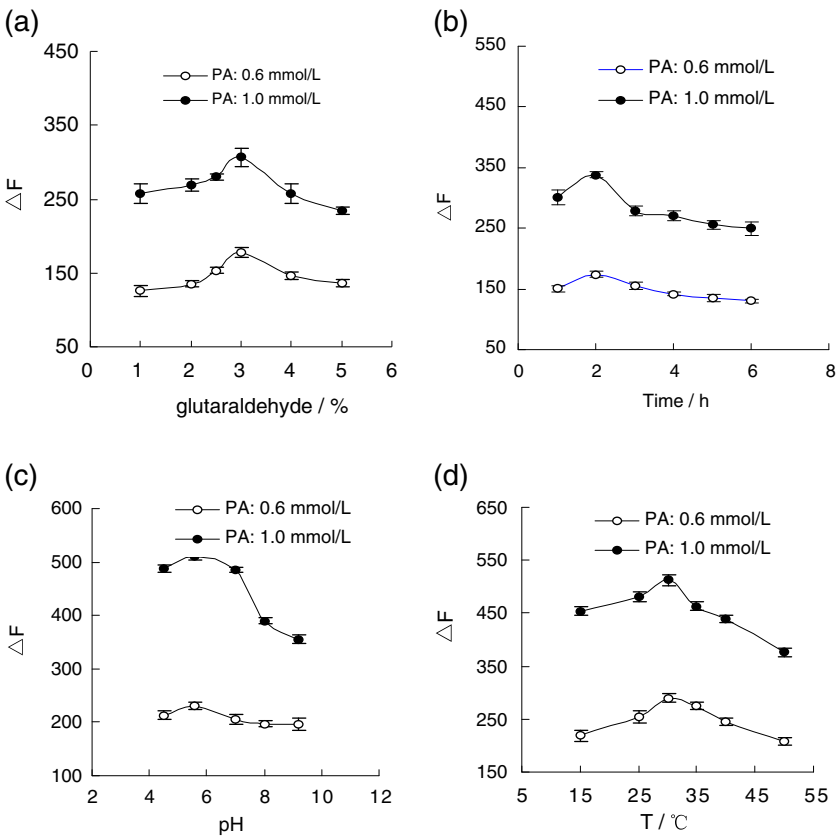

Fig. 5 Effects of concentration and temperature as well as pH on capillary aldehydation

showed that the ΔF of the PA/NADH mixture in the finished capillary biosensor has the maximum at 30 °C. So, the aldehydation temperature for the capillary was selected 30 °C.

Active Concentration of LDH and Its Modification Method

The active concentration of LDH for the capillary modification was investigated. The aldehydated glass capillaries were respectively immersed in 20, 40, 50, 60, 80 and 100 kU/L of LDH for 12 h to complete the enzyme modification and to form the capillary biosensors. And then, the ΔF of the PA/NADH mixture in the capillary biosensor was detected, in which the PA standard was used as the test sample. The results in Fig. 6 showed that when the active concentration of the LDH for the modification on the capillary was less than 50 kU/L, the ΔF was increased with the heightening of the active concentration, but when the active concentration exceeded 50 kU/L, the ΔF does not change. So, the LDH active concentration for modifying was selected at 50 kU/L.

Besides, static and dynamic circulation methods for the LDH modification on the capillary were also evaluated respectively. 0, 0.2, 0.4, 0.6, 1.0 mM of PA standards were used as the test samples. For the dynamic modification, a circulation system was designed in Fig. 7a. The aldehydated capillaries were mutually connected with some short and soft peristaltic pump tubings (i.d. 0.5 mm), and an inlet and an outlet of capillaries connecting in series together were simultaneously inserted in a vial in which was containing the LDH solution, and then the LDH solution was pumped by a peristaltic pump into these capillaries and started to circulate. The pump (flow rate: 0.2 ml/min) was placed between the outlet of the LDH solution and the inlet of the first capillary in the series capillaries. Whole circulation system was fixed in a refrigerator at 4 °C to prevent the LDH deactivation. Obtained results in Fig. 7b displayed

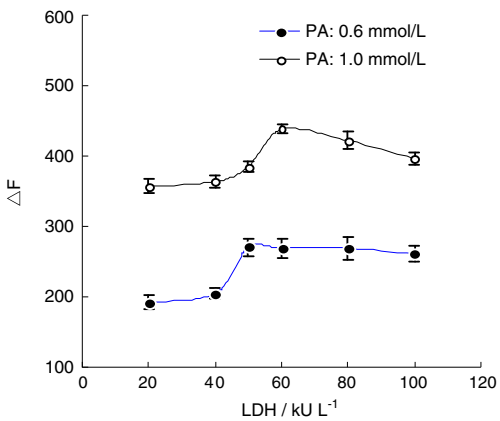

Fig. 6 Effect of the LDH active concentration on performance of the finished capillary biosensor

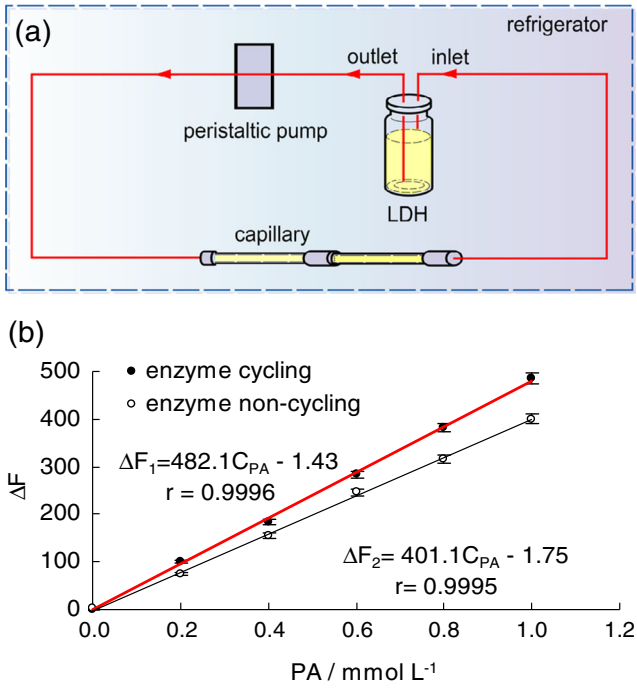

Fig. 7 Effects of active concentration and method of LDH for modification on the capillary biosensor construction

that the capillary biosensor sensitivities obtained by the dynamic ($\Delta F_1 = 482.1c_{PA} - 1.43$, $r = 0.9996$) and static modifications ($\Delta F_2 = 401.1c_{PA} - 1.75$, $r = 0.9995$) were respectively 482 ΔF/μM and 401 ΔF/μM. Obviously, the sensitivity of the former was higher than that of the latter, so this test proved that the dynamic method for the LDH modification on the capillary was better than the static method.

Comparison between PA/LA Biosensor Performance before and after Optimization

Different mixtures containing the PA (or LA) standard and NADH (or NAD^+) reagent were inhaled in order into the capillary biosensors which were made under optimized and un-optimized conditions [27] and ΔF of the mixtures were detected. And then, calibration curves for PA in Fig. 8a and for LA in Fig. 8b were plotted by using the obtained fluorescence values.

The results exhibited that the slope (428 ΔF/mM) of the PA standard curve from the capillary biosensor made under optimum conditions was more than 3.5 times (to 138 ΔF/mM) of the biosensor un-optimized. Similarly, the slopes for the LA standard curves optimized and unoptimized approximately differed 6 times (399/65.5). Apparently, the sensitivity of the optimized capillary biosensor has been improved greatly. This make known that the capillary biosensor performance has been markedly enhanced after endeavours above-mentioned.

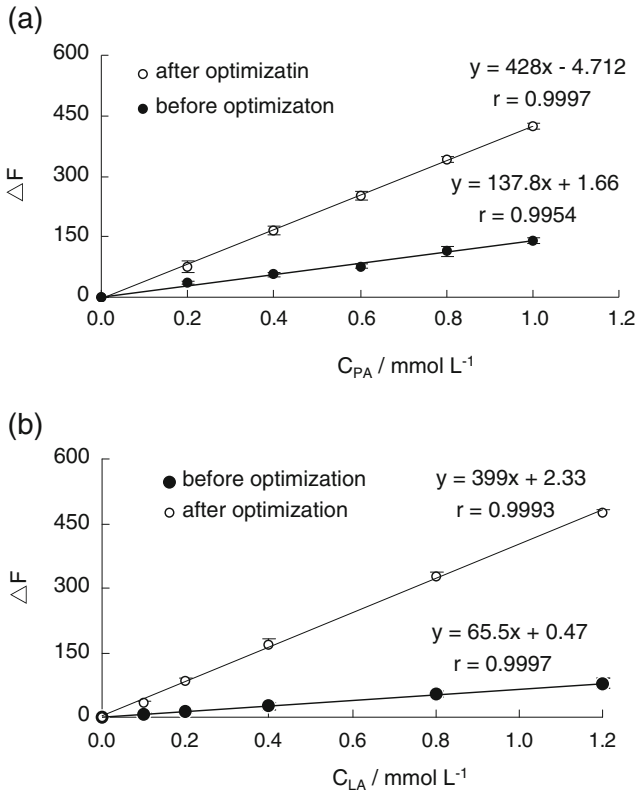

Fig. 8 Performance comparisons between the fluorescence capillary biosensors before and after the optimization for determing the PA (**a** curves) and LA (**b** curves)

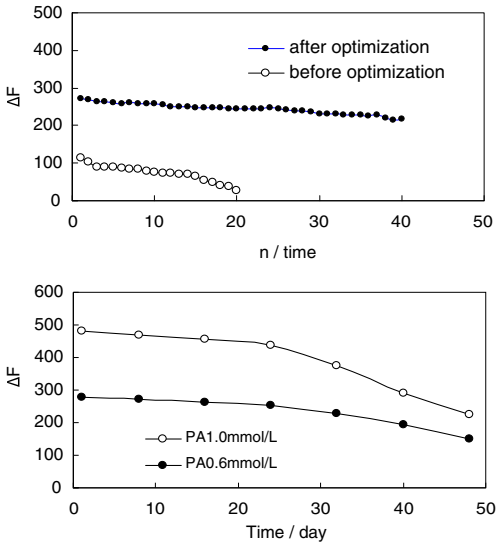

Fig. 9 Service life (the figure above) and its long-term stability (the figure below) for capillary biosensors before and after optimization

Service Life for Fluorescence Capillary Biosensors

Intraday service-life of the biosensors was evaluated by repeatedly detecting ΔF of the PA/NADH reaction mixture in the capillary biosensors made under the optimization conditions, and the obtained data was in Fig. 9. The result shown that the biosensors made by the optimized condition can continuously use for 40 times, with 3.0% of the relative standard deviation (RSD) ($n = 20$), but the biosensors un-optimized can only use for 10 times (RSD = 11%, $n = 10$).

Subsequently, to observe long-term stability of the capillary biosensors stored at approximately 4 °C, the ΔF's variation of the PA/NADH mixture in the capillary biosensors made in the identical batch was discontinuously examined once a week. The below curves Fig. 9 showed that ΔF of the capillary biosensors was essentially stable within 32 days (RSD = 5.1%, $n = 4$), namely, the service-life and deposited time of the capillary biosensors made by the optimization conditions was prolonged markedly.

Effect of Other Co-Existing Substances in Serum

It is complicated in composition of serum, in which contains many other coexisting substances, such as NaCl, glucose, ascorbic acid, etc. Therefore, the possible interference with determination of the LA and PA existing in human serums were investigated. Due to the PA and LA concentrations in normal human serums are in ranges 40–100 μM and 0.56–2.22 mM, respectively, so in the interference investigation, 50 μM PA and 0.20 mM LA standards were selected as matrix samples. Different concentrations of NaCl (50–800 mM), K^+ (0.5–20 mM), Mg^{2+} (0.50–10 mM), Cu^{2+} (0.02–0.2 mM), glucose (1.0–20 mM), ascorbic acid (0.02–2.0 mM), vitamin B_1 (0.01–1.0 mM), urea (1.0–20 mM), L-glutamate (0.01–1.0 mM), and L-tyrosine acid (0.02–2.0 mM) were respectively added into the two matrix samples, and their fluorescent change values were detected by using the capillary biosensors. Obtained results demonstrated that the added substances did not interfere with the biosensor determination, and recoveries of the PA or LA standards containing the added constituents were in 95–106% which was satisfactory.

Fluorescent Interference from Unknown Constituent in Real Serum

In addition to the evaluated interference factors above-mentioned, real human serums also contain some unknown constituent which influences the fluorescence intensity of assaying PA or LA, therefore, the effects of unknown constituent in serum were investigated.

The hollow circle curve in Fig. 10a indicated that the fluorescence intensity for the NADH mixing the untreated serum was gradually decreased with increasing in the detecting time, namely, unknown constituent in the serum caused the fluorescent quenching of the NADH. This will produce serious interference with determination of PA. The solid circle curve in Fig. 10b indicated that the fluorescence intensity of the NADH in the serum was no longer changed with time by using the

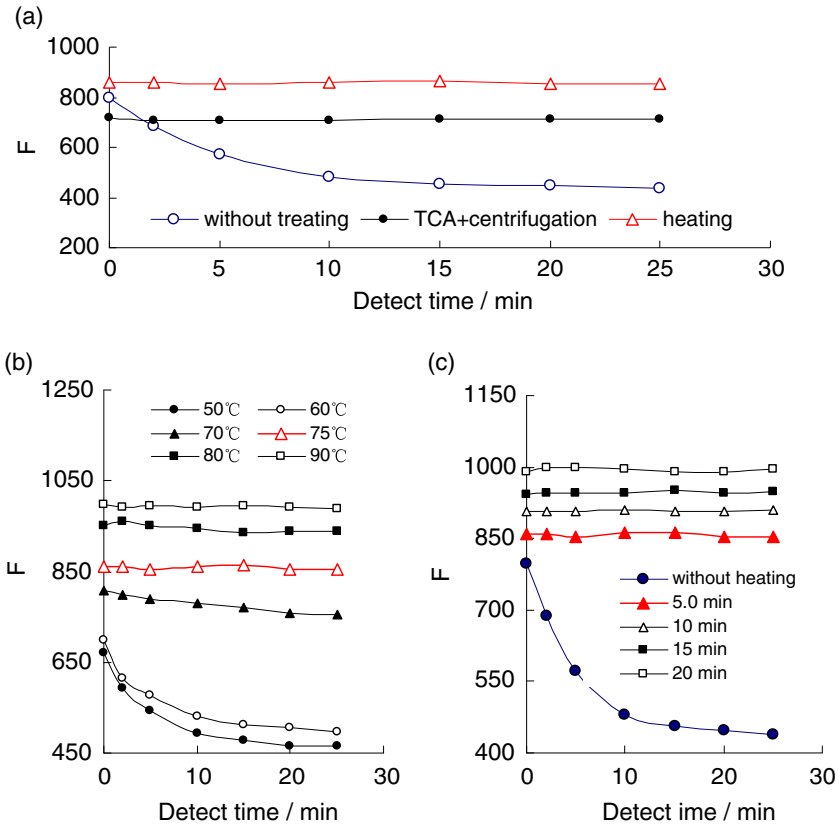

Fig. 10 Examination of pretreatment conditions for serum sample. **a**, **b** and **c** sub-graphs respectively were the optimized results of three methods, and heating temperature and time for pretreating serums

Table 1 Determination results of LA and PA contents in serum samples obtained by the biosensors (n = 3)

Serum (lactate)[a]	F_{S1}'	F_{S2}'	ΔF_{LA}	RSD / %	found conc.[b]/ μmol L^{-1}
No.1	369.4	545.9	176.5		
	358.2	540.8	182.6	1.86	0.45 ± 0.01
	366.8	543.9	177.1		
No.2	316.9	414.1	97.2		
	317.3	416.6	99.3	1.62	0.24 ± 0.01
	319.6	415.8	96.2		
No.3	324.1	458.9	134.8		
	329.7	468.1	138.4	1.45	0.34 ± 0.01
	322.0	457.2	135.2		
Serum (pyruvate)[a]	F_{S1}	F_{S2}	ΔF_{PA}	RSD / %	found conc.[c]/ μmol L^{-1}
No.1	933.4	851.8	81.6		
	926.6	843.5	83.1	1.53	12.6 ± 0.2
	931.8	847.6	84.2		
No.2	946.2	823.5	122.7		
	940.2	820.3	119.9	1.13	18.5 ± 0.2
	945.7	824.1	121.6		
No.3	909.5	841.8	67.7		
	915.8	846.7	69.1	2.03	10.5 ± 0.2
	914.7	844.2	70.5		

[a] Samples were heated for 5 min at 75 °C after be diluted to 5 times

[b] $\Delta F = 399.55\ c_{LA} + 0.0242$, r = 0.9991, linear range: 0.10–1.2 mmol/L

[c] $\Delta F = 6.571 c_{PA} + 0.109$, r = 0.9997, linear range: 4-120 μmol/L

Table 2 Recovery determinations of lactate and pyruvate in the serums by the biosensor assay

Serums (lactate)	dilution multiple	sample conc. (before dilution) /mM	added conc. /mM	calculate conc. /mM	found conc. /mM	recovery/ %
No.1	2	0.22 (0.45)	0.2	0.42	0.41	98
			0.6	0.82	0.83	100
No.2	2	0.12 (0.24)	0.2	0.32	0.31	99
			0.6	0.72	0.76	106
No.3	2	0.17(0.34)	0.2	0.37	0.35	97
			0.6	0.77	0.79	103
Serums (pyruvate)	dilution multiple	sample conc. (before dilution) /μM	added conc. /μM	calculate conc. /μM	found conc. /μM	recovery/ %
No.1	2	6.3 (12.6)	10	16.3	16.1	99
			40	46.3	45.8	99
No.2	2	9.2 (18.5)	10	19.2	20.1	104
			40	49.2	47.8	97
No.3	2	5.3 (10.5)	10	15.3	16.1	105
			40	45.3	45.8	101

"trichloroacetic acid (TCA) + centrifugation" step to pretreat the serum. This suggesting that, unknown constituents resulting in the NADH's fluorescence quenching has been removed. The hollow triangle curve in Fig. 10a showed that the effectiveness of heating the serum to remove interference of unknown constituent was the same with one of the TCA treatment.

Consequently, temperature and time for heating the serum was examined. Six identical serum samples which were respectively heated for 10 min at 50, 60, 70, 75, 80 and 90 °C were cooled to the room temperature and mixed with the NADH, and then using an empty capillary respectively inhaled the mixture solutions to detect its fluorescence changes. Fig. 10b showed that heating the serum sample over 75 °C for 20 min, the fluorescent intensity of NADH which was mixing the preheat-treated serum no longer changed with time. So, the temperature of preheating the serum was selected at 75 °C. Fig. 10c indicated that when the preheating time of the serum was greater than 5.0 min, the NADH's fluorescence will not be quenched.

Besides, in Fig. 10a-c, there was a phenomenon that heightening the preheat temperature will increase the fluorescence initial value of the NADH in the serum. This was because unknown constituents resulting in the fluorescence quenching was thermally decomposed in heating the serum. This phenomenon is advantageous to save the NADH reagent. So, this simplified pre-treatment method was introduced in our capillary biosensor assay to remove interferences of unknown constituents in serum samples.

Real Serum Sample Quantification

A serum samples were first pretreated at 75 °C for 5 min, and other handling steps were identical with "Procedures for sequential assay PA and LA standards" Section. Finally, ΔF_{PA} (F_{S2}-F_{S1}) for quantifying PA and ΔF_{LA} (F_{S2}' - F_{S1}') for quantifying LA were obtained. In the linear response range of the capillary biosensor assay, c_{PA} and c_{LA} values in the serum samples were calculated according to the formula: $c_{PA} = \Delta F_{PA} \cdot c_{SPA}/\Delta F_{SPA}$ and $c_{LA} = \Delta F_{LA} \cdot c_{SLA}/\Delta F_{SLA}$. If utilizing the calibration curve method, ΔF_{PA} and ΔF_{LA} will be respectively substituted to the linear equations of PA or LA standard curves ($\Delta F_{PA} = kc_{PA} + b$, or $\Delta F_{LA} = kc_{LA} + b$) for finding c_{PA} and c_{LA}. All the samples were analyzed in triplicate.

If discharging the solution within the capillary biosensor, let the biosensor bottom gently touched absorbent papers, the

Table 3 Results comparison of determination of PA and LA contents by our method (FCA) and UV methods

Serums (PA)	FCA / μM	UV / μM	FCA RSD/%	UV RSD/%
No.4	63.0 ± 0.7 (62.0, 63.2, 63.9)	63.6 ± 1.0 (63.2, 62.6, 65.1)	1.53	2.07
No.5	92.3 ± 0.8 (93.3, 91.2, 92.5)	92.1 ± 1.0 (91.0, 91.8, 93.7)	1.13	1.51
No.6	52.5 ± 0.8 (51.4, 52.5, 53.6)	53.3 ± 0.7 (53.4, 52.4, 53.3)	2.03	1.77
No.7	116.0 ± 1.7 (117, 118, 114)	116.8 ± 1.0 (118, 116, 117)	1.91	1.21
Serums (LA)	FCA / mM	UV / mM	FCA RSD/%	UV RSD/%
No.4	2.24 ± 0.04 (2.21, 2.28, 2.22)	2.30 ± 0.03 (2.29, 2.26, 2.34)	1.86	1.77
No.5	1.22 ± 0.02 (1.22, 1.24, 1.21)	1.28 ± 0.02 (1.31, 1.28, 1.26)	1.62	2.08
No.6	1.70 ± 0.02 (1.69, 1.73, 1.70)	1.75 ± 0.02 (1.78, 1.73, 1.75)	1.45	1.52
No.7	2.94 ± 0.03 (2.98, 2.91, 2.95)	3.01 ± 0.03 (3.00, 3.05, 2.97)	1.31	1.37

solution in the biosensor will be automatically effused into the papers. This emptied biosensor can be regenerated by inhaling the buffer II (pH 7.5) for PA and the buffer I (pH 9.0) for LA. The capillary biosensor can be also disposable. Besides, if multiple samples have to be analyzed, also multiple capillary biosensors can be simultaneously used to suck different reaction mixture solution and to detect in sequence.

Real Serum Assay and Recovery Test as well as Accuracy Comparison

First, calibrations curves for LA and PA were obtained by the capillary biosensor, in which the calibrations curves were in range 4–120 μM for PA and in 0.1–1.2 mM for LA. After regress-calculating, two regression equations for PA and LA were obtained: $\Delta F_{PA} = 6.57c_{SPA} + 0.11$ ($r = 0.9997$) for PA and $\Delta F_{LA} = 399.55c_{SLA} + 0.024$ ($r = 0.9991$) for LA. And then, the capillary biosensors were employed to determine two analyte concentrations in serum samples which were collected after overnight fasting from a hospital in Chengdu. All these samples were preheated and pre-diluted five-folds. Determined results were listed in Table 1.

To evaluate the assay accuracy, 20 μM and 40 μM of the PA standards and 0.2 μM and 0.8 mM of the LA standards were added respectively into the serum samples, the recovery tests were carried out. Related data in Table 2 indicated that the got recoveries were in 97–105% for the LA and in 97–106% for the PA and were satisfactory.

Afterwards, to further judge the assay accuracy, the quantification results for PA and LA by the assay were compared with one of the traditional ultraviolet spectrophotometry [23, 24]. Compared results in Table 3 indicated that the got recoveries were in 97–105% for the LA and in 97–106% for the PA and were satisfactory.

Reproducibility and Detection Limit of Biosensor

Reproducibility testing of the capillary biosensor was actualized in the linear range, and the obtained RSD for 0.2 mM LA was 3.3% which was calculated from the average value (93.7 ± 3.1 a.u., $n = 11$) of the fluorescent enhancement, and the RSD for 10 μM PA was 2.92% which was computed from the mean value of the fluorescent quenching (64.7 ± 1.9 a.u., $n = 11$). The mean deviations and RSDs in Table 3 displayed that there was not significant difference between the measured results from two methods.

On the basis of this, Detection limits ($D_L = 3S/k$) of the capillary biosensor assay were calculated, where the S is the standard deviation of the background and the k refers to the slope of the linear equation for the calibration curves. The obtained D_L for LA was 0.023 μM and for PA was 0.87 μM, respectively.

Above results clearly demonstrate that our assay is credible for determining PA and LA concentrations in serums.

Conclusions

Based on optimized experiments for the bidirectional reaction and the LDH modification on the capillary, the fluorescence capillary biosensor assay has been developed, and it was successfully used for the quantification of PA in 4–120 μM and LA in 0.10–1.2 mM in human serums. The method's advantages were that the LDH immobilizing on capillary realized the reuse of expensive enzymes in fluorospectrophotometry, the consumption of serum samples or the NAD^+ (or NADH) reagent were decreased to 9 μL in per assay, the analytes no needed to preseparation and it also are accurate and reliable.

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